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A BAR domain-mediated autoinhibitory mechanism for RhoGAPs of the GRAF family

Alexander EBERTH*[1], Richard LUNDMARK†[2], Lothar GREMER*‡, Radovan DVORSKY*, Katja T. KOESSMEIER*, Harvey T. McMAHON†[2] and Mohammad Reza AHMADIAN*[3]

*Institute of Biochemistry and Molecular Biology II, Heinrich Heine University Medical Center, Universitätsstrasse 1, 40225 Düsseldorf, Germany, †Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K., and ‡Max-Planck Institute of Molecular Physiology, Department Structural Biology, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany

The BAR (Bin/amphiphysin/Rvs) domain defines an emerging superfamily of proteins implicated in fundamental biological processes by sensing and inducing membrane curvature. We identified a novel autoregulatory function for the BAR domain of two related GAPs' (GTPase-activating proteins) of the GRAF (GTPase regulator associated with focal adhesion kinase) subfamily. We demonstrate that the N-terminal fragment of these GAPs including the BAR domain interacts directly with the GAP domain and inhibits its activity. Analysis of various BAR and GAP domains revealed that the BAR domain-mediated inhibition of these GAPs' function is highly specific. These GAPs, in their autoinhibited state, are able to bind and tubulate liposomes *in vitro*, and to generate lipid tubules in cells. Taken together, we identified BAR domains as *cis*-acting inhibitory elements that very likely mask the active sites of the GAP domains and thus prevent down-regulation of Rho proteins. Most remarkably, these BAR proteins represent a dual-site system with separate membrane-tubulation and GAP-inhibitory functions that operate simultaneously.

Key words: autoregulation, autoinhibited state, Bin/amphiphysin/Rvs (BAR) domain, GTPase-activating protein (GAP), GTP hydrolysis, GTPase reaction, Rho protein family.

INTRODUCTION

Members of the Rho family of small guanine-nucleotide-binding proteins are key regulatory molecules that couple changes in the extracellular environment to intracellular signal transduction. They act as intracellular molecular switches by cycling between active (GTP-bound) and inactive (GDP-bound) states [1]. Activation of Rho proteins results in their association with effector molecules that subsequently activate a wide variety of downstream signalling cascades and regulate many important processes in all eukaryotic cells, including motility and endocytic trafficking [2]. The activity of Rho proteins – at a specific time and at a specific site in the cell – is strictly controlled by three classes of regulatory proteins: the GDIs (guanine-nucleotide-dissociation inhibitors), the GEFs (guanine-nucleotide-exchange factors) and the GAPs (GTPase-activating proteins) [1]. These regulators safeguard two distinct cycles: membrane/cytosol partition of the Rho proteins and exchange/hydrolysis of the bound nucleotide.

The GTP-hydrolysis reaction is a fundamental process in living cells and represents an important timer in intracellular processes. The rate of Rho protein-mediated GTP hydrolysis is intrinsically low. Direct interaction with GAPs specific for Rho proteins accelerates the reaction by several orders of magnitude [3]. The RhoGAP functions are critical for the termination of signal transduction [4]. Thus mutations in genes encoding GAPs of the Rho proteins have drastic consequences and underlie several human diseases. *OPHN1* (oligophrenin-1) mutations frequently cause X-linked mental retardation that is associated with cerebellar hypoplasia [5]. Another GAP that regulates endocytosis via the CLIC (clathrin-independent carrier)/GEEC (glycosylphosphatidylinositol-enriched endosomal compartment) pathway [6] and has been implicated as a tumour-suppressor gene of acute myelogenous leukaemia and myelodysplastic syndrome is GRAF1 (GTPase-regulator associated with focal adhesion kinase-1) [7]. These RhoGAPs together with PSGAP [PH (pleckstrin homology) and SH3 (Src homology 3) domain-containing RhoGAP] [8] and GRAF3 (G. Doherty, personal communication) constitute a subfamily of structurally related regulatory proteins. They share an N-terminal BAR (Bin/amphiphysin/Rvs)-like domain, followed by a PH domain and a RhoGAP-related domain (Figure 1A). Nadrin (Rich1, ARHGAP17) also has similar architecture but lacks the central PH domain between the BAR and the GAP domain [9]. The BAR domain superfamily of proteins have emerged as important players in membrane-remodelling processes (http://www.bar-superfamily.org). These domains dimerize to sense, and often to induce, membrane curvature [10–12]. In addition, the BAR domain of arfaptin2/POR1 (partner of Rac1) has been reported to bind differently to ADP-ribosylation factors and Rac1 [13].

Although the molecular mechanisms of Rho protein regulation by GAPs are well characterized [4], our understanding of the GAP regulation itself is an open and challenging issue. Recently, we

Abbreviations used: aa, amino acids; ABR, active BCR (breakpoint cluster region)-related gene; BAR, Bin/amphiphysin/Rvs; BAR^N, nadrin BAR domain (aa 1–241); $BAR\text{-}GAP^N$, nadrin BAR domain–GAP domain (aa 1–499); GAP, GTPase-activating protein; GAP^N, nadrin GAP domain (aa 241–499); GRAF, GTPase regulator associated with focal adhesion kinase; PH domain, pleckstrin homology domain; $BAR\text{-}PH^G$, GRAF1 BAR domain–PH domain (aa 1–382); GAP^G, GRAF1 GAP domain (aa 361–576); GST, glutathione transferase; ITC, isothermal titration calorimetry; OPHN1, oligophrenin-1; $BAR\text{-}PH^O$, OPHN1 BAR domain–PH domain (aa 1–366); $BAR\text{-}PH\text{-}GAP^O$, OPHN1 BAR domain–GAP domain (aa 1–572); GAP^O, OPHN1 GAP domain (aa 360–572); $PH\text{-}GAP^O$, OPHN1 PH domain–GAP domain (aa 231–572); tamraGTP, tetramethylrhodamine-conjugated GTP.

[1] Present Address: Division of Structural Biology, Helmholtz Centre for Infection Research, Inhoffenstrasse 7, D-38124 Braunschweig, Germany.
[2] Present Address: Medical Biochemistry and Biophysics, Umeå University, 901 87 Umeå, Sweden.
[3] To whom correspondence should be addressed (email reza.ahmadian@uni-duesseldorf.de).

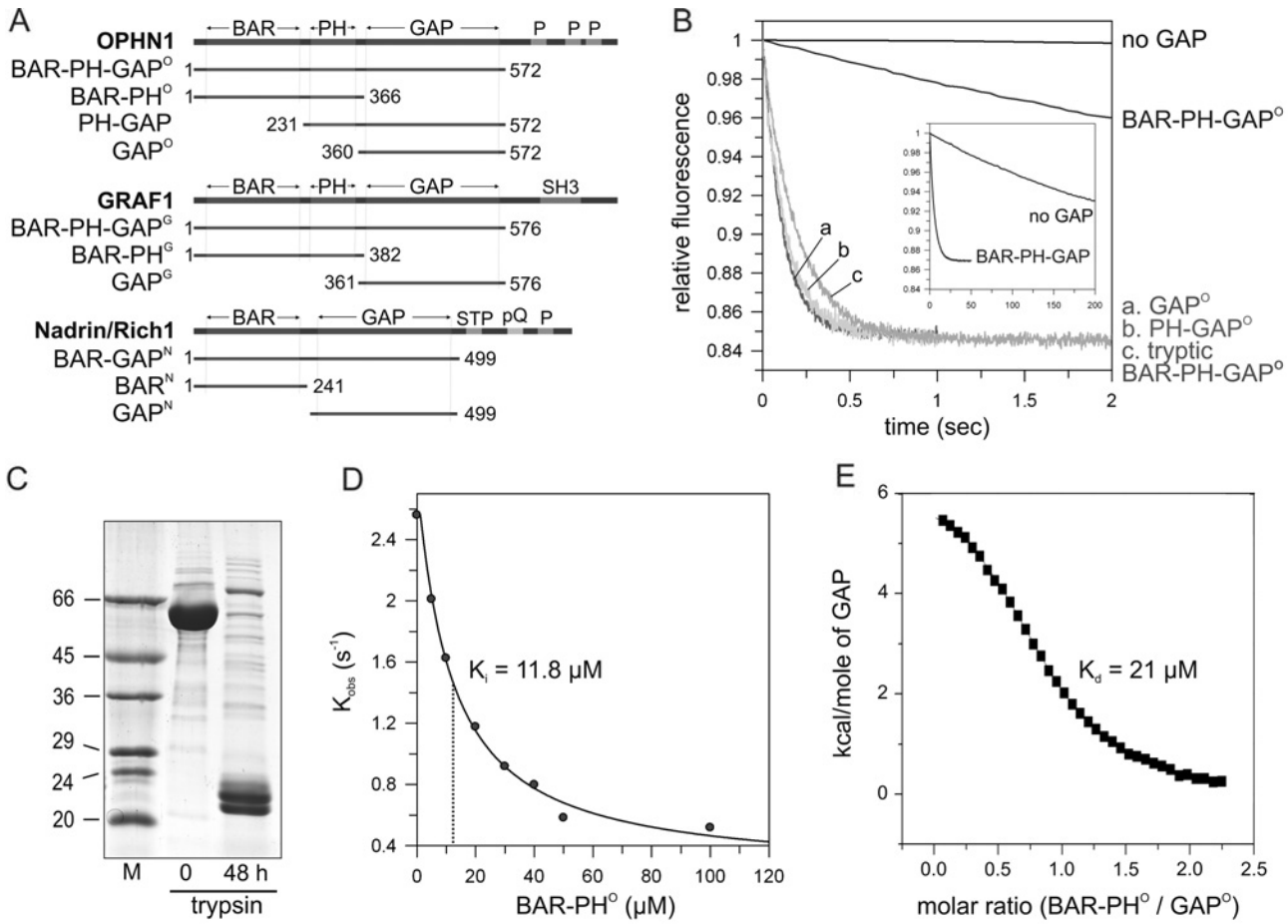

Figure 1 BAR-mediated autoinhibition of OPHN1 GAP activity

(**A**) Schematic representation of the BAR domain-containing regulators of Rho proteins and constructs used in the present study. Abbreviations not explained in the text are: P, proline-rich sequence; pQ, polyglutamine sequence; SH3, Src homology 3 domain; STP, serine/threonine/proline-rich domain. (**B**) In contrast with GAP^0 or PH-GAP^0, the BAR-PH-GAP^0 represents an autoinhibited state of OPHN1, as it exhibited a strongly reduced GAP activity towards Cdc42 · tamraGTP (0.2 μM). The concentration of the GAP proteins was 1 μM. The inset shows the complete time course of the GTPase reaction in the absence (intrinsic; no GAP) or in the presence of BAR-PH-GAP. The observed rate constants (k_{obs}) obtained by single exponential fitting of the curves were 0.0031 s^{-1} for the intrinsic reaction (no GAP), 0.18 s^{-1} for BAR-PH-GAP^0, 9.1 s^{-1} for GAP^0, 8.6 s^{-1} for PH-GAP^0 and 6.3 s^{-1} for the tryptic product of the BAR-PH-GAP^0. Values represent the average of at least five different experiments. (**C**) Coommassie Blue-stained SDS gel showing purified BAR-PH-GAP^0 protein before (0 h) and after (48 h) tryptic digestion. M stands for marker proteins, with molecular mass shown on the left-hand side in kDa. (**D**) Inhibition of the GAP-stimulated tamraGTP hydrolysis reaction of Cdc42 (0.2 μM) was measured in the presence of 0.5 μM GAP^0 and various amounts of the BAR-PH^0 domain (5–100 μM). The IC_{50} value of 11.8 μM was calculated by hyperbolic fitting of the binding curves. (**E**) Calorimetric titration of 150 μM BAR-PH^0 with 1500 μM GAP^0. Fitting of the isothermal curve yielded a molar ratio of 0.87 and a K_d value of 21 μM.

have found that the N-terminal region of OPHN1 seems to effect its GAP function and suggested that this part of the protein itself could act as a regulator of GAP activity either by an autoinhibitory mechanism or by binding of a second inhibitory protein [14]. In the present study we investigated a potential role of the N-terminal domains of GRAF1 and OPHN1 on their GAP activities. We describe a novel function for the BAR domains of the GRAF subfamily, which inhibit the activity of the GAP domains and remodel simultaneously lipid membranes.

MATERIALS AND METHODS

Constructs

Human Cdc42 [aa (amino acids) 1–178; GenBank® accession number NM_001791], Rac1 (aa 1–184; M29870) and RhoA (aa 1–181; L25080) were cloned as described previously [15]. BAR-PH-GAP (aa 1–572), BAR-PH (aa 1–366), PH-GAP (aa 231–572) and GAP (aa 360–572) of OPHN1 (NM_002547), BAR-PH-GAP (aa 1–576), BAR-PH (aa 1–382) and GAP (aa 361–576) of GRAF1 (Y10388), BAR-GAP (aa 1–499), BAR (aa 1–241) and GAP (aa 245–499) of Rich1/nadrin (BC003259), BAR (aa 116–341) of arfaptin2 (NM_012402), BAR (aa 1–256) of endophilin-A1 (Q99962), GAP (aa 1250–1513) of p190RhoGAP (M94721), GAP (aa 559–882) of ABR [active BCR (breakpoint cluster region)-related gene] (L19704), GAP (aa 198–439) of p50RhoGAP (NM_004308) and BAR-PH (aa 1–384) of β2-centaurin (NP_055729) were cloned in pGEX vectors.

Proteins and fluorescent nucleotides

All proteins were produced and prepared as described in [16]. Briefly, the proteins were produced as GST (glutathione transferase) fusion proteins in *Escherichia coli* BL21 (DE3) or Rosetta (DE3). GSH–Sepharose (Pharmacia) was used as the first purification step. After protease cleavage of the GST tags, the proteins were applied to a gel-filtration column (Superdex 75 or 200, Pharmacia) and a subsequent GSH–Sepharose column as the final step to obtain a purity of at least 95 %. Nucleotide-free GTPases as well as fluorescent nucleotide-bound GTPases

were prepared, and concentration and quality were determined as described in [16]. TamraGTP (tetramethylrhodamine-conjugated GTP) was synthesized according to protocols previously established in our laboratory [3,16].

Proteolytic cleavage

Purified BAR-PH-GAP was treated with trypsin at a protease/substrate ratio of 1:4000 at room temperature (25 °C) for up to 48 h. Aliquots from the proteolysis reaction were withdrawn at the indicated time points. The reaction was terminated with 50 μg/ml leupeptin for the trypsin reaction. BAR-PH-GAP cleavage was analysed on SDS/polyacrylamide gels.

Fluorescence measurements

The stopped-flow instrument (Applied Photophysics SX18MV) was routinely utilized for analysis of rapid kinetics, such as GAP-stimulated GTPase reactions (single turnover conditions) as described in [16]. All fluorescence measurements were performed in 30 mM Tris/HCl, pH 7.5, 10 mM KH_2PO_4/K_2HPO_4, 10 mM $MgCl_2$, 50 mM NaCl and 3 mM dithioerythritol at 25 °C.

Isothermal titration microcalorimetry

Titration experiments were carried out at 10 °C with the isothermal titration microcalorimeter VP-ITC (isothermal titration calorimetry) system (Microcal, Northampton, MA, U.S.A.). All protein solutions were prepared in 20 mM Tris/HCl (pH 7.5) by size-exclusion chromatography. The protein concentrations in the calorimetric cell were between 50 and 400 μM. Injections of ligand solution (1–3.5 mM) into the calorimetric cell were carried out at time intervals of 200 s with injection volumes of 8 μl. The injection volume was 8 μl, except for the first step (2 μl). The total number of injections was 60. Spacing time between injections was 4 min. Analysis of the experimental data was carried out with Origin 7.0 software (Microcal). Binding parameters such as number of binding sites (n), the association constant (K_a) and the binding enthalpy ($\Delta H°$) were determined as parameters of the fitted experimental binding values.

Liposome co-sedimentation assay

Liposomes were generated from total brain lipids (Folch fraction I) (Sigma–Aldrich) or synthetic lipids [10 % phosphatidylinositol 4,5-bisphosphate, 10 % cholesterol, 40 % phosphatidylethanolamine, 40 % phosphatidylcholine (Avanti Polar Lipids)] and filtered to the specified diameter as previously described [10]. Briefly, the lipid mixtures were dried under a stream of nitrogen and resolved in assay buffer before sonication to generate spherical liposomes. Liposome-binding assays were performed essentially as described in [10]. Briefly, proteins were incubated together with liposomes followed by centrifugation and analysis of the pellet and supernatant on SDS/PAGE.

Transfection and membrane staining

HeLa cells were grown in DMEM (Dulbecco's modified Eagle's medium) media (GIBCO) supplemented with 10 % foetal bovine serum, and transfected for transient protein expression using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. For immunofluorescence analysis, HeLa cells were fixed in 3 % (w/v) paraformaldehyde in PBS for 15 min at 22 °C, then washed and blocked in PBS containing 5 % goat serum and 0.1 % saponin before staining with rabbit anti-Myc antibodies (Cell Signaling Technology) in 1 % goat serum, 0.1 % saponin in PBS and secondary Alexa Fluor® 488-conjugated anti-rabbit antibodies (Invitrogen) using standard protocols. Epifluorescence images were taken using a Zeiss Axioimager Z1 system with AxioVision software.

RESULTS AND DISCUSSION

BAR domain is a *cis*-acting inhibitory element

In a previous study, we found that full-length OPHN1 showed a much weaker GAP activity compared with the GAP domain alone when overexpressed in cells [14]. To investigate a potential inhibitory mechanism for the catalytic GAP domain, we purified various protein fragments of OPHN1 (Figure 1A) and determined their activities in a real-time fluorescence-based GTP-hydrolysis assay [3,16]. The central tool of this assay is a tamraGTP, which is sensitive towards the active state of the Rho proteins and thus can be used to monitor not only the intrinsic but also the GAP-stimulated hydrolysis reactions. In contrast with OPHN1 GAP (denoted GAP^O) and $PH\text{-}GAP^O$, which were able to rapidly stimulate the intrinsic GTP-hydrolysis reaction of Cdc42 to the same extent, the GAP activity of $BAR\text{-}PH\text{-}GAP^O$ was drastically reduced (50-fold, under the conditions tested here; Figure 1B). Similar results were obtained when RhoA or Rac1 were used (results not shown). We used Cdc42 as a model protein for all experiments since GAP^O revealed the highest activity towards Cdc42 when compared with that of RhoA and Rac1 (Supplementary Figure S1, at http://www.BiochemJ.org/bj/417/bj4170371add.htm).

To prove that the drastic reduction of the $BAR\text{-}PH\text{-}GAP^O$ activity is caused by a specific domain–domain interaction rather than by protein instability or misfolding, we subjected $BAR\text{-}PH\text{-}GAP^O$ to partial proteolysis. Two fragments of 24 and 22 kDa remained stable after limited trypsin cleavage for 48 h (Figure 1C; Supplementary Figure S2A, at http://www.BiochemJ.org/bj/417/bj4170371add.htm). Trypsin treatment of GAP^O or $BAR\text{-}PH^O$, however, resulted in a complete digestion of $BAR\text{-}PH^O$, but GAP^O was cleaved to a 22 kDa fragment that remained resistant against trypsin (Supplementary Figure S2B). A comparison of the trypsin treatment of GAP^O and $BAR\text{-}PH\text{-}GAP^O$ clearly indicated that the tryptic products of the latter cover GAP^O, as it retained full GAP activity (Figure 1B). Moreover, $BAR\text{-}PH\text{-}GAP^O$ cleavage at 30 min was significantly reduced in comparison with $BAR\text{-}PH^O$, which is entirely trypsinized after 15 min (Supplementary Figure S2B). It appears that the presence of GAP^O in $BAR\text{-}PH\text{-}GAP^O$ protects $BAR\text{-}PH^O$ against trypsin digestion. These results provided the first evidence for a domain–domain interaction of OPHN1, indicating that $BAR\text{-}PH\text{-}GAP^O$ exists in a 'closed' autoinhibited state, and tryptic degradation of $BAR\text{-}PH^O$ allowed the release of the autoinhibitory function.

Next we examined whether the autoinhibited state of OPHN1 can be reconstituted *in vitro* using isolated GAP^O and $BAR\text{-}PH^O$. As shown in Figure 1(D), increasing amounts of $BAR\text{-}PH^O$ led to an incremental inhibition of the GAP-stimulated tamraGTP hydrolysis of Cdc42, which is indicative of the direct association between $BAR\text{-}PH^O$ and GAP^O. An IC_{50} (concentration causing 50 % inhibition of GAP activity) value of 11.8 μM was obtained by hyperbolic fitting of individual observed rate constants (k_{obs}) plotted against the $BAR\text{-}PH^O$ concentrations used. Addition of $BAR\text{-}PH^O$ to GAP^O was able to reduce the GAP activity to a level similar to that of $BAR\text{-}PH\text{-}GAP^O$. To validate this result, the equilibrium dissociation constant (K_d) for the interaction between GAP^O and $BAR\text{-}PH^O$ was determined to be 21.3 μM using ITC (Figure 1E; see also Supplementary Figure S3, at http://www.

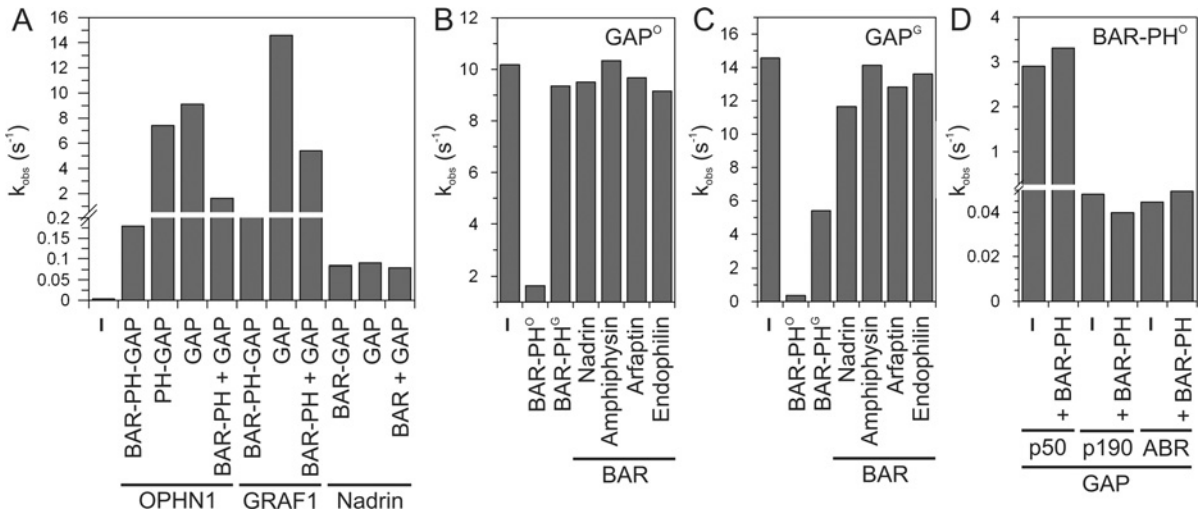

Figure 2 Inhibitory activity of BAR domains of the OPHN1/GRAF1 BAR/GAP domains

(**A**) Comparison of GAP activities of different proteins from OPHN1, GRAF1 and nadrin. All catalytic fragments of GAPs have been used at 1 μM concentration and inhibitory BAR^N or BAR-PH$^{O/G}$ domains were applied at 50 μM concentration. (**B**) Inhibitory effect of different BAR domain-containing proteins on GAP^O activity. Concentrations have been used as in (**A**). (**C**) Inhibitory effect of different BAR domain-containing proteins on GAP^G activity. Similar concentrations were used as in (**A**) and (**B**). (**D**) Influence of BAR-PH^O on the activity of different GAP catalytic fragments from p50RhoGAP, p190RhoGAP and ABR. All k_{obs} values represent the average of 5–7 different experiments.

BiochemJ.org/bj/417/bj4170371add.htm). The difference between the K_i and K_d values is most probably based on different temperature conditions (see the Materials and methods section). In addition, a 1:1 stoichiometry was derived from the ITC curve for this intermolecular interaction. On the basis of the recent dimeric BAR and BAR-PH structures ([11,18] and references therein), this result can also be interpreted as a 2:2 complex between BAR-PH and GAP.

In summary, our results showed that OPHN1 exists in an autoinhibited state that is characterized by physical interactions between the BAR-PH and the GAP domains. As a consequence, BAR-PH competitively interferes with complex formation between the GAP domain and cognate Rho protein (Cdc42 · GTP in this case), thereby inhibiting the stimulation of the GTP-hydrolysis reaction. This result adds a novel function to the rapidly expanding knowledge of the BAR protein superfamily.

A conserved autoregulatory mechanism for the GRAF subfamily

GRAF proteins (GRAF1, 2 and 3) are BAR domain-containing RhoGAPs from the same family as OPHN1 (Figure 1A). This is why we have also examined the impact of the corresponding GRAF1 BAR-PH (BAR-PH^G) on the activity of GAP^G. Similarly to OPHN1, BAR-PH-GAP^G also resided in an autoinhibited state. Its activity in stimulating GTP hydrolysis of Cdc42 was far below that of GAP^G alone (Figure 2A). In addition, GAP^G activity was also inhibited *in vitro* by rapidly mixing BAR-PH^G and GAP^G in stopped-flow experiments, although higher concentrations were required to achieve an equal inhibition compared with OPHN1 (results not shown). The observed lower efficiency of GRAF1 inhibition is probably caused by a lower binding affinity of BAR-PH^G for its GAP^G domain, as a quantitative analysis of BAR-PH^G-mediated GAP^G inhibition revealed an IC_{50} value of 43.6 μM. Titration of BAR-PH^G with GAP^G in ITC yielded an approximate K_d value of 111 μM for this bimolecular interaction (Supplementary Figure S3). Our data showed that OPHN1 and GRAF1 adopted an autoinhibited state that is provided by an interaction between the BAR-PH and the GAP domains. This novel mechanism is general for proteins in this subfamily.

In contrast with the BAR-PH-containing GRAF subfamily members, stopped-flow experiments with nadrin BAR-GAP (BAR-GAP^N) revealed approximately the same activity as measured for the isolated GAP^N domain (Figure 2A). Even a 50-fold molar excess of the isolated BAR^N above the GAP^N concentration did not inhibit the GAP-stimulated tamraGTP hydrolysis at all (Figure 2A). These results led us to the conclusion that BAR^N is unable to inactivate GAP^N and thus may not be directly involved in the regulation of nadrin GAP activity. It is also important to note that nadrin is not as efficient as OPHN1 and GRAF (Figure 2A). Comparison with other members of the Rho family revealed that nadrin exhibited the highest GAP activity for Rac1 (results not shown). Using tamraGTP hydrolysis by Rac1 and GAP^N, BAR^N and BAR-GAP^N (Supplementary Figure S4, at http://www.BiochemJ.org/bj/417/bj4170371add.htm), we could show that nadrin GAP activity is not regulated by the N-terminal BAR domain.

There are two possible explanations for this finding. The first and perhaps most probable explanation for the lack of BAR-mediated regulation of nadrin GAP activity may rely on the nature of the BAR^N structure that is different from the classical BAR domains [10,11,17]. Nadrin belongs to a subclass of BAR domains that contain an N-terminal amphipathic helix (called N-BAR), which works as functional unit to promote dimerization and membrane curvature generation [18,19]. It is also necessary to note that nadrin lacks the central PH domain in contrast with GAPs of the GRAF subfamily (Figure 1A). Although the PH-GAP^O showed no significant change in GAP-stimulated hydrolysis (Figures 1B and 2A), the possibility that the PH domain might be important for autoinhibition should not be excluded. The isolated BAR domain of OPHN1 or GRAF1 could shed light on this subject, but we have not been successful so far in preparing a stable BAR domain of OPHN1 or GRAF1.

BAR-mediated GAP inhibition is highly specific

After we identified the BAR domain of OPHN1 and GRAF1 as an autoinhibitory element that potently suppresses the GAP activity of these regulators, we next examined whether such an

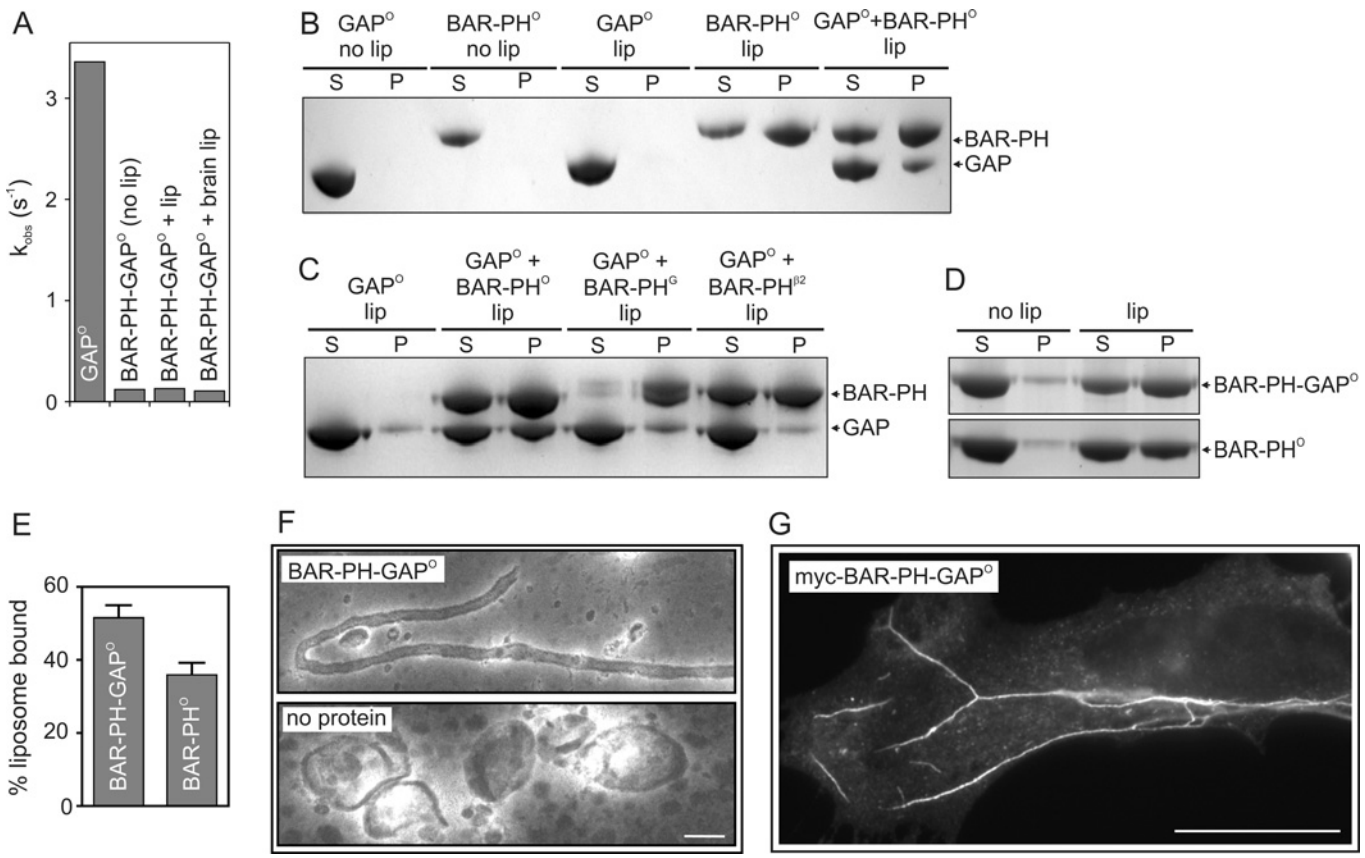

Figure 3 Simultaneous operation of membrane tubulation and GAP inhibitory functions

(**A**) Effect of lipid membranes on the autoinhibited state of OPHN1. Comparison of GAP activities of BAR-PH-GAP^0 measured in the absence (no lip) and presence (lip) of phosphatidylinositol 4,5-bisphosphate-enriched liposomes and liposomes generated from total brain lipids (brain lip). Values represent the average of at least five different experiments. (**B**–**D**) Coomassie Blue-stained gels of liposome co-sedimentation assays. Indicated proteins (4 μM) were incubated with liposomes (lip) or without liposomes (no lip) for 20 min and centrifuged, and the supernatant (S) and pellet (P) fractions were analysed by SDS/PAGE. Note that BAR-PH^0 (**B**–**C**), but not BAR-PH^G or β2-centaurin BAR-PH (BARPH$^{\beta 2}$) (**C**), specifically recruits the GAP^0 to the liposomes and that GAP^0 does not inhibit membrane binding of the BAR-PH^0 domains *in trans* (**C**) or *cis* (**D**). (**E**) Quantification of experiments as shown in (**D**) using densitometry ($n = 4$). The histogram shows percent of bound protein, calculated as the amount of protein in the pellet divided by the sum of protein in the supernatant and pellet. Error bars represent S.D. (**F**) Membrane tubulation by BAR-PH-GAP^0. Electron micrograph of liposomes incubated in the presence (upper panel) or absence (lower panel) of protein. Note the lipid tubule created from spherical liposomes by the addition of BAR-PH-GAP^0 (15 μM); scale bar = 0.1 μm. (**G**) Confocal micrograph of HeLa cell expressing myc-tagged OPHN1 BAR-PH-GAP. Scale bar = 10 μm.

inhibitory activity is interchangeable among other BAR domains. To address this question, we determined the k_{obs} values of the GAP-stimulated tamraGTP hydrolysis reaction of Cdc42 in the presence of various BAR domains, including OPHN1, GRAF1, nadrin, amphiphysin, arfaptin-2 and endophilin-A1. As shown in Figure 2(B), GAP^O activity is exclusively inhibited by a 50-fold molar excess of the BAR-PH^O. Even the closely homologous BAR-PH^G was not able to reduce the GAP^O activity. Similarly, BAR domains were not able to bind to GAP^G and inhibit its stimulatory GTPase activity, except for BAR-PH^G and BAR-PH^O (Figure 2C) that, most remarkably, appeared to be a more potent inhibitor than BAR-PH^G itself. This result is consistent with higher binding affinity of BAR-PH^O for the GAP^O domain and indicates that OPHN1 and GRAF1 autoregulation operates through a similar molecular mechanism.

Another issue that we addressed was whether the BAR-binding site of the GAP domain is specific among different GAPs for the Rho family. Therefore we measured stimulation of Cdc42-mediated tamraGTP hydrolysis by the catalytic domain of p50GAP, p190GAP and ABR in the absence and in the presence of BAR-PH^O. Figure 2(D) shows that the activity of these GAPs is not affected at all and emphasizes that the BAR domain-mediated autoinhibition of OPHN1 is highly specific. These results also indicate that the BAR–GAP interaction utilizes signatures, which are selective for the GRAF subfamily of GAPs.

Simultaneous dual-site of action of the BAR domain

The next step of the investigation was to examine the impact of lipid membrane on the autoinhibited state of these GAPs. We repeated the real-time fluorescence measurements of the tamraGTP hydrolysis reaction of Cdc42 in the presence of liposomes and lipid membranes of different size. Representative results are shown in Figure 3(A). It is evident that the activity of the autoinhibited BAR-PH-GAP was not altered at all. This prompted us to address the question of whether the autoinhibited state of OPHN1 influences membrane-binding and -tubulating abilities of the BAR domain, which was reported previously [10]. Using a liposome co-sedimentation assay, we found that the GAP^O domain could be co-sedimented with the membranes if BAR-PH^O was present, again showing the *trans*-interaction. The robust binding of BAR-PH^O to liposomes both in the absence and presence of GAP^O (Figures 3B and 3C) suggests that the *trans*-interaction between these domains did not inhibit the membrane-binding ability of the BAR-PH^O. GAP^O was also able to

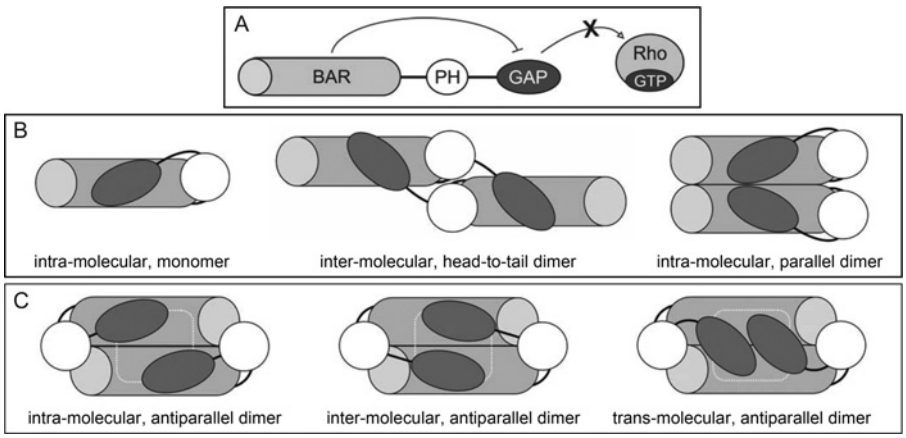

Figure 4 Conceptual models for BAR–GAP interaction of the GRAF protein family

Possible and probable binding modes of how the BAR domain of GRAF1 or OPHN1 binds to the GAP domain and inhibits its activity towards Rho protein (**A**) is illustrated schematically (**B**, **C**); see the text for details. The dotted square indicates the membrane-binding region at the reverse site.

co-sediment with BAR-PH^{G}, but to a much lower extent compared with BAR-PH^{O} (Figure 3C). This is in agreement with results described above and again indicates that BAR-PH^{G} exhibits a much weaker GAP-inhibitory effect. Similar results were obtained using β2-centaurin BAR-PH as a control. To investigate further whether membrane binding is influenced in the context of autoinhibited protein, we compared the liposome-binding activity of BAR-PH^{O} and BAR-PH-GAP^{O}. As shown in Figure 3(D) and 3(E), instead of an inhibitory action, the presence of the GAP domain yielded an even higher amount of BAR-PH-GAP^{O} bound to the liposomes compared with that of BAR-PH^{O}. This result shows that the presence of the GAP domain does not perturb membrane binding but may facilitate the interaction of the BAR domain with the lipid membrane. Moreover, BAR-PH-GAP^{O} was also able to generate lipid tubules when incubated together with liposomes (Figure 3F) and to localize to membrane tubules when overexpressed in cells (Figure 3G). These data show that BAR-PH^{O} domain was unaffected in its membrane-binding and -tubulating activity, regardless of its interaction with the C-terminal GAP domain. The BAR domain represents a dual-site system, which simultaneously interacts with the GAP domain and the lipid vesicles. In addition, interaction between the GAP and BAR domains seems to potentiate the BAR-mediated ability of membrane binding and remodelling.

CONCLUSIONS

BAR domains recently took centre stage in their area because of their function as membrane curvature-sensing and -stabilizing protein modules [11,17,19–21]. Our work has uncovered a novel autoinhibitory function for BAR domain-containing RhoGAPs of the GRAF subfamily. This is a stringent control mechanism to suppress efficiently and locally the GAP activity of GRAF proteins. Similar autoregulatory modes have been implicated for other RhoGAPs, including DLC1 (deleted in liver cancer 1) and p50RhoGAP. Whereas the mechanism of the autoinhibitory domain remained unknown for DLC1 [22], an N-terminal Sec14/BCH (Bnip2 and Cdc42 GAP homology) domain has been shown to be essential for the regulation of the GAP activity of p50RhoGAP [23].

The present study has provided the first evidence for dual-site action of a BAR domain that exhibits simultaneously membrane- and protein-binding capabilities with the latter being an inhibitory function on GAPs of the GRAF subfamily. One of the most crucial questions of how the BAR domain of GRAF1 or OPHN1 binds to the GAP domain and inhibits its activity (Figure 4A) still remains a subject for further research. Biochemical data from the present and other studies along with the structural determination of BAR domain-containing proteins have shown that BAR-like domains form elongated, banana-shaped, α-helical homodimers in an antiparallel orientation [11]. Therefore intramolecular interactions of a monomer or of a parallel dimer and even an intermolecular head-to-tail dimer (Figure 4B) can be excluded. The fact that the BAR domain directly inhibits the GAP-stimulated GTPase reaction of Rho proteins supports the notion that it may bind to the Rho-binding region of the GAP domain and thus mask its catalytic residues, including the arginine finger [24]. Moreover, it has been reported that BAR dimers bind membranes electrostatically through their positively charged concave surface [10]. But simultaneous interactions of the BAR domain of OPHN1 with the membrane and the GAP domain suggests that the GAP domain may bind to the convex surface of a banana-shaped homodimer of the BAR domain. Accordingly, an intramolecular interaction between the domains of one protein or an intermolecular interaction between the domains across the two proteins or a transmolecular interaction of one GAP domain and two BAR domains are three alternative symmetrical binding modes of how these domains approach one another (Figure 4C). The latter model of an autoinhibited state supports the idea of GAP-induced stabilization of the dimerization state of the BAR domain.

The role of the BAR domain, in conjunction with other domains including the PH domain, is to localize the dormant autoinhibited GAP protein to the membrane. As we know now, this is not sufficient to release the GAP domain and induce GAP activity. Thus an obvious next step of the investigation is to understand the release mechanism of the autoinhibited state of these GAPs. Additional interactions are likely to displace the autoinhibiting BAR domain and to release the GAP domain for specific binding to an adjacent Rho protein at the membrane. This will drive the progression of cellular processes in which GAPs of the GRAF subfamily are required, including dendritic spine morphogenesis and axon growth [25]. Identification of functional modules that inhibit the GAP activity, however, was an important first step towards elucidating underlying cellular mechanism of these critical regulators.

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REFERENCES

1 Dvorsky, R. and Ahmadian, M. R. (2004) Always look on the bright site of Rho: structural implications for a conserved intermolecular interface. EMBO Rep. **5**, 1130–1136
2 Etienne-Manneville, S. and Hall, A. (2002) Rho GTPases in cell biology. Nature **420**, 629–635
3 Eberth, A., Dvorsky, R., Becker, C. F. W., Beste, A., Goody, R. S. and Ahmadian, M. R. (2005) Monitoring the real-time kinetics of the hydrolysis reaction of guanine nucleotide-binding proteins. Biol. Chem. **386**, 1105–1114
4 Scheffzek, K. and Ahmadian, M. R. (2005) GTPase activating proteins: structural and functional insights 18 years after discovery. Cell. Mol. Life Sci. **62**, 3014–3038
5 Billuart, P., Bienvenu, T., Ronce, N., des Portes, V., Vinet, M. C., Zemni, R., Crollius, H. R., Carrie, A., Fauchereau, F., Cherry, M. et al. (1998) Oligophrenin-1 encodes a RhoGAP protein involved in X-linked mental retardation. Nature **392**, 923–926
6 Lundmark, R., Doherty, G. J., Howes, M. T., Cortese, K., Vallis, Y., Parton, R. G. and McMahon, H. T. (2008) The GTPase activating protein GRAF1 regulates the CLIC/GEEC endocytic pathway, Curr. Biol. **18**, 1102–1108
7 Borkhardt, A., Bojesen, S., Haas, O. A., Fuchs, U., Bartelheimer, D., Loncarevic, I. F., Bohle, R. M., Harbott, J., Repp, R., Jaeger, U. et al. (2000) The human GRAF gene is fused to MLL in a unique t(5;11)(q31;q23) and both alleles are disrupted in three cases of myelodysplastic syndrome/acute myeloid leukemia with a deletion 5q. Proc. Natl. Acad. Sci. U.S.A. **97**, 9168–9173
8 Ren, X. R., Du, Q. S., Huang, Y. Z., Ao, S. Z., Mei, L. and Xiong, W. C. (2001) Regulation of CDC42 GTPase by proline-rich tyrosine kinase 2 interacting with PSGAP, a novel pleckstrin homology and Src homology 3 domain containing RhoGAP protein. J. Cell Biol. **152**, 971–983
9 Harada, A., Furuta, B., Takeuchi, K., Itakura, M., Takahashi, M. and Umeda, M. (2000) Nadrin, a novel neuron-specific GTPase-activating protein involved in regulated exocytosis. J. Biol. Chem. **275**, 36885–36891
10 Peter, B. J., Kent, H. M., Mills, I. G., Vallis, Y., Butler, P. J. G., Evans, P. R. and McMahon, H. T. (2004) BAR domains as sensors of membrane curvature: the amphiphysin BAR structure. Science **303**, 495–499
11 Gallop, J. L. and McMahon, H. T. (2005) BAR domains and membrane curvature: bringing your curves to the BAR. Biochem. Soc. Symp. **72**, 223–231
12 Frost, A., De Camilli, P. and Unger, V. M. (2007) F-BAR proteins join the BAR family fold. Structure **15**, 751–753
13 Shin, O. H. and Exton, J. H. (2005) Assays and properties of arfaptin 2 binding to Rac1 and ADP-ribosylation factors (ARFs). Methods Enzymol. **404**, 359–367
14 Fauchereau, F., Herbrand, U., Chafey, P., Eberth, A., Koulakoff, A., Vinet, M. C., Ahmadian, M. R., Chelly, J. and Billuart, P. (2003) The RhoGAP activity of OPHN1, a new F-actin-binding protein, is negatively controlled by its amino-terminal domain. Mol. Cell. Neurosci. **23**, 574–586
15 Hemsath, L., Dvorsky, R., Fiegen, D., Carlier, M. F. and Ahmadian, M. R. (2005) An electrostatic steering mechanism of Cdc42 recognition by Wiskott-Aldrich syndrome proteins. Mol. Cell **20**, 313–324
16 Hemsath, L. and Ahmadian, M. R. (2005) Fluorescence approaches for monitoring interactions of Rho GTPases with nucleotides, regulators, and effectors. Methods **37**, 173–182
17 Dawson, J. C., Legg, J. A. and Machesky, L. M. (2006) BAR domain proteins: a role in tubulation, scission and actin assembly in clathrin-mediated endocytosis. Trends Cell Biol. **16**, 493–498
18 Richnau, N., Fransson, A., Farsad, K. and Aspenstrom, P. (2004) RICH-1 has a BIN/Amphiphysin/Rvsp domain responsible for binding to membrane lipids and tubulation of liposomes. Biochem. Biophys. Res. Commun. **320**, 1034–1042
19 Gallop, J. L., Jao, C. C., Kent, H. M., Butler, P. J. G., Evans, P. R., Langen, R. and McMahon, T. (2006) Mechanism of endophilin N-BAR domain-mediated membrane curvature. EMBO J. **25**, 2898–2910
20 Zimmerberg, J. and McLaughlin, S. (2004) Membrane curvature: how BAR domains bend bilayers. Curr. Biol. **14**, R250–R252
21 Habermann, B. (2004) The BAR-domain family of proteins: a case of bending and binding? EMBO Rep. **5**, 250–255
22 Healy, K. D., Hodgson, L., Kim, T. Y., Shutes, A., Maddileti, S., Juliano, R. L., Hahn, K. M., Harden, T. K., Bang, Y. J. and Der, C. J. (2008) DLC-1 suppresses non-small cell lung cancer growth and invasion by RhoGAP-dependent and independent mechanisms. Mol. Carcinog. **47**, 326–337
23 Moskwa, P., Paclet, M. N., Dagher, M. C. and Ligeti, E. (2005) Autoinhibition of p50 Rho GTPase-activating protein (GAP) is released by prenylated small GTPases. J. Biol. Chem. **280**, 6716–6720
24 Longenecker, K. L., Zhang, B. L., Derewenda, U., Sheffield, P. J., Dauter, Z., Parsons, J. T., Zheng, Y. and Derewenda, Z. S. (2000) Structure of the BH domain from graf and its implications for Rho GTPase recognition. J. Biol. Chem. **275**, 38605–38610
25 Govek, E. E., Newey, S. E., Akerman, C. J., Cross, J. R., Van der Veken, L. and Van Aelst, L. (2004) The X-linked mental retardation protein oligophrenin-1 is required for dendritic spine morphogenesis. Nat. Neurosci. **7**, 364–372

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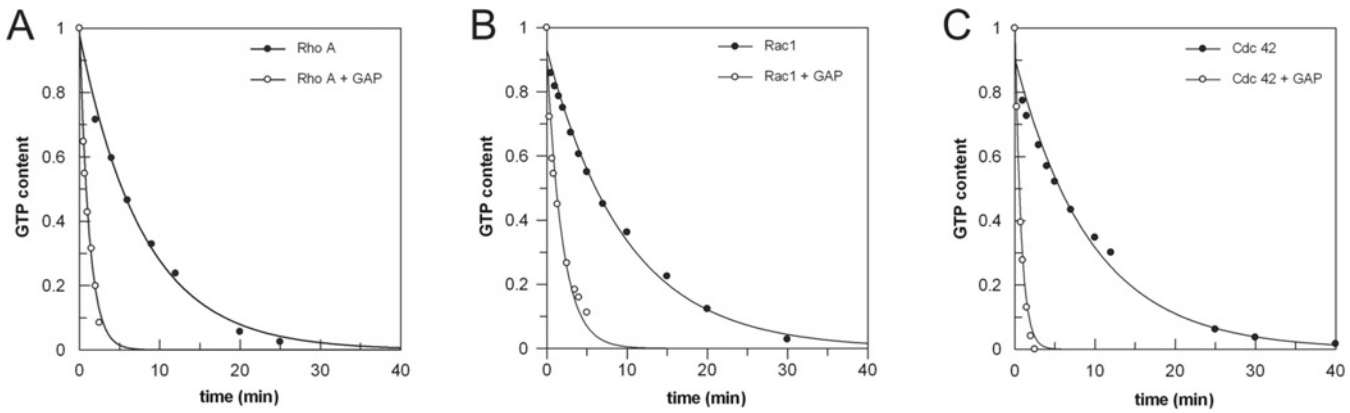
online data

SUPPLEMENTARY ONLINE DATA

A BAR domain-mediated autoinhibitory mechanism for RhoGAPs of the GRAF family

Alexander EBERTH*[1], Richard LUNDMARK†[2], Lothar GREMER*‡, Radovan DVORSKY*, Katja T. KOESSMEIER*, Harvey T. McMAHON†[2] and Mohammad Reza AHMADIAN*[3]

*Institute of Biochemistry and Molecular Biology II, Heinrich Heine University Medical Center, Universitätsstrasse 1, 40225 Düsseldorf, Germany, †Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K., and ‡Max-Planck Institute of Molecular Physiology, Department Structural Biology, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany



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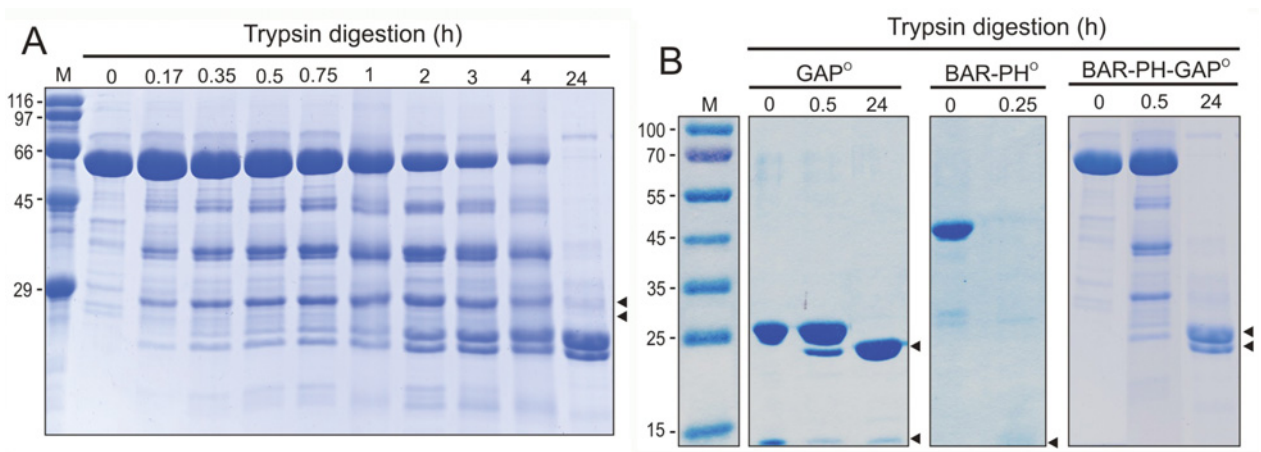

Figure S2 Proteolytic cleavage of the OPHN1 protein fragments

(**A**) Time course of trypsin treatment of the autoinhibited BAR-PH-GAP^0 protein. Purified BAR-PH-GAP^0 was mixed with trypsin at a protease/substrate ratio of 1:4000 at 25 °C. Aliquots from the proteolysis reaction mixture were withdrawn at the indicated time points and analysed by SDS/PAGE after termination of the reaction with 50 μg/ml leupeptin. (**B**) The effects of proteolytic digestion by trypsin on various OPHN1 proteins. Partial cleavage of GAP^0 (24.6 kDa) for 24 h resulted in a trypsin-resistant 22 kDa fragment, whereas BAR-PH^0 (43.2 kDa) is completely digested after 15 min. Trypsin treatment of BAR-PH-GAP^0 (66.8 kDa) led to the fragments of 24 and 22 kDa that correspond to the size of the GAP domain and its tryptic product. These proteins exhibit a similar GAP activity as determined for the GAP^0 and PH-GAP^0 fragments (Figure 1B, main text). Arrowheads highlight tryptic products of the GAP, BAR-PH and BAR-PH-GAP proteins.

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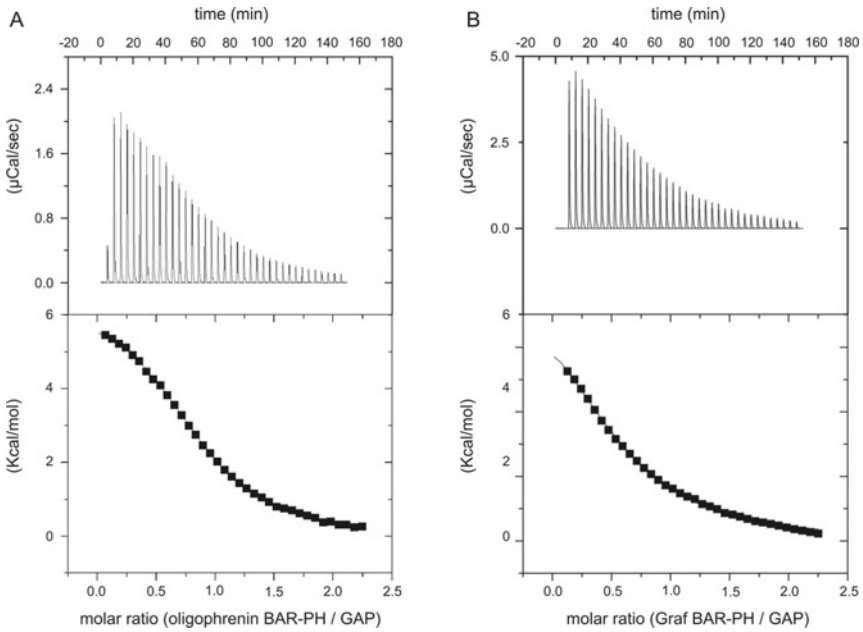

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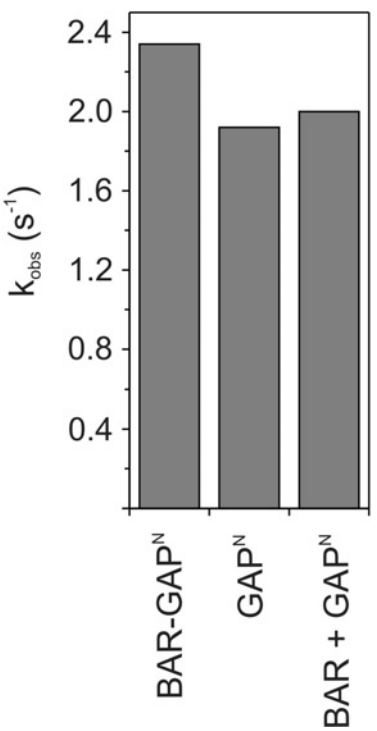

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REFERENCES

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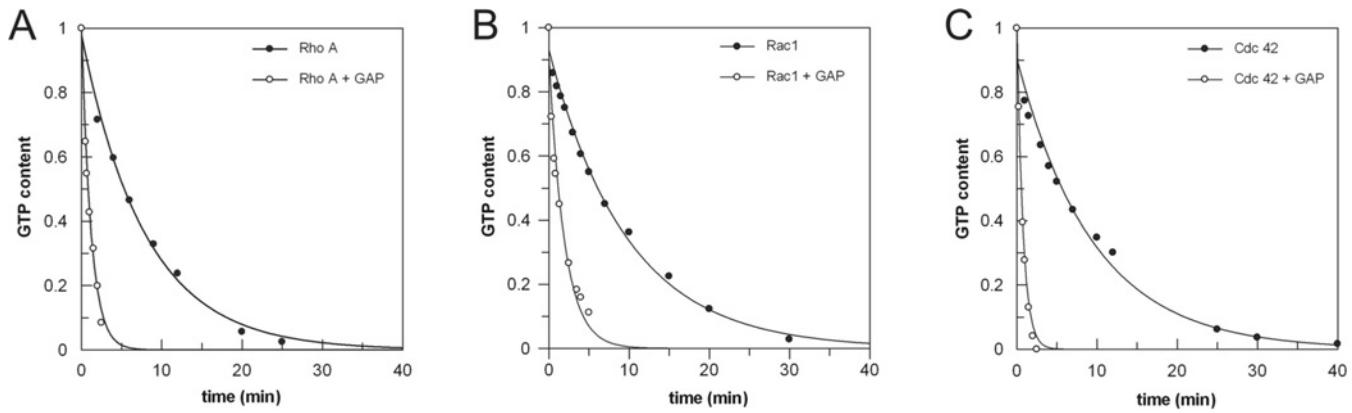

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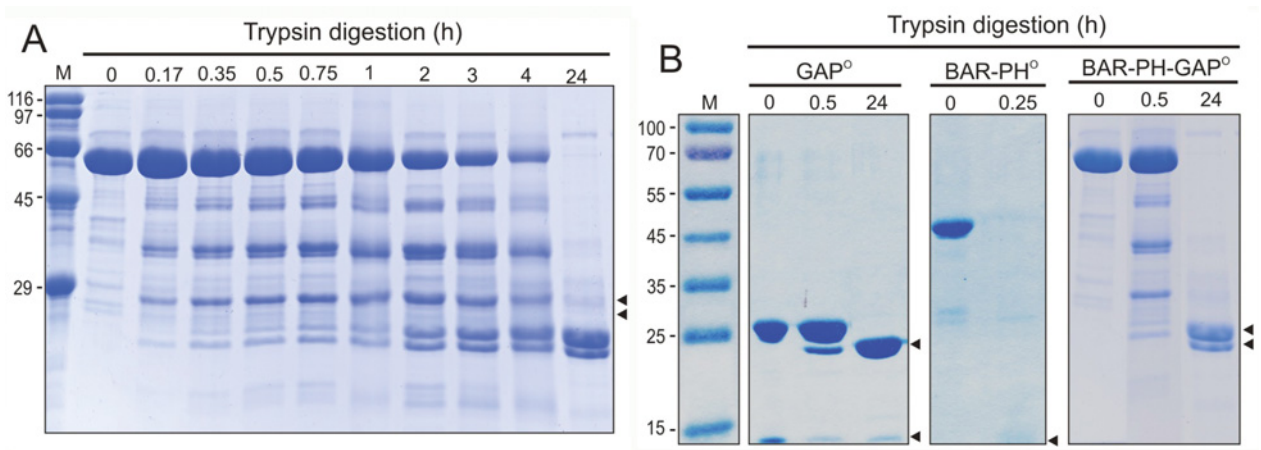

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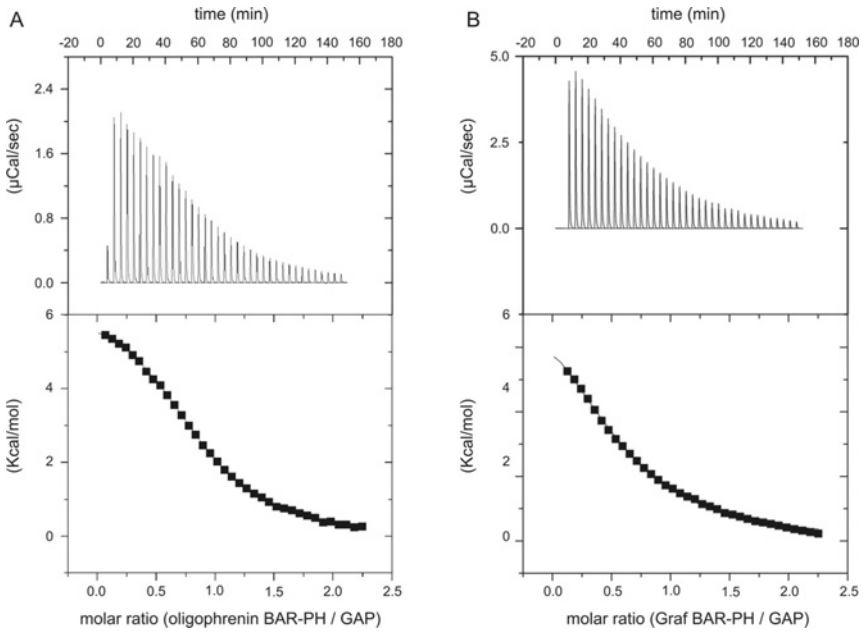

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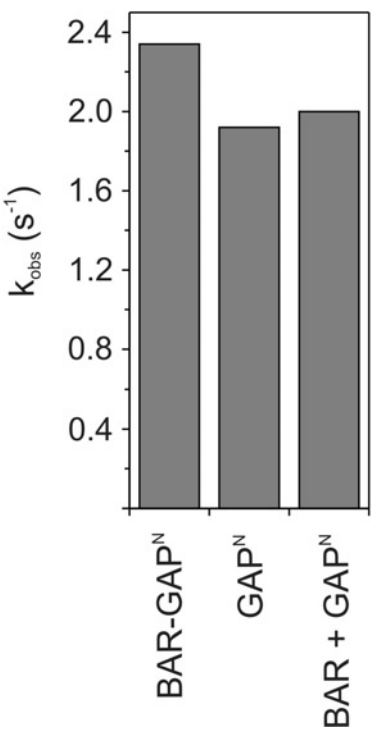

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