Concerted Regulation of Cell Dynamics by BNIP-2 and Cdc42GAP Homology/Sec14p-like, Proline-rich, and GTPase-activating Protein Domains of a Novel Rho GTPase-activating Protein, BPGAP1*

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RhoA, Cdc42, and Rac1 are small GTPases that regulate cytoskeletal reorganization leading to changes in cell morphology and cell motility. Their signaling pathways are activated by guanine nucleotide exchange factors and inactivated by GTPase-activating proteins (GAPs). We have identified a novel RhoGAP, BPGAP1 (for BNIP-2 and Cdc42GAP Homology (BCH) domain-containing, Proline-rich and Cdc42GAP-like protein subtype-1), that is ubiquitously expressed and shares 54% sequence identity to Cdc42GAP/p50RhoGAP. BPGAP1 selectively enhanced RhoA GTPase activity in vivo although it also interacted strongly with Cdc42 and Rac1. “Pull-down” and co-immunoprecipitation studies indicated that it formed homophilic or heterophilic complexes with other BCH domain-containing proteins. Fluorescence studies of epitope-tagged BPGAP1 revealed that it induced pseudopodia and increased migration of MCF7 cells. Formation of pseudopodia required its BCH and GAP domains but not the proline-rich region, and was differentially inhibited by coexpression of the constitutively active mutant of RhoA, or dominant negative mutants of Cdc42 and Rac1. However, the mutant without the proline-rich region failed to confer any increase in cell migration despite the induction of pseudopodia. Our findings provide evidence that cell morphology changes and migration are coordinated via multiple domains in BPGAP1 and present a novel mode of regulation for cell dynamics by a RhoGAP protein.

Cells undergo dynamic changes as part of their adaptation and response to stimuli. These include their abilities to proliferate, differentiate, or execute death. Many of these processes are controlled by a series of signaling events relayed via a cascade of molecular interaction that are normally associated with the enzymatic or structural modifications of target proteins. Furthermore, there exist various checkpoints that serve to fine-tune the amplitude, duration, as well as the integration of such circuity response.

One of the relatively well characterized signaling circuits in eukaryotic system is the Ras small GTP-binding protein (GTPase) superfamily (1–3) that binds and slowly hydrolyzes GTP to GDP, which is still bound to the proteins. The GDP-bound form assumes an active conformation that allows interaction with downstream effectors, thus the “on-switch,” whereas its conversion to the GDP-bound form keeps the proteins in an “off-switch” mode and renders the GTPase inactive. The balance of these two forms determines the final execution of the pathway. This is regulated by two other important classes of proteins, one that helps enhance its GTPase activity, termed GTPase-activating proteins (GAPs)1 and the other, termed guanine nucleotide exchange factors (GEFs) that activate the protein by catalyzing its exchange of GDP for GTP.

Many members of the small GTPases have already been identified, and they can further be subdivided into various families or subfamilies according to the similarities in their primary sequences. Members from different families exhibit diverse functions ranging from the control of intracellular trafficking to cytoskeletal rearrangements and cell cycle progression. The degree of specificity is further extended to even closely related members within the same families. For example, in the Rho family, the Cdc42 plays an important role in the formation of filopodia, whereas RhoA and Rac1 activation results in the formation of stress fibers and membrane ruffles respectively (4). In addition, there is a hierarchy of network in certain cell types where activation of one member can affect the activity of another. For example, activation of Cdc42 leads to filopodia formation, which could in turn activate Rac1 (5, 6), whereas Rac1 activation leads to inactivation of RhoA in NIH3T3 resulting in the epithelioid phenotype (7–9). In contrast, in Swiss 3T3 fibroblasts, Rac1 activates RhoA instead (10). With an increasing number of known GTPases, there remain key questions as to how each one of them can be regulated by their GEFs, GAPs, or other regulators in vivo.

The human genome is predicted to encode at least 50 members of the GAP family (11, 12). Current data show that various GAP domains exhibit overlapping substrate specificity both in vitro and in vivo but all involve a common mechanism of action by utilizing an “arginine-finger” motif in trans to stabilize the transition state of GTP hydrolysis (13, 14). For example, the p50RhoGAP (also known as Cdc42GAP) (15, 16) and p122RhoGAP (17) bind and inactivate mainly Cdc42 and RhoA, respectively. In comparison, p200RhoGAP targets RhoA and Rac1 but not Cdc42 (18) while p115RhoGAP confines its action mainly to RhoA (19). Therefore, it appears that there is no specific GAP for a single GTPase. Instead, there exists a GAP

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* This work was funded from a grant from the Academic Research Fund, National University of Singapore. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: GAPs, GTPase-activating proteins; EST, expressed sequence tag; HA, hemagglutinin; ANOVA, analysis of variance; LD, longest diameter; SD, shortest diameter; IP, immunoprecipitation; IB, immunoblotting; FL, full-length; GFP, green fluorescent protein; GST, glutathione S-transferase; WCL, whole cell lysate.
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that recognizes more than one GTPase, and a single GTPase can be a target of multiple GAPs. The molecular basis for such distinctive or overlapping recognition profile remains to be understood. Furthermore, most of these GAPs possess multiple signaling modules that could couple their activities to other signaling pathways. This could have far reaching consequences for the regulation of Rho and other small GTPase signals, and remains to be seen how, where, and when any subsets or combinations of these cellular counterparts will co-exist and exert their effects.

In order to understand the specificity versus redundancy nature of the RhoGAPs as well as the roles of their various signaling modules, we have set out to study novel proteins that harbor the GAP domain together with other protein domains. Bioinformatic searches through the human genome public databases revealed a striking number of sequences that encode putative GAP proteins and with various arrays of domain organizations. One of the family proteins that we are interested in has the organization that is similar to that of the Cdc42GAP, yet exhibiting diversified sequences in other regions. Here we report the cloning and functional characterization for such a member in this family that harbors (from the proximal N terminus) a BNIP-2 and Cdc42GAP Homology (BCH)/Sec14p-like domain that we first described (20–23), a proline-rich sequence, and a functional GAP domain. We showed that BPGAP1 differentially modulates RhoA, Cdc42, and Rac1 signaling pathway by a mechanism that required cooperation between the BCH and GAP domain. When expressed in non-metastatic human breast epithelial cancer cell lines MCF7 cells, BPGAP1 induced cell protrusions/pseudopodia that required its GAP activity as well as the BCH domain, but not the proline-rich sequence. However, the proline-rich region was required for ensuring cell migration following the morphological changes induced by both GAP and BCH domains. These results indicate the unique interplay by different domains of BPGAP1 in exerting cell dynamics and confirm that changes in cell morphology is a prerequisite but not necessarily the only determinant for cell migration, it requires the input of other factor(s) as well. Our findings also emphasize the need to address functions of distinct protein domains in various RhoGAP families in order to have a better understanding of their physiological functions and regulation.

MATERIALS AND METHODS

Bioinformatics—To identify novel proteins containing GAP domains, the peptide sequence of the RhoGAP domain of p50RhoGAP/Cdc42GAP (GenBank™ accession number: Q07960; residues: 260–289) and the peptide sequence of the RhoGAP domain of p50RhoGAP/Cdc42GAP (GenBank™ accession number: Q07960; residues: 260–439) was used as query sequence in the “position-specific iterative BLAST” against the current non-redundant sequence as well as human and mouse EST databases (www.ncbi.nlm.nih.gov/). Progress of the identification was described in the text. Multiple sequence alignments were generated using Vector NTI suite (InforMax, Inc.).

RT-PCR Cloning of BPGAP1 Isolomorph and Plasmid Constructions—To obtain the full-length cDNA of BPGAP1, total RNA was isolated from MCF7 cells using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. 5 μg of this RNA was subjected to the first-strand cDNA synthesis with Expand Reverse Transcriptase Kit (Roche Applied Science) primed with oligo(dT) (Operon) for 60 min at 42 °C in a total volume of 20 μl. 0.5 μg of this cDNA was then amplified by the high fidelity, long-template PCR by the high fidelity, long-template using specific primers corresponding to the putative sequence BAA91614. PCR conditions were: initial denaturation 94 °C, 2 min; subsequent cycling (30 cycles) at 94 °C, 10 s; annealing at 50 °C, 30 s; extension at 68 °C, 2 min; and final extension at 68 °C, 7 min. These PCR primers contained HindIII and XhoI restriction sites on the forward and reverse primers, respectively, to facilitate their subsequent cloning. The full-length PCR products were gel-purified (Qiagen) and cloned into a FLAG epitope-tagged or GFP-tagged expression vector, pXJ40 (Dr. E. Manser, Institute of Molecular and Cell Biology, Singapore). Sequence unique to BPGAP1 was obtained (GenBank™ AF544240), and fragments encoding its various domains were generated from the full-length template using specific primers in a standard PCR and then gel-purified for cloning. For each construct, clones were chosen and sequenced entirely in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems). All plasmids were purified using Qiagen miniprep kit for subsequent use in transfection experiments. For generation of deletion mutants, inverse-PCR was carried out to exclude region of interest whereas point mutation R220A was performed by site-directed mutagenesis as previously described (21). Escherichia coli strain DH5α was used as host for the propagation of the clones. Reagents used were of analytical grade, and standard protocols for molecular manipulations and media preparation were as described (24).

Semi-quantitative RT-PCR—To distinguish the mRNA expression level of BPGAP1 and Cdc42GAP in various cells and tissues, RT-PCR using the oligo-dT primers was employed. Total RNA was isolated using the RNeasy kit (Qiagen) from either various cultured cell lines or from various organs obtained from a 2-week-old male mouse and primed for the first-strand cDNA synthesis as described above. Equal amounts of the reverse transcription product were then subjected to PCR amplification for BPGAP1 and Cdc42GAP. The full-length PCR products of BPGAP1 were then subjected to internal amplification using primers that encompass BPGAP1-specific BCH region that contained the unique insertion (see text). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the level of expression. The results were verified in at least two independent experiments with varying numbers of PCR cycles to ensure near-linear amplification.

Cell Culture and Transfection—Human breast cancer MCF7, human embryonic kidney epithelial cells 293T, human stomach cancer lines MKN45 and KMN74 were all grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from HyClone), and maintained at 37 °C in a 5% CO2 atmosphere. Human cervical cancer epithelial HeLa cells were grown in Dulbecco’s modified Eagle’s medium (high glucose), whereas human colon epithelial HT29 and HCT116 were grown in McCoy’s medium (Sigma). Cells at 90% confluence in 100-mm plates or 6-well plates were transfected with 5 or 2 μg of either GFP-tagged plasmids using Lipofectamine 6 transfection reagent, according to the manufacturer’s instructions (Roche Applied Science).

Precipitation/Pull-down Studies and Western Blot Analyses—Control 293T cells or cells transfected with expression plasmids were lysed in 1 ml of lysis buffer (150 mM sodium chloride, 50 mM Tris, pH 7.3, 0.25 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and a mixture of protease inhibitors (Roche Applied Science)). The lysates were directly analyzed, either as whole cell lysates (25 μg) or aliquots (500 μg) used in affinity precipitation/pull-down experiments with various GST fusion proteins (5 μg), as previously described (21). Samples were run in SDS-PAGE gels and analyzed by Western blotting with FLAG monoclonal antibody (Sigma).

Immunofluorescence—Cells were seeded on coverslips in 6-well plates, transfected with various expression constructs for 16–20 h, and then stained for immunofluorescence detection as previously described (25). Fluorescent images were taken with a confocal laser microscopy system (Fluoview, FV300, Olympus). FLAG-tagged proteins were detected with monoclonal anti-FLAG, followed by Texas Red® dye-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch). For cells co-expressing GFP-tagged recombinants and HA-tagged proteins, HA-tagged constructs were detected with polyclonal anti-HA, followed by Texas Red® dye-conjugated goat anti-rabbit IgG. For cells expressing only GFP-tagged recombinants, the morphology of cells was examined using a fluorescent microscope after the transfection for 16–20 h as previously described (23).

Preparation of GST Fusion Proteins—GST fusion proteins were purified using glutathione-agarose beads. In brief, E. coli cells were lysed by sonication in a HEPES buffer, pH 7.5, 150 mM NaCl, 1 mM EDTA, multiple protein inhibitors (Roche Applied Science), 0.1% (w/v) d-mercaptoethanol, and 0.1% (v/v) Triton X-100. Following centrifugation (10,000 rpm, 30 min, 4 °C), the supernatants of lysates were mixed with glutathione-agarose beads (Amersham Biosciences) and incubated at 4 °C for overnight. Beads were washed three times with 10 ml of HEPES buffer. When needed, fusion proteins were eluted with 10 mM glutathione solution in the HEPES buffer. Protein concentrations were measured by using Bradford assay (Bio-Rad). In Vivo GTPase Activity Assay—GTPase activity assays were performed with the Enz-check™ Phosphate Assay kit (E-6646, Molecular Probes) to monitor the rate of phosphate release from GTP hydrolysis catalyzed by recombinant Cdc42, RhoA, or Rac1 (pGEX plasmids of...
these and Cdc42GAP are gifts from Dr. A. Hall, University College London, United Kingdom) in the presence of GST control or GST-BPGAP1 full-length, domains, or its mutant. For these assays, we used a previously described protocol (26) with some modifications. In brief, 0.5 mM of purified GST-BPGAP1 full-length, domains, or mutant proteins (in a volume of 15 μl) was mixed in a cuvette with 10 μl of 0.2 mM GTP, 0.2 ml of 2-amino-6-mercapto-7-methylurine ribonucleoside, 10 μl (1 unit) of purine nucleotide phosphatase, and 0.76 ml of HEPES buffer (pH 7.5). The cuvette was immediately placed in the spectrophotometer to monitor absorbance at 360 nm (A360). 10 μl of 1 mM MgCl2 solution was added to 0.25 mM of eluted GST, GST-Cdc42, GST-RhoA, or GST-Rac1 fusion proteins and incubated for 10 min at room temperature. When the first multiple turnover reached an equilibrium at A360, the second mixture of small GTPase solution was added to initiate the reaction. The reading at A360 was recorded every 10 s.

In Vivo GTPase Activity and Binding Assay—GST-bound Cdc42, Rac1, or RhoA was determined by specific binding to the p21-binding domain of PAK1 (GST-PBD) (27) or rothonin (GST-RBD) (28) (all kindly provided by Dr. Simone Schoenwaelder; Monash University, Australia). In brief, cell lysates expressing HA-tagged wild-type small GTPases (Cdc42, Rac1, or RhoA) with or without FLAG-tagged BPGAP1 were incubated with 5 μg of recombinant GST-PBD or GST-RBD conjugated with glutathione-Sepharose beads for 1 h at 4 °C, washed with buffer (50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1.5 mM magnesium chloride, 5 mM EGTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, a mixture of protease inhibitors and 5 mM sodium orthovanadate) and separated on SDS-PAGE. Bound Cdc42, Rac1, or RhoA was analyzed by Western blotting using anti-HA antibodies (Roche Applied Science). Western blots were also analyzed for the presence of expressed Cdc42, Rac1, RhoA, and BPGAP1 for normalization. For detecting binding of endogenous Rho GTPases, the following antibodies were used: polyclonal anti-Cdc42 (Santa Cruz Biotechnology), polyclonal anti-RhoA, and monoclonal anti-Rac1 (both from Upstate Biotechnology).

Co-immunoprecipitation—293T cells were transfected with expression vectors for FLAG-BPGAP1 full-length alone or together with either HA-BPGAP1, HA-Cdc42GAP, HA-BNIP-2 or HA-GTPases. Lysates were immunoprecipitated (IP) with anti-FLAG M2 beads (Sigma) and the associated proteins separated on SDS-PAGE, and probed with anti-Cdc42, RhoA, Rac1, or HA antibodies to reveal the binding of targets.

Cell Measurement—MCFT cells were transfected with GFP control or GFP-tagged BPGAP1 full-length, NP, or PC domains. After 20 h, the center of cells and perpendicular to each were measured (29). The total cell areas and the length of the cell protrusion (PT) were also measured after image capturing as previously described and analyzed using the Leica IM 1000 software. Measurements were means and S.D. from three separate experiments, each time with at least 30 different cells. Statistical comparison was made using ANOVA (StatsDirect). p values of <0.01 indicate significant difference compared with the vector control.

Cell Migration Assay—The ability of cells to migrate through coated filters was measured with a modified Boyden chamber (24-well Transwell, Corning Costar; 8-μm pore size) as previously described (30). The lower surface of the filters was coated with 5-μg film (Sigma) as a chemotactrant. MCFT cells transiently transfected with GFP vector, GFP-BPGAP1 full-length, different fragments, or mutants were seeded at a density of 3 × 104 cells in 100 μl of RPMI 1640 with 0.2% bovine serum albumin. The lower compartment was added with 600 μl of RPMI 1640 containing 10% fetal bovine serum. After incubation for 1 day at 37 °C in 5% CO2, the cells that did not penetrate the filters were completely wiped off with cotton swabs, and the cells that had migrated to the lower surface of the filter were fixed with methanol and counted. Three independent experiments were performed for each experimental condition. The data were represented as the means of three independent experiments with S.D. indicated. Statistical comparison was made using ANOVA (StatsDirect). p values of <0.01 indicate significant difference compared with the vector control.

RESULTS

Identifying Novel GTPase-activating Proteins—To identify novel GTPase-activating proteins (GAPs) encoded in the human genome and to gain an insight on how they might regulate various cellular processes through their various protein modules, we undertook bioinformatics approach and employed the Conserved Domain Architecture Retrieval Tool (CDART) (www.ncbi.nlm.nih.gov/BLAST/) with the well characterized GAP domain of Cdc42GAP/p50RhoGAP as the query sequence. We have identified in silico many classes of proteins across species that harbor the homologous GAP domain together with other unique signaling protein domains. Some of them include the Pleckstrin homology domain, Src homology-3 domain, Fes/ CIP4 homology domain, Rho guanine nucleotide exchange factor domain, and the p21 Rho binding domain. One of these classes is represented by several putative members that resemble the organization of the Cdc42GAP protein. They are typified by the presence, at the proximal N terminus, of the newly identified BNIIP-2 and Cdc42GAP homology (BCH)/Sec14p-like domain that we first described in the BNIIP-2 family (20–23) and a well conserved GAP domain at its distal C terminus. Present in between these two domains is a proline-rich moiety. Based on the predicted open reading frame from one of the putative sequences deposited, BAA91614, several conserved primers were designed and used in reverse-transcription-based PCR to isolate the full-length cDNA from human MCF7 cells. Interestingly, one unique sequence of cDNA was repeatedly identified (Fig. 1A), which codes for a protein that differs from BAA91614 by lacking 31 amino acids (Fig. 1B, upper line). The protein also differs at the N terminus, from two putative proteins encoded from the same human ARHGAP8 locus (GenBankTM accession numbers: Q9NSG0 and AF195968). Despite using primers specific to those variants, we had not identified the full contigs for such transcripts in all samples examined thus far. Many classes of GAPs have been identified from the human genome and labeled ARHGAP1–12. However, they are not related to each other as each one carries different types and numbers of other associated protein domains. To provide meaningful reference to the specific subclass of GAP with its unique domain organization, we propose to name this family of proteins BPGAPs (for BCH domain-containing, proline-rich, and Cdc42GAP-like proteins) with their notable three-domain organization. We further sought to understand how one novel member we identified here, BPGAP1 (GenBankTM accession number: AF544240), regulates cellular processes via these protein domains. Efforts are underway to isolate the full contigs for other putative isoforms, BPGAP2 (represented by BAA91614), BPGAP3 (AF195968), and the longest subtype, BPGAP4 (Q9NSG0). It is believed that these isoforms could be derived from alternative RNA splicing of the same gene. A mouse homolog with 88% similarity to human BPGAP1 was also identified from the genome data base (encoded by accession NP_087231 or AI430858).

Compared with Cdc42GAP, BPGAP1 displays unique divergence at various regions. Notably, the BPGAP1 has a much shorter sequence at the N terminus but a much longer C tail than Cdc42GAP (Fig. 2A). To understand the degree of similarity or divergence for the BCH and GAP domains, more detailed comparisons were made with similar domains found in other proteins. The BCH domain of BPGAP1 is more closely related to that of Cdc42GAP (84% similarity) (Fig. 2B) while its GAP domain also shares the highest degree of homology with that of Cdc42GAP (Fig. 2C). More importantly, BPGAP1 contains an invariant arginine at residue 232 (Fig. 2C, indicated by an arrow). This residue in other functional GAPs is known as an “arginine finger” and shown to be critical for acting as a catalytic residue in-trans (13, 14, 31). In addition, BPGAP1 possesses several more proline residues in the proline-rich sequence, which is very similar to those identified in RNB6, ena-VASP-like and cdc-related proteins (Fig. 2D). It could comprise more than one putative binding sites for either the Src homology 3 or WW domains (32, 33), the physiologic target(s) of which remains to be identified.
BPGAP1 Forms Homophilic and Heterophilic Complex with BCH-containing Proteins—To gain an insight into the potential cellular function(s) of BPGAP1, we examined the general expression profile of BPGAP1 and the binding repertoire of the various protein domains it harbors. Various human cell lines were maintained in the presence of serum and RNA isolated for the semi-quantitative approach of RT-PCR using gene-specific primers. In strong contrast to Cdc42GAP whose expression was restricted to mostly cells of epithelial origin such as breast cancer MCF7, cervical cancer HeLa, and kidney 293T, the expression of BPGAP1 appeared more ubiquitous (Fig. 3A).

Similarly, BPGAP1 expression can be detected in all mouse tissues/organs tested, including the lung, liver, heart, kidney, brain, and testis (data not shown).

For binding and functional studies, various deletion constructs were made as FLAG epitope-tagged recombinant that would express fragments of proteins that contain either the BCH, proline-rich sequence, or GAP domains. Cells were transfected with these constructs, lysed, and analyzed for their expression by Western blot as described under "Materials and Methods." Fig. 3B shows that full-length and deletion mutants all expressed equally well and were suitable for subsequent GST "pull-down" experiments or cellular studies. We recently showed that BCH domain confers a novel protein interaction domain (20–23). To test whether BPGAP1 could indeed interact with other BCH domain-containing proteins, the lysates were subjected to pull-down with GST recombinants of the full-length Cdc42GAP or BNIP-2, or their respective BCH domains. Bound BPGAP1 fragments were resolved in SDS-PAGE and determined by anti-FLAG Western blot analyses. Fig. 3C
shows that full-length BPGAP1 was a target of Cdc42GAP or its BCH domain, but it failed to interact with BNIP-2 full-length or its BCH domain in vitro. Interestingly, when fragment NP that contains the BCH domain of BPGAP1 was used, BNIP-2 full-length or its BCH domain did not bind to any of the GST recombinants. To further confirm these interactions, the reciprocal pull-downs were performed. Cdc42GAP and BNIP-2 were expressed as FLAG-tagged proteins in the mammalian cells and subjected to GST-BPGAP1 binding. Fig. 3D shows that, as expected, FLAG-Cdc42GAP and BNIP-2 were precipitated by GST-BPGAP1. Likewise, full-length BPGAP1 and its BCH domain could also be precipitated with GST-BPGAP1. Consistent with this is our observation that HA-tagged BPGAP1, Cdc42GAP, and BNIP-2 could also be co-immunoprecipitated with FLAG-tagged BPGAP1 when co-expressed (Fig. 3E). These results confirmed that BPGAP1 could interact with other BCH domain-containing proteins in vitro and in vivo.

**BPGAP1 Targets RhoA, Cdc42, and Rac1 Differentially via Their BCH and GAP Domains**—With the conserved GAP domain that includes the invariant arginine-finger motif (Fig. 2C), BPGAP1 was predicted to bind and confer catalysis toward the GTP hydrolysis of certain Rho GTPases. To examine this, in vitro and in vivo GTPase activity assays were performed. For the in vitro GTPase activity assays, purified recombinant of GST-RhoA, Cdc42, or Rac1 were loaded with GTP and the level of GTP hydrolysis determined in the absence or presence of BPGAP1 full-length or its deletion mutants using the enzyme-coupled spectrophotometric assays (26) as described under “Materials and Methods.” Fig. 4A shows that BPGAP1 augmented GTPase activity of Cdc42 and RhoA in vitro, by 7-fold and 2.5-fold, respectively. In contrast, it showed no significant activation toward Rac1 GTPase activity. These effects were mediated via its GAP domains, as evidenced by the same magnitude of activation from the PC fragment (that carried the GAP domain) and the lack of effect from the NP fragment that carried the BCH domain, but devoid of the GAP domain. Furthermore, such activation was abrogated after introducing a mutation R232A at the invariant arginine residue (see Fig. 2C).

To compare the significance of such differential activity in vitro, we next examined how the activity of the Rho GTPases inside the cells could be regulated by BPGAP1 in vitro. Cells were cotransfected with HA-tagged Cdc42, RhoA, or Rac1 together with either the vector control or FLAG-BPGAP1. The activity of these GTPases in vivo was then determined by their magnitude of binding to the respective effector domains, as described under “Materials and Methods.” Fig. 4B shows that, in vivo, RhoA binding to its effector domain (RBD) was reduced in the presence of BPGAP1. In contrast, the binding status of Cdc42 and Rac1 to their effector domain (PBD) remained unaffected, and at times they seemed to be modestly increased instead. These results indicate that BPGAP1 exerts its GAP domain activity toward RhoA GTPase in vivo, whereas it has no significant effect on Cdc42 and Rac1 GTPases.
activity on RhoA, but not on Cdc42 or Rac1 in vivo. Next, we examined more closely the binding status of endogenous Rho GTPases to the full-length, BCH, and GAP domains of BP-GAP1. Fig. 4C shows that endogenous Cdc42, despite their very low expression level (detectable in the whole cell lysate only upon prolonged exposure of film), could be greatly precipitated by BPGAP1. Interestingly, the endogenous RhoA was not readily bound by BPGAP1 unless its level was elevated by overexpression (Fig. 4D). Consistent with this was the observation that endogenous RhoA could be co-immunoprecipitated with overexpressed BPGAP1 (Fig. 4E), and this level of interaction was further increased when RhoA itself was also overexpressed (Fig. 4F). Similar to the Cdc42, endogenous and overexpressed Rac1 interacted strongly with BPGAP1 in either the pull-down or co-immunoprecipitation assays (Fig. 4, C–F). Given that BPGAP1 could associate with Cdc42GAP strongly inside the cells, there still exists the possibility that some of these Rho GTPases might interact indirectly with BPGAP1 via Cdc42GAP. Taken together, our results confirm that BPGAP1 regulates RhoA, but not Cdc42 or Rac1 activities in vivo and that it still retained its ability to form a complex with Cdc42 and Rac1.

BPGAP1 Induces Pseudopodia via Its BCH and GAP Domains—To further understand the physiological significance of BPGAP1 interaction via its different domains, we set out to investigate their role(s) in regulating one of the key biological responses elicited by Rho GTPases, i.e. the control of cell dynamics. We specifically wish to elucidate how BPGAP1 would...
affect the morphology as well as the migration potentials of the target cells, and to examine whether changes in cell morphology alone is directly linked and sufficient to induce cell migration.

We had chosen to use the human breast epithelial cancer cells MCF7 as the model because of its relative ease in transfection, monitoring of its cell morphology and it is non-metastatic with minimal cell migration. MCF7 cells were transfected with expression plasmids of either FLAG-tagged BPGAP1, or vector control. Sixteen hours after the transfection, samples were processed for indirect immunofluorescence as described under “Materials and Methods.” Fig. 5A shows that while control cells with vector alone exhibited regular cuboidal feature of an epithelium and with diffused staining of the tag peptide, the expression of BPGAP1 was shown to be mainly localized in the cytosol and it induced unique long and short protrusions (collectively referred as pseudopodia) in the transfected cells (arrows). To further confirm such observations and to monitor the dynamics directly without resorting to fixing the cells, we opted for direct fluorescence with green fluo-
rescent protein (GFP) fusion of BPGAP1 and its various deletion mutants (refer to Fig. 3B). A quantitative measure of the cell dynamics could be performed by identifying certain types of changes in cell morphology and their relative percentages determined (Fig. 5, B and C). MCF7 cells expressing the vector control were mostly cuboidal in shape and less than 5% of them had some background with irregular shapes including short protrusions. However, when expressed, full-length GFP-BPGAP1 induced pseudopodia in almost 60% of the transfected cells, a unique feature that was normally typified by long

**Fig. 5.** BPGAP1 induces pseudopodia via BCH and GAP domains. A, MCF7 cells were transfected with FLAG-tagged expression plasmids for BPGAP1 full-length or control vector. Cells were then fixed, permeabilized, and incubated with anti-FLAG monoclonal, followed by Texas Red dye-conjugated rabbit anti-mouse IgG for immunofluorescence detection as described under “Materials and Methods.” Arrows indicate pseudopodia formation. Bar, 10 μm. B, MCF7 cells transfected for 16 h with GFP expression plasmids for BPGAP1 full-length, domains, mutants, or GFP vector alone were visualized for GFP expression by direct fluorescence detection. The yellow appearance was due to increased exposure set to allow better detection of pseudopodia. Arrows indicate features described in the text. C, percentage of cells exhibiting pseudopodia in the presence of various GFP constructs (from experiment B above) were determined and represented as a bar graph. Results are averages ± half the ranges for two determinations that are representative of at least three separate experiments. Asterisk indicates only short pseudopodia. All differences are significant at \( p < 0.01 \) versus GFP vector control. D, BPGAP1-induced morphological changes are protrusions/pseudopodia and not retraction fibers. MCF7 cells were transfected with expression plasmids for GFP-tagged BPGAP1 full-length, NP, and PC domains or GFP vector control. After the transfection for 20 h, the total cell areas, LD (the longest diameter that goes through the center of cells, showed by the bold line), SD (the shortest diameter that goes through the center of cells, showed by the gray line), and PT (the lengths of protrusion) were measured and analyzed as described under “Materials and Methods.” Measurements are means ± S.D. for 30 determinations. Results are representative of three separate experiments. Statistical comparison was made using ANOVA (StatsDirect). Asterisk indicates significant difference at \( p < 0.01 \) when compared with the vector control.
projections of more than 30 micron from the opposing ends of the cell bodies, accompanied by occasion branching off the main pseudopods (Fig. 5B). Interestingly, when fragments NNP (with BCH, but without proline-rich region) or NP (with BCH and proline-rich region) were tested, long pseudopodia were no longer observed. Instead, between 25 and 30% of the transfected cells caused many shorter protrusions (less than 10 micron) with “microspike-like” features surrounding the cell peripheries. In comparison, the presence of the GAP domain in the PC fragment was sufficient to cause mainly long, and very few short pseudopodia. This was further supported by the R232A inactive GAP mutant that when expressed, resulted in the features resemblance to that induced by the NNP or NP fragments alone. In contrast, deletion of the entire proline-rich region (amino acids 176–189; see Fig. 2D) of BPGAP1 (P1 mutant) did not affect the overall formation of protrusions by the cells. To confirm that the formation of protrusions induced by BPGAP1 was not the result of cell body retraction or shrinkage (yielded as retraction fibers), various parameters for cell dimensions were also measured as described under “Materials and Methods.” These include the LD and the SD that bisected the center of cells and perpendicular to each other, the total areas of the cell bodies and also the average lengths of long pseudopods (Fig. 5D). The results show that when compared with the GFP control, BPGAP1 full-length, NP or PC mutants all induced similar morphological changes without changes in the total cell areas, the longest or the shortest diameters, indicating that there was no cell shrinkage or retraction. Instead, they only caused the lengthening of pseudopodia. Taken together, these results confirm that BPGAP1 indeed induces unique pseudopodia formation via the BCH and GAP domains independently of the proline-rich region. Induction of pseudopodia by BPGAP1 was also observed in 293T and HeLa cells (data not shown).

BPGAP1-induced Pseudopodia Involve Inactivation of RhoA but Activation of Pathways Downstream of Cdc42/Rac1—Since BPGAP1-induced pseudopodia required its GAP domain and the BCH domain, and that in vivo BPGAP1 inactivated only the RhoA but not Cdc42 and Rac1, we postulated that such formation of pseudopodia would involve inactivation of RhoA but not the inactivation of Cdc42 or Rac1. Furthermore, activation of RhoA could inhibit such process whereas persistent activation of Cdc42 or Rac1 might potentiate morphological changes elicited by BPGAP1. To test these two hypotheses, cells were cotransfected with BPGAP1 along with either the wild type, constitutively active, or dominant negative mutants of the RhoA, Cdc42, or Rac1. Indirect immunofluorescence studies using confocal microscopy shows that expression of wild type or G14V constitutively active mutant of RhoA prevented the formation of long pseudopodia with some short protrusions still remained detectable. However, its dominant negative mutant T19N did not affect any of the process (Fig. 6A). These results are consistent with the earlier observations that GAP domain mediated long pseudopodia and its absence or its inactive arginine mutant resulted in only the short pseudopodia (see Fig. 5, B and C). Consistent with the requirement of the inactivation of RhoA was the loss of stress fibers detectable by phallloidin staining for the filamentous actin in cells expressing BPGAP1 (data not shown).

In contrast, wild type or G12V mutant Cdc42 allowed propagation of cell shapes that saw many more short protrusions with branching formed, whereas the T17N negative mutant of Cdc42 completely blocked the effect of BPGAP1 (Fig. 6B). As for Rac1, expression of the wild-type Rac1 ensured persistence of the lamellipodia despite the presence of BPGAP1. However, when present together with BPGAP1, its G12V constitutive active mutants resulted in very extensive and thin pseudopodia, resemblance the general features for a “neurite-like” outgrowth (Fig. 6C). Intriguingly, in these structures, there were various sprouting of “sub-branches” at quite regular intervals while the main pseudopods appeared to contact the similar structures from adjacent cells. Unlike Cdc42 T17N, the Rac1 T17N could only block the formation of long pseudopodia, with remnant short protrusions still seen in certain cells. In order to understand the mechanism of the neurite-like induction by active Rac1 and BPGAP1, Rac1 G12V mutant was coexpressed with the NP domain (containing the BCH domain) or the PC domain (with GAP domain) of BPGAP1 followed by confocal microscopic examination. Fig. 6D shows that BCH domain together with the Rac1 G12V resulted in extensive pseudopods with clear “nodule-like” structures (inset (i)) whereas the GAP domain caused similar extensive pseudopods but with lesser extents of “nodule-like” structures (inset (ii)). These drastic morphological changes provide strong evidence that BCH and GAP domain are indeed involved in the regulation of cell dynamics possibly involving many other downstream effectors of Cdc42 and Rac1 without directly affecting the intrinsic GTPase activity of Cdc42 or Rac1 per se.

BPGAP1 Promotes Cell Migration via Coupling of BCH and GAP Domains with the Proline-rich Region—While BPGAP1 induces drastic changes to cell morphology, it remains a key question as to what the physiological outcomes that might accompany such effects. Changes in cell morphology are often associated with cell motility as exemplified in macrophage action and in numerous metastatic cancer cells (8, 34). We went on to examine if induction of pseudopodia in MCF7 cells were indeed necessary for their ability to promote cell migration and to test if this event was directly linked to cell motility, at least within the context of BPGAP1 effect. Cells were transfected with either the vector control or plasmids encoding either full-length, NP, or PC domain of BPGAP1 or the P1 mutant. Transfected cells were monitored for their potential to migrate across the modified Boyden chamber toward fibronectin-coated surfaces, as described under “Materials and Methods.” Fig. 7 shows that around 45% of the cells transfected with the full-length BPGAP1 had migrated to the fibronectin-coated surfaces, 2-fold over the control cells, whereas NP or PC domains did not elicit any significant increase in their migration potential despite the induction of pseudopodia (compare with Fig. 5, B and C). These results imply that BCH or GAP domains alone, despite their positive effects on morphological changes, is not sufficient to propagate cell migration. Interestingly, the “P1” mutant, despite having the intact BCH and GAP domains and the ability to induce morphological changes, had failed to confer any increase in the cell migration. These results indicate that cell morphological changes induced by BPGAP1 through the BCH and GAP domains, are required but still not sufficient for mediating cell migration. It required further input of signals via the proline-rich sequence. The significance of the interplay between BCH domain, GAP domain and proline-rich sequence in regulating cell dynamics is discussed below.

DISCUSSION

The current studies described the identification and characterization of BPGAP1, a novel Cdc42GAP/p50RhoGAP-like protein that plays an important role in regulating cell dynamics. Biochemical and cellular functions of its three protein domains, namely BCH domain, proline-rich region, and the GAP domain were delineated either singly, in combination or as a whole protein under in vitro and in vivo conditions. We discovered that BCH and GAP domains induced short and long pseudopodia, respectively, that were subsequently needed to trigger cell migration only when coupled to its proline-rich region.
The ability of the BCH and GAP domains of BPGAP1 in mediating different extents of pseudopodia is intriguing and it potentially involves different mechanisms. Although BPGAP1 functions biochemically as a GAP for RhoA in vitro and in vivo, it also targets Cdc42 and Rac1. These observations raise the possibility that despite not being the substrates, Cdc42 and
Rac1 could still be involved in BPGAP1 signaling. Indeed, our results demonstrated that BPGAP1-induced pseudopodia formation could be completely blocked by the dominant negative mutants of Cdc42 and partially inhibited by dominant negative mutants of Rac1 (affecting only the long pseudopodia). However, it was unaffected by the corresponding mutant of RhoA (Fig. 8). Conversely, RhoA activation inhibited formation of long pseudopodia whereas activating Cdc42 or Rac1 potentiated the cell shape changes further, in particularly, the drastic neurite-like outgrowth feature seen with Rac1 G12V. Furthermore, this feature could be associated with extensive branching off the existing protrusions and with an apparent “nodule” appearance, as conferred by BCH or GAP domains. In this regard, it is possible that Cdc42 or/and Rac1 could be recruited to BPGAP1 for other cellular component(s) to interact with, so as to further propagate the Cdc42/Rac1 signaling needed in causing those pseudopodia. Work is currently under way to test this hypothesis further. These results strongly indicate the involvement of BPGAP1 in differentially regulating distinctive pathways of Rho GTPases that could have important physiological bearings, including the cell migration featured in this study.

The current model where BPGAP1 regulates cell dynamics via two discrete domains of BCH and GAP that are adjacent to each other also represents a novel combination and mode of regulation for GAP proteins. Analysis through Conserved Domain Architecture Retrieval Tool at NCBI showed that such unique combination of BCH with GAP domains are also conserved in several proteins from various eukaryotic organisms, including the Caenorhabditis elegans, Anopheles gambiae, Plasmodium falciparum, Drosophila melanogaster, zebrafish Danio rerio, but not in plants. To date, we have discovered and characterized at least one member of the BPGAP family. Its GAP domain specifically inactivates RhoA pathway and induces long pseudopodia whereas the BCH domain leads to the formation of short pseudopodia via a mechanism that is yet to be identified. Formation of pseudopodia can be inhibited at different points by mutants of Rho GTPases as indicated. It is believed that the GAP domain can cross-talk to the BCH domain as exemplified by the ability of both domains to separately induce similar neurite-like features when Rac1 is active. Collectively, both BCH and GAP domains, but not the proline-rich region, confer unique pseudopodia, which are necessary but not sufficient to exert cell migration in the absence of a functional proline-rich region. It is therefore likely that protein(s) that harbor the proline-targeting domains such as SH3 or WW domains are involved in linking cell morphological changes to its migration.

Various GAP proteins have been identified to regulate cell morphology but little is known about the coupling of cell morphology to cell migration via their protein domains in cis. BPGAP1 provides an example for such an intricate process. With multiple signaling modules, BPGAP1 is poised to target different classes of signaling molecules and thus could play a pivotal role in the integration of several signaling events. Indeed, our results here indicate that cell morphological changes induced by BPGAP1 through the BCH and GAP domains are required but still not sufficient for mediating cell migration. It requires an additional input from the proline-rich region that specifically couples the control of cell movement to the morphological changes that precede the event (Fig. 8). This stringent requirement of multi-domain interplay is different from several other RhoGAPs whose function was predominantly dependent upon the functional GAP domains. For example, overexpression of the p190RhoGAP wild-type GAP domain alone decreased RhoA activity, promoted the formation of membrane protrusions, and enhanced motility (35). Likewise, DEP1/ASAP1 (the GAP for ADP-ribosylation factor-1, ARF-1) enhanced cell motility via a GAP-dependent mechanism (36) but another ArfGAP protein, ARAP1 (which also includes a rhoGAP domain besides the ArfGAP domain) utilizes its RhoGAP domain to cause cell rounding independently of the other domains (37). In addition, the RhoGAP domain of

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B. C. Low, unpublished results.
p122RhoGAP could only induce an extensive cell rounding and detachment of adherent cells (38).

It is also interesting to note that the proline-rich region of BPGAP1 is very similar to those identified in RNB6 and ena/VASP-like and could comprise more than one putative binding sites for either SH3 (39) or WW (33) domains (Fig. 2D). RNB6 and ena/VASP-like protein are members of the Ena/VASP family proteins that are associated with microfilaments, adherents type cell matrix and cell-cell junctions, and highly dynamic membrane regions (39, 40). Given the complex nature of the proline-rich region and the likelihood of this being a target(s) for several SH3 and/or WW domain-containing proteins, identifying the real interacting partner(s) that mediate this and other biological processes remains a challenging but exciting prospect for future work. This will help elucidate the functional significance of BPGAP1 in controlling the specificity, redundancy, and regulation of small GTPase signaling in cell dynamics control.

Acknowledgments—We thank Bee Leng Lua and Dr. Jan Buschdorf for critically reviewing this manuscript and Yun Ping Lim for assistance in Vector NTH Suite.

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Concerted Regulation of Cell Dynamics by BNIP-2 and Cdc42GAP Homology/Sec14p-like, Proline-rich, and GTPase-activating Protein Domains of a Novel Rho GTPase-activating Protein, BPGAP1

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J. Biol. Chem. 2003, 278:45903-45914.
doi: 10.1074/jbc.M304514200 originally published online August 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304514200

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