FUNCTIONAL STUDIES OF BPGAP1, A NOVEL BCH DOMAIN-CONTAINING RHOGAP PROTEIN

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献给我最亲爱的妈妈，感谢她对我的养育和爱护。

妈妈的爱和鼓励是我的精神支柱和完成学业的最大动力。

Dedicated to my dearest mother

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Shang Xun

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CHAPTER 1 INTRODUCTION

SUMMARY

Rho GTPases are small molecular switches of 21-25 kDa that cycle between GTP-bound active form and GDP-bound inactive form. They control a wide variety of signal transduction pathways that regulate cytoskeletal reorganization, leading to changes in cell morphology and cell motility. Cdc42, RhoA and Rac1 are among the most well-studied members of these small GTPases They are activated by guanine nucleotide exchange factors (GEFs) which catalyze the exchange from GDP to GTP and inactivated by GTPase-activiting proteins (GAPs) that accelerate GTP hydrolysis. In this study, we present the cloning of a novel RhoGAP, BPGAP1 (BNIP-2 and Cdc42GAP Homology (BCH) domain-containing, Proline-rich and Cdc42GAP-like protein subtype-1), its expression and functional characterization in mammalian cell signaling.

Full length BPGAP1 cDNA was isolated by reverse transcription-polymerase chain reaction. BPGAP1 is ubiquitously expressed and shares 54% sequence identity to Cdc42GAP/p50RhoGAP, one of the first RhoGAPs identified. GTPase assays and protein binding assays were carried out to investigate the Rho GTPase interaction and activities of BPGAP1 towards Cdc42, RhoA and Rac1 both *in vivo and in vitro*. BPGAP1 selectively enhanced RhoA GTPase activity, but not those of Cdc42 (excepting *in vitro*) and Rac1, despite interacting with its GAP domain. In contrast, the BCH domain, which is a protein-protein interaction domain, preferentially targeted Cdc42. Pull-down and co-immunoprecipitation studies indicated that BPGAP1 formed homophilic or heterophilic complexes with other BCH domain

containing proteins such as Cdc42GAP, BNIP-2 and itself *via* its BCH domain and could assume an intramolecular interaction between its BCH and GAP domain. Furthermore, its proline-rich sequence targeted various SH3 and WW domains including p85α, PLC-γ, c-Src and Nedd4. These protein-protein interactions imply the involvement of BPGAP1 in multiple cell signaling pathways.

Fluorescence studies of epitope-tagged BPGAP1 revealed that it induced pseudopodia and increased migration of human breast adenocarcinoma (MCF7) cells. Formation of pseudopodia required its GAP and BCH domains but not its proline-rich region, and was inhibited by co-expression of constitutive active mutant of RhoA G14V, dominant negative mutants of Cdc42 T17N or Rac1 T17N. Interestingly, with BPGAP1, constitutive active mutant of Cdc42 G12V caused intensed microspikes whereas Rac1 G12V induced drastic “neurite-like” feature. However, mutant devoid of the proline-rich region failed to confer any increase in cell migration despite the induction of pseudopodia.

Further experiments also showed that BPGAP1 interacted with endogenous Nedd4, a ubiquitin ligase, both *in vivo* and *in vitro*. Ubiqutination assays showed that BPGAP1 was ubiqutinated in the Nedd4-dependent manner. These findings provided a possible mechanism for the turn-over of BPGAP1, hence down-regulation of signaling induced by BPGAP1.

The present study reports both the biochemical features and cellular functions of BPGAP1, and provides evidence that cell morphology changes and migration are coordinated *via* multiple domains in BPGAP1. The results present a novel mode of

regulation for cell dynamics by a RhoGAP protein and its possible involvement in multiple signaling pathways.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of Variance

Arp2/3: Actin-Related Proteins 2 and 3

ATP: Adenosine Triphosphate

BCH domain: BNIP-2 and Cdc42GAP Homology domain

BNIP-2: BCL2/adenovirus E1B 19kD Interacting Protein 2

BNIP-S: BNIP-2 Similar

BPGAP1: BNIP-2 and Cdc42GAP homology (BCH) domain-containing, proline-rich and Cdc42GAP-like protein subtype-1

BSA: Bovine Serum Albumin

CDART: Conserved Domain Architecture Retrieval Tool

Cdc42: Cell Division Cycle 42

EDTA: Ethylenediamine Tetraacetic Acid

GAP: GTPase-Activating Protein

GDI: Guanine Nucleotide Dissociation Inhibitor

GDP: Guanosine Diphosphate

GEF: Guanine Nucleotide Exchange Factors

GFP: Green Fluorescent Protein

GST: Glutathione S-transferase

GTP: Guanosine Triphosphate

GTPases: Guanosine Triphosphatases

HEPES: 50mm 4-(2-hydroxyethyl)-1-Piperazineethanesulfonic Acid

MESG: 2-Amino-6-Mercapto-7-Methylpurine Riboside

mRNA: Messenger RNA

Nedd4: Neural precursor cell Expressed, Developmentally Down-regulated 4

PAK: p21-Activated Kinase

PBD: p21-Binding Domain of PAK1

Pi: Inorganic Phosphate

PI3K: Phosphatidylinositol 3' Kinase

PLC-γ: Phospholipase C-γ

PtdIns-(3,4,5)P3: Phosphatidylinositol 3,4,5-Triphosphate

Rac1: Ras-related C3 Botulinum Toxin Substrate 1

Ras: Retrovirus Associated Sequence

RBD: p21-Binding Domain of Rhotekin

RhoA: Ras Homologous member A

ROK: Rho Kinase

RT-PCR: Reverse Transcription-Polymerease Chain Reaction

SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Ub: Ubiquitin

WASP: Wiskott-Aldrich Syndrome Protein

WAVE: WASP-like Verprolin-homologous protein

WCL: Whole Cell Lysates

Wt: Wild type

Chapter 1

Introduction

1.1 Rho GTPases regulate actin cytoskeleton dynamics and cell molitity

Cells undergo dynamic changes as part of their adaptation and response to extracellular stimuli. These adaptation and response include their abilities to proliferate, differentiate, migrate or execute death (Hall, 1998). Actin cytoskeleton reorganization plays an important role in the regulation of cell dynamics in all eukaryotic cells. It is a major determinant of cell morphology and polarity. The assembly and disassembly of filamentous actin structures provides a driving force for dynamic process such as cell motility, phagocytosis, growth con guidance and cytokinesis. Rho family of small GTPases Rho, Rac, and Cdc42 play central roles in signal transduction pathways that link plasma membrane receptors to the organization of the actin cytoskeleton (Hall and Nobes, 2000). They are also the key regulators of cell migration, cell cycle progression, vascular transportation, gene transcription, cell polarity and microtubule dynamics (Jaffe and Hall, 2003; Moon and Zheng, 2003). Three types of regulators have been identified to control the "on/off" switch of GTPases, including guanine nucleotide exchange factors, GTPase-activating proteins and guanine nucleotide dissociation inhibitors. Multiple down stream effectors of Rho GTPases such as ROK, WASP and WAVE functions to relay signals to actin cytoskeleton, thus to regulate cell dynamics and cell migration.

1.1.1 Rho GTPases

Rho GTPases are members of the Ras superfamily of monomeric 21-25 kDa GTP-binding proteins. Rho is for "Ras Homology" and GTPases are for "Guanosine triphosphatases". So far, at least 18 different mammalian Rho GTPases have been

identified, some with multiple isoforms. They inculde: Rho(A,B,C isoforms), Rac (1,2,3 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), Rnd1/Rho6, Rnd2/Rho7, Rnd3/RhoE, RhoD, RhoG, TC10, TTF. They share around 50-55% identity to each other. Phylogenetic analysis has been done to show their evolutional relationship (Figure 1.1). The most extensively characterized members are Rho, Rac and Cdc42 (Bishop and Hall, 2000; Hall and Nobes, 2000; Wherlock and Mellor, 2002).

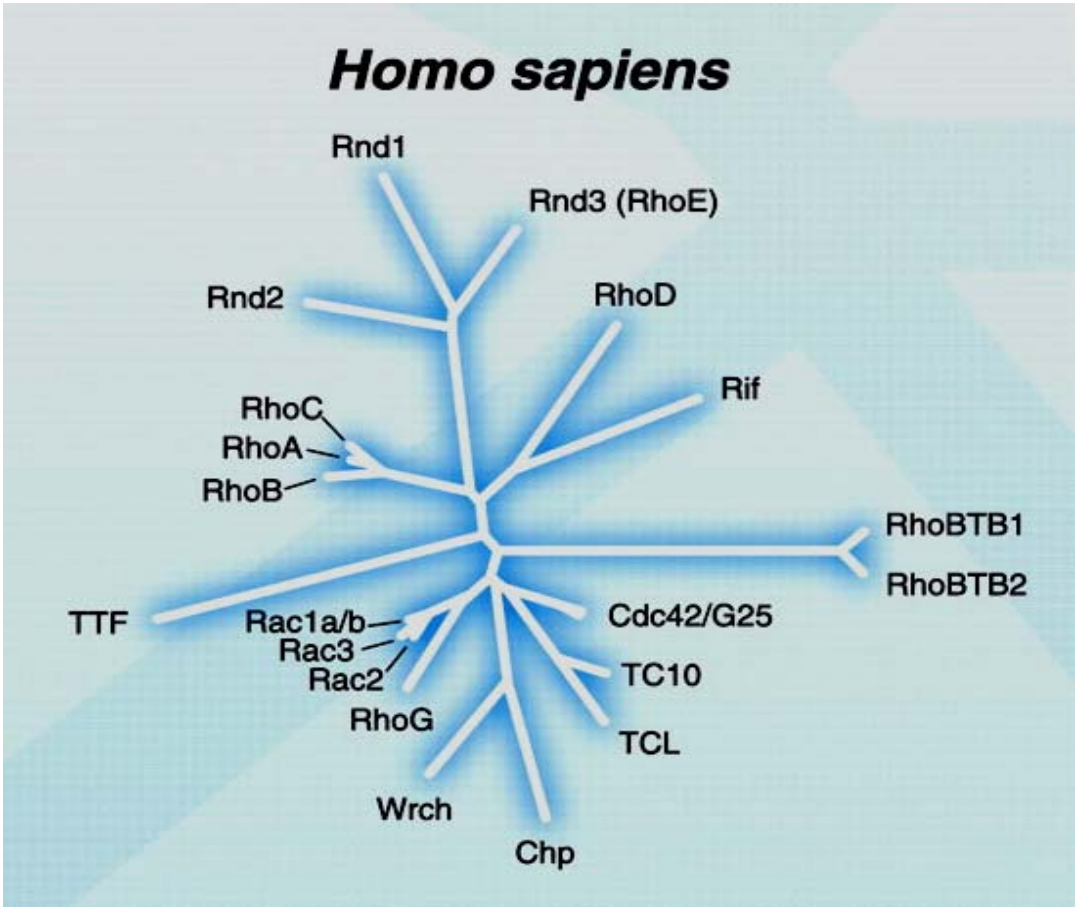

Figure 1.1 Phylogenetic tree of Rho small GTPases subfamily (adapted from Wherlock and Mellor, 2002)**.**

Rho GTPases are small GTP binding proteins that serve as molecular switches to control a wide variety of signaling pathways. They are known principally for their pivotal role in regulating the actin cytoskeleton. By switching on a single GTPase, several distinct signaling pathways can be coordinately activated. They use a simple biochemical

strategy to control complex cellular processes (Figure 1.2). They cycle between two conformational states: one bound to GTP which is in the "active state", the other bound to GDP which is in the "inactive state". In the active (GTP) state, GTPases recognize target proteins and generate a response until GTP hydrolysis returns the switch to the inactive state (Etienne-Maneville and Hall, 2002). This signaling paradigm has been elaborated throughout evolution, which is confirmed in mammalian cells as well as in yeast, flies, worms and plants.

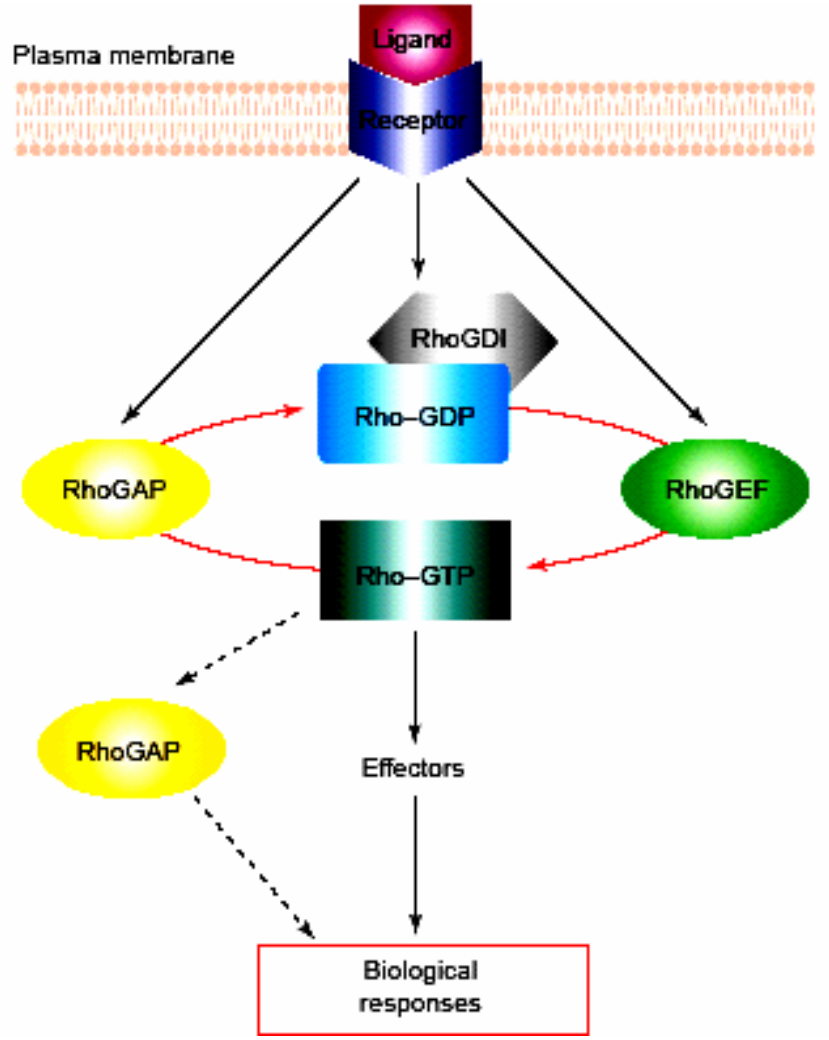

Figure 1.2 The Rho GTPase cycle. The cycle is between an active (GTP-bound) and an inactive (GDP-bound) conformation. The cycle is highly regulated by three classes of protein: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide exchange inhibitors (GDIs) (adapted from Moon and Zheng, 2003).

1.1.2 Rho GTPases regulate actin cytoskeleton organization

The actin cytoskeleton regulates a variety of essential biological functions in all eukaryotic cells. In addition to providing a structural framework around which cell shape and polarity are formed, its dynamic properties provide the driving force for cells to move and to divide. Understanding the biochemical mechanisms that control the organization of actin is thus a major goal of contemporary cell biology, which also have implications for health and disease (Hall, 1998).

The actin cytoskeleton is composed of actin filaments and many specialized actin-binding proteins (Small *et al*., 1994; Stossel *et al*., 1993; Zigmond *et al*., 1996). Filamentous actin is generally organized into a number of discrete structures including : actin stress fibers which are bundles of actin filaments that traverse the cell and are linked to the extracellular matrix through focal adhesions; lamellipodia which are thin protrusive actin sheets that dominate the edges of cultured fibroblasts and many migrating cells; membrane ruffles observed at the leading edge of the cell result from lamellipodia that lift up off the substratum and fold backward; and filopodia which are fingerlike protrusions that contain a tight bundle of long actin filaments in the direction of the protrusion. They are found primarily in motile cells and neuronal growth cones. Therefore, it is important that the polymerization and depolymerization of cortical actin be tightly regulated. In most cases, this regulation of actin polymerization is regulated by Rho GTPases, Rho, Cdc42 and Rac.

Members of the Rho family of small GTPases have been studied as key regulators of the actin cytoskeleton. It is showed that in fibroblasts Rho can be activated by the addition of extracellular stimulation such as lysophosphatidic acid (LPA), and that activation of Rho causes the bundling of actin filaments into stress

fibers and the clustering of integrins and associated proteins into focal adhesions complexes (Hall, 1998; Ridley and Hall, 1992; Kozma *et al*., 1997). Rac can be activated by a distinct set of agonist (for example, platelet-derived growth factor or insulin), leading to the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles. And activation of Cdc42 is shown to trigger actin polymerization to form filopodia or microspikes (Mackay and Hall, 1998; Ridley and Hall, 1992; Ridley *et al*., 1992; Nobes and Hall, 1995; Kozma, 1995; Machesky and Hall, 1997). With similar to Rho, the cytoskeletal changes induced by Rac and Cdc42 are also associated with distinct, integrin-based adhesion complexes (Figure 1.3a; Figure1.3b). Moreover, there is significant cross-talk between GTPases of the Ras and Rho subfamilies: Ras can activate Rac, thus Ras induces lamellipodia; Cdc42 can activate Rac, therefore filopodia are intimately associated with lamellipodia (Nobes and Hall, 1995; Kozma *et al*., 1995); Rac1 can inactivate RhoA in NIH3T3 cells resulting in epithelioid phenotype (Sander *et al*., 2000; Zondag *et al*., 2000; Evers *et al*., 2000); In contrast, in Swiss 3T3 fibroblasts, Rac1 activates RhoA instead (Ridley *et al*., 1992).

From the observations above, it can be concluded that members of the Rho GTPase family are the key regulatory molecules that link surface receptors to the organization of the actin cytoskeleton. And this conclusion is further confirmed in a wide variety of mammalian cell types as well as in yeast, flies and worms (Etienne-Manneville and Hall, 2002).

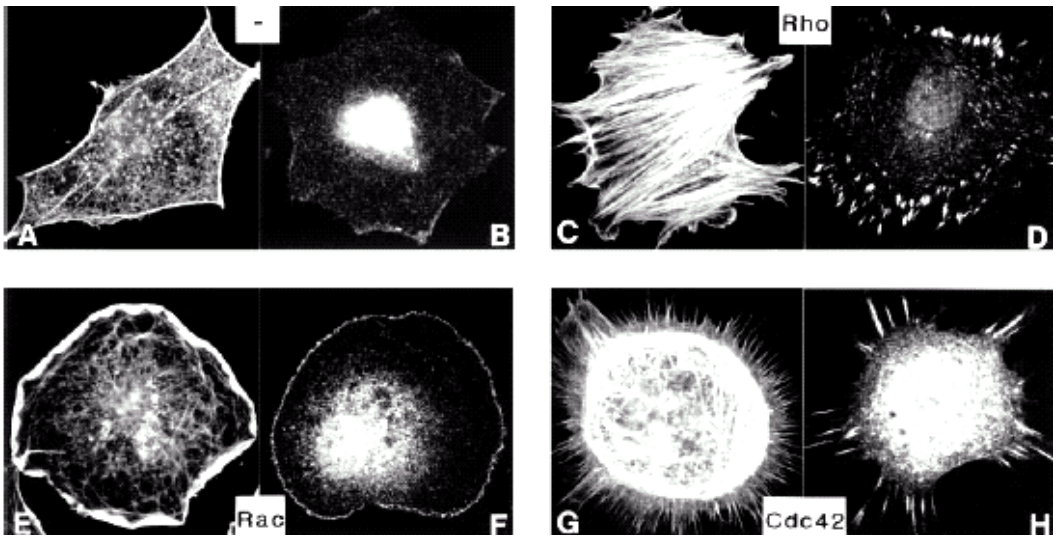

Figure 1.3a Rho, Rac, and Cdc42 control the assembly and organization of the actin cytoskeleton. In fibroblast, activation of Rho causes the bundling of actin filaments into stress fibers and the clustering of integrins and associated proteins into focal adhesions complexes; activation of Rac leads to the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles; activation of Cdc42 is shown to trigger actin polymerization to form filopodia or microspikes (adapted from Hall, 1998).

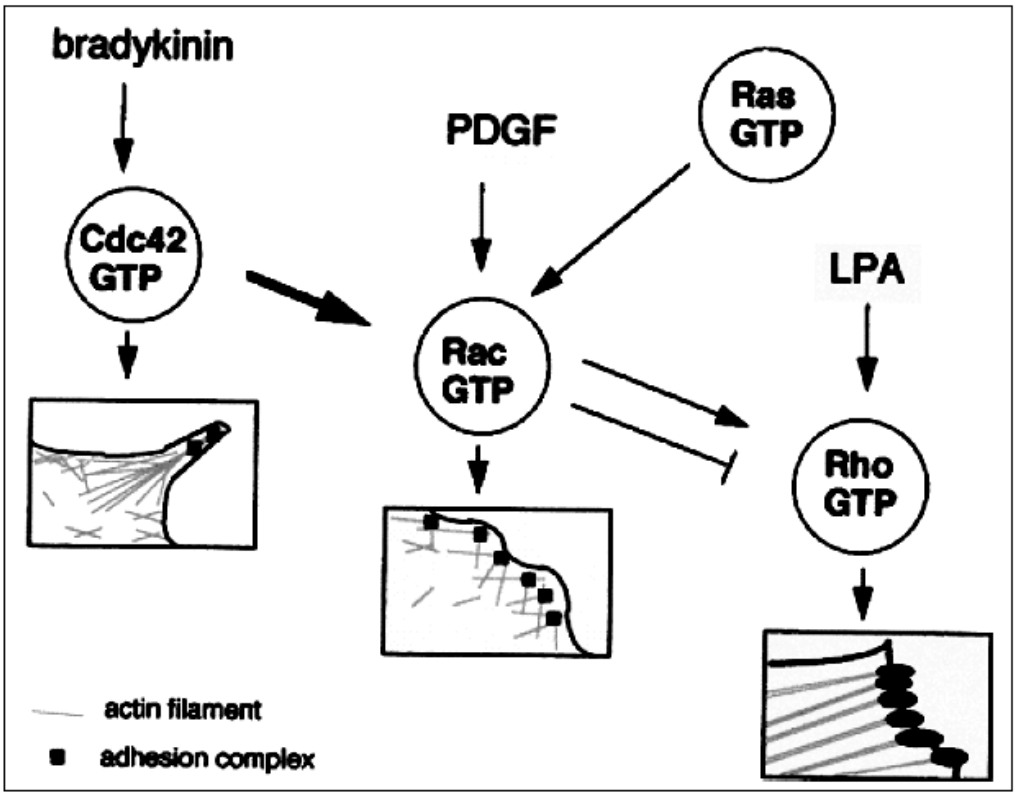

Figure 1.3b Activation of Rho, Rac, and Cdc42 by extracellular agonists and the regulation on actin cytoskeleton. LPA (a major constituent of tissue culture serum) can activate Rho, leading to the assembly of actin-myosin stress fibers and associated integrin adhesion complexes (focal adhesions). Rac can be activated by PDGF or insulin, inducing actin polymerization at the cell periphery causing lamellipodial extensions and membrane ruffling activity. Bradykinin activates Cdc42 to produce filopodia or microspikes and associated integrin complexes. There is a lot of crosstalk within and between the Ras and Rho GTPase families (adapted from Mackay and Hall, 1998).

1.1.3 Rho GTPases regulate cell migration

1.1.3.1 Cell migration

In multicellular organisms, cell migration is essential to normal development, and is required throughout life for responses to tissue damage and infection. Cell migration also occurs in chronic human diseases; in cancer, atherosclerosis and chronic inflammatory diseases such as rheumatoid arthritis, thus preventing the migration of specific cell types could significantly inhibit disease progression.

Cell migration is a multistep process including changes in the cytoskeleton, cell-substrate adhesions and the extracellular matrix (Figure 1.4). Many cell types migrate as individual cells, involving leukocytes, lymphocytes, fibroblasts and neuronal cells, but epithelial cells and endothelial cells often move as sheets or groups of cells - for example, in duct development, in healing a wound and in angiogenesis (Ridley, 2001; Ridley *et al*., 2003).

Cell migration is usually initiated in response to extracellular cues including diffusible factors, signals on neighbouring cells, and/or signals from the extracellular matrix. These signals then stimulate transmembrane receptors to initiate intracellular signaling. Many different intracellular signaling molecules have been implicated in

cell migration, including small GTPases, Ca2+-regulated proteins, mitogen activated protein kinase (MAPK) cascades, protein kinases C, phosphatidylinositide kinases, phospholipases C and D, and tyrosine kinases.

Rho family GTPases could regulate cell migration as they mediate the formation of specific actin cytoskeleton organizations (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Rho proteins have also been found to regulate several other processes relevant to cell migration, including cell-substrate adhesion, cell-cell adhesion, protein secretion, vesicle trafficking and transcription.

The actin cytoskeleton is a major determinant of cell morphology and polarity. This assembly and disassembly of filamentous actin structures may act as a driving force for the dynamic process such as cell migration, phagocytosis, growth cone guidance and cytokinesis. Since changes in cell morphology are often associated with cell migration as exemplified in macrophage action and in a variety of metastatic cancer cells, Rho GTPases are also functioned as the main regulators for cell migration (Hall and Nobes, 2000).

Cell migration requires the asymmetrical organization of cellular activities. It can be divided into four mechanistically separate steps: lamellipodium extension, formation of new adhesions, cell body contraction, and tail detachment. The front of the migrating cell generates protrusive activities, generally associated with the extension of a lamellipodium in the direction of cell movement. Meanwhile, the new cell adhesion to the extracellular substrate is developed. But that is not sufficient for cell to move. In addition, the cell contractility is also necessary to allow the body and the rear of the cell to follow the extending front (Ridley, 2001; Ridley *et al*., 2003; Jaffe and Hall, 2003).

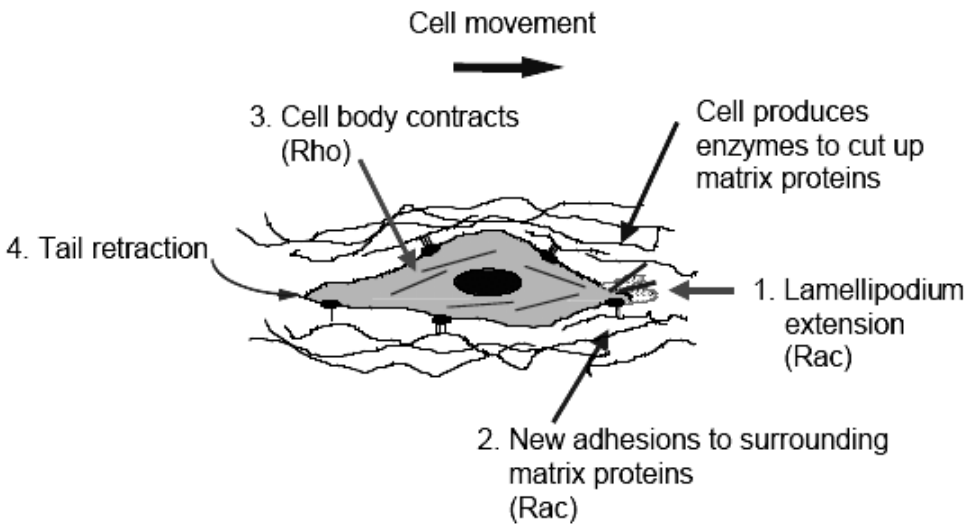

Figure 1.4 A model for the steps of cell migration. A migrating cell extends a lamellipodium at the front and this extension is stabilized through the formation of new adhesions to the extracellular matrix, which is induced by activated Rac and Cdc42. Then the activated Rho is required for the control of both the cell body to contract and move forward and the tail of the cell to detach from the substratum and retracts. Migrating cells also secrete proteases that cut up extracellular matrix proteins, and this is important for cell movement (adapted from Ridley, 2001).

1.1.3.2 Role of Rho GTPases in cell migration

1.1.3.2.1 Rac induces lamellipodium extension

Lamellipodium extension involves actin polymerization, and it is widely believed that lamellipodia consist of branching filament networks formed through the actin-nucleating activity of the Arp2/3 complex (Pollard *et al*., 2000). Rac is required for lamellipodium extension induced by growth factors, cytokines and extracellular matrix components, and videomicroscopy experiments show that cells cannot migrate if Rac activity is inhibited (Allen *et al*., 1998; Nobes and Hall, 1999; Knight *et al*., 2000).

Studies have been focused on the possible mechanism that controls the protrusive activity required for cell migration. It has been reported that RacGTP levels are the hightest at the leading edge of a migrating cell. Integrin-matix interaction probably plays an important role in regulating the activity of Rac (Kraynov *et al*., 2000).

Rac induced actin polymerization and integrin adhesion complex assembly at the cell periphery leads to membrane protrusion. This is essential for the migration of all cell types based on the current data (Small *et al*., 2002). As for the biochemical mechanism that Rac catalyses actin polymerization, four Rac effectors are implicated including IRSp53 (Insulin receptor substrate p53), phosphatidylinositol-4-phosphate 5-kinase, p65Pak (p21 activated kinase) and LIM kinase. Through these effectors, Rac regulates the nucleation of actin polymerization and the formation of new filament branches (Condeelis *et al*., 2001).

Rac is postulated to act through several downstream targets to regulate F-actin accumulation at the leading edge of cells in lamellipodia. It stimulates Arp2/3-complex-induced actin polymerization by interacting with a complex of IRSp53 and WAVE (Wiskott-Aldrich syndrome protein family verprolin-homologous protein) proteins. This leads to the formation of a branched filament network, because the Arp2/3 complex preferentially nucleates new actin filaments on the sides of existing filaments. Rac can also induce actin filament uncapping by generating phosphatidylinositol 4,5-bisphosphate locally, generating extra sites for actin polymerization. Finally, Rac acts *via* PAKs to stimulate LIMK, which inhibits cofilin-induced actin depolymerization, allowing increased accumulation of polymerized actin at the leading edge of cells. PAK may also contribute to migration in other ways by regulating myosin function and focal complex turnover. Crosstalk of Rac with

Cdc42 *via* IRSp53 and/or PAKs may regulate the level of Rac signalling.

1.1.3.2.2 Cdc42 directs and stablizes Rac activity during cell migration

Cell migration is normally directed and controlled by extracellular stimulation. Many cells adopt a polarized morphology with a front and a rear, and then migrate. But this is only a transient state, leading to a random migration named Chemokinesis. The stabilization of directional movement named Chemotaxis requires the external cues, which is controlled by Cdc42. In the study of macrophage cells moving up a gradient of a chemotactic factor, when Cdc42 is inhibited, the macrophage can only migrate in random directions. And when Rac is inhibited, all cell movements are inhibited (Allen *et al*., 1998). In this case, Cdc42 maybe function to direct and /or stabilize Rac activity at the cell front.

1.1.3.2.3 Rho promotes assembly of actin-myosin filaments cell body contraction

Cell body contraction is dependent on actomyosin contractility (Mitchison and Cramer, 1996) and can be regulated by Rho. Rho has been shown to be involved in the regulation of cell contractility. In motile monocytes, Rho is responsible for contraction and retraction within the trailing cell body, suggesting that RhoGTP is restrictedly localized in the cell body while not at the leading edge (Worthylake *et al*., 2001).

Rho acts *via* ROCKs (also known as Rho-kinases) to affect MLC (Myosin light chain) phosphorylation, through inhibiting MLC phosphatase and phosphorylating MLC (Kaibuchi *et al*., 1999; Amano *et al*., 2000). MLC phosphorylation is also regulated by MLC kinase (MLCK), which is activated by

calcium, and stimulated by the ERK (Extracellular-signal regulated kinase) MAPKs (mitogen-activated protein kinase) (Hansen *et al*., 2000). It is possible that ROCKs and MLCK act in the opposite to regulate different aspects of cell contractility, because ROCKs seem to be required for MLC phosphorylation associated with actin filaments in the cell body, whereas MLCK is required at the cell periphery (Totsukawa *et al*., 2000).

In a brief, cells move through the polarized and dynamic reorganization of the actin cytoskeleton, which involves a protruding force at the front, combined with a contractile force in the cell body. This contractile activity leads to retraction of the rear of the cell as the adhesions are lost. Rho GTPases are the main regulators to control this whole process. Rac regulates actin polymerization at the front to promote protrusion. Cdc42 acts at the front to control direction in response to extracellular cues. Rho stimulates actin-myosin contraction in the cell body (Etienne-Maneville and Hall, 2002; Mackay DJG and Hall, 1998; Hall, 1998; Hall and Nobes, 2000). In conclusion, cells move through differentially regulating the activities and localizations of Rho GTPases.

1.1.4 Regulators of Rho GTPases

There are mainly three types of regulators for the "on/off" switch of GTPases. Activation of the GTPase, through GDP-GTP exchange, is stimulated by guanine nucleotide exchange factors (GEFs), whereas the inactivation is catalyzed by GTPase-activating proteins (GAPs). Rho Guanine nucleotide dissociation inhibitor (Rho-GDI) stabilize the inactive, GDP-bound form of the protein (Mackay and Hall, 1998; Moon and Zheng, 2003; Figure 1.2).

1.1.4.1 Guanine nucleotide exchange factors (GEFs)

So far, a large family (>30) of Rho GEFs has been identified, each of which shares two common motifs: the Dbl homology domain, which is involved in the encoding the catalytic nucleotide exchange activity; and a pleckstrin homology domain, which might function to determine subcellular localization. Some GEFs seems specific for individual Rho GTPase. For example, Lbc for Rho, Tiam1 for Rac and FGD1 (faciogenital dysplasia gene product) for Cdc42, whereas others have activities towards all the three, *e.g.* Vav and Dbl (Mackay and Hall, 1998; Van Aelst and D'Souza-Schorey, 1997; Cerione and Zheng, 1996).

1.1.4.2 GTPase-activating proteins (GAPs)

Numerous GAPs have also been identified. The lifetime of active state is determined by the combination of slow intrinsic GTPase activity and the activity of GTPase-activaing proteins (GAPs), which can accelerate GTP hydrolysis by up to five orders of magnitude (Gamblin and Smerdon, 1998; Gideon *et al*., 1992; Lamarche and Hall, 1994). The RhoGAP family is defined by the presence of a conserved RhoGAP domain in the primary sequences that consists of about 150 amino acids and shares at least 20% sequence identity with other family members (Moon and Zheng, 2003). More comprehensive introduction about RhoGAPs can be referred at Chapter 1.3.

1.1.4.3 Guanine nucleotide dissociation inhibitors (GDIs)

The guanine nucleotide dissociation inhibitors (GDIs) sequester the GDP-bound form of Rho GTPases by the formation of a Rho-GDI complexs. The dissociation of the

GTPase from the Rho-GDI complex is likely to be another key feature of the activation mechanism. GEFs added to a GTPase-GDI complex *in vitro* are unable to stimulate nucleotide exchange, and so a dissociation signal appears to be required. It could even be that this is the rate-limiting step for GTPase activation *in vivo*. GDI may also be involved in the regulation of the intracellular localization of Rho GTPases (Moon and Zheng, 2003; Mackay and Hall, 1998).

1.1.5 Effectors of Rho GTPases

At least 30 potential effector proteins have been identified that interact with members of the Rho family (Bishop and Hall, 2000). Since the major function of Rho GTPases is to regulate the assembly and organization of the actin cytoskeleton, effectors involved in the actin reorganization has been well identified.

1.1.5.1 Effectors of Rho

At least two effectors, ROK (Rho kinase) and Dia, are required for Rho-induced assembly of stress fibers and focal adhesions (Bishop and Hall, 2000). ROK is a kind of Ser/Thr kinase. The activity of ROK is enhanced after binding to the Rho-GTP and when expressed in cells, it has been reported to induce stress fibers independent of Rho (Mackay and Hall, 1998; Leung *et al*., 1996; Amano *et al*., 1997; Ishizaki *et al*., 1997). Two substrates of ROK, Myosin light chain (MLC) and myosin-binding subunit (MBS) of MLC phosphatase, are likely to be the key regulators of the formation of actomysin assembly and contraction (Amano *et al*., 1996; Kawano *et al*., 1999). Another ROK target is LIM kinase (LIMK). When LIMK is phosphorylated, it is able to inhibit cofilin,

leading to stabilization of filamentous actin structures (Maekawa *et al*., 1999; Bamburg *et al*., 1999). When ROK alone does not induce correctly organized stress fibres, it has been reported that when ROK combined with Dia, another effector of Rho, stress fibres are induced (Watanabe *et al*., 1999; Nakano *et al*., 1999; Watanabe *et al*., 1997). Dia can interact with the actin monomer binding protein, profilin, and therefore it plays a part in linking Rho to the actin cytoskeleton.

1.1.5.2 Effectors of Cdc42

WASP (Wiskott-Aldrich syndrome protein) and N-WASP are both the effectors of Cdc42. It was observed that overexpression of N-WASP and Cdc42 induce long microspikes, like an exaggeration of Cdc42 activity, indicating that these proteins may be involved in the formation of filopodia downstream of Cdc42 (Miki *et al*., 1998). N-WASP binds to profilin, and both WASP and N-WASP bind to actin monomers, which directly induce actin polymerization (Machesky and Insall, 1998; Miki and Takenawa, 1998; Suetsugu *et al*., 1998; Eden *et al*., 2002). Cdc42 also interacts with two Ser/Thr kinases that are involved in actin reorganization and filopodia formation, MRCKs α and β.

1.1.5.3 Effectors of Rac

So far, there are several possible targets of Rac which have been implicated in actin reorganization including WAVE, PI-4-P5K and PAK and *et al.* (Bishop and Hall, 2000). WAVE is for WASP-like Verprolin-homologous protein. It induces actin nucleation and polymerization by activating and interacting with its downstream

Profilin, G-actin and Arp2/3 complex (Machesky and Insall, 1998; Machesky *et al*., 1999; Zigmond *et al*., 1997; Eden *et al*., 2002). Rac interacts directly with PI-4-P5K, and this interaction is not GTP-dependent (Tolias *et al*., 1998). Upon the interaction and activation of Rac, PIP_2 level is increased, capping proteins are released, and finally leads to actin-filament assembly (Hartwig *et al*., 1995; Tolias *et al*., 2000). PAK 1,2,3 are Ser/Thr kinase, which are the common target proteins utilized by both Rac and Cdc42 in the induction of lamellipodia and filopodia respectively.

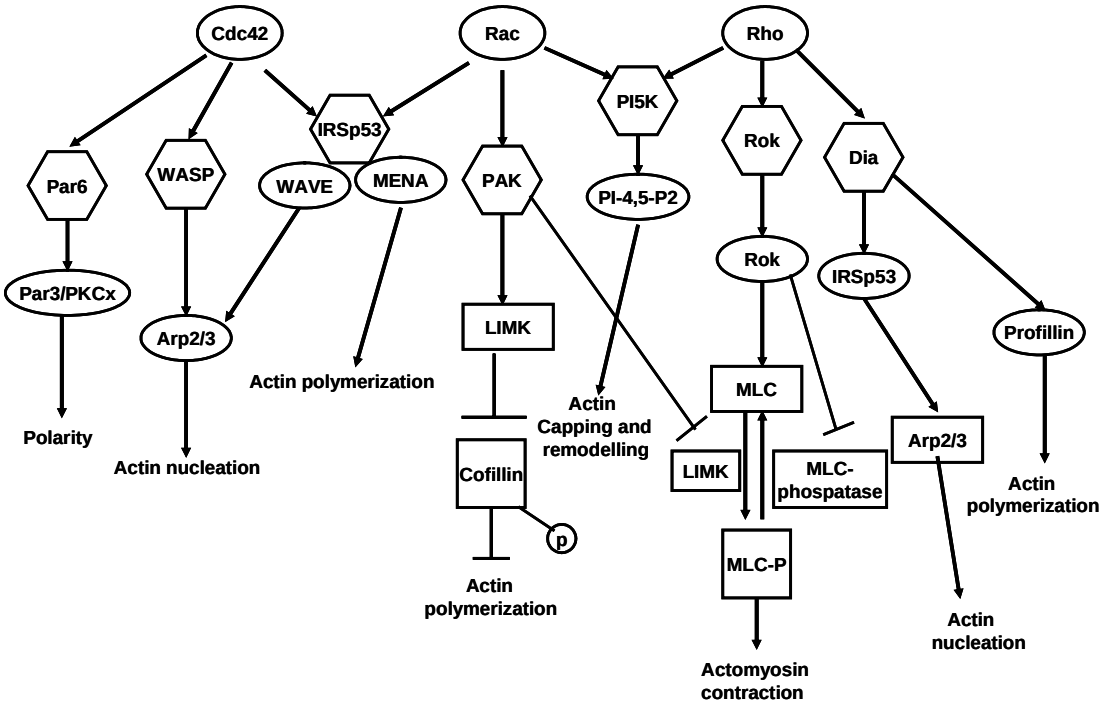

Figure 1.5 Rho GTPases regulate cell dynamics *via* their down stream effectors during cell migration (adapted from Van Aelst and Symons, 2002).

1.1.6 The role of Rho GTPases in disease development

The functionality and efficacy of Rho GTPase signaling is critical for various biological processes. Due to the integral nature of these molecules, the dysregulation of their activities can result in diverse aberrant phenotypes. Dysregulation is based on an altered signaling strength at the level of a specific regulator or that of the

respective GTPase itself. Alternatively, effector pathways induced by a specific Rho GTPase may be under- or over-activated. The steadily growing list of genetic alterations that specifically impinge on proper Rho GTPase function corresponds to pathological categories such as cancer progression, mental disabilities and a group of quite diverse and unrelated disorders (Boettner and Van Aelst, 2002).

There is a variety of disease-causing mutations in genes that have been associated with Rho GTPase signaling by using functional prediction or insights obtained by direct biochemical analysis. These include GEFs, GAPs and effector proteins that appear to be part of quite diverse signaling networks. Surprisingly, aberrations in only a single gene encoding a Rho GTPase itself, namely the RhoH gene, have been described to putatively induce lymphoma development (Preudhomme *et al*., 2000; Pasqualucci *et al*., 2001). Other mutations that may inactivate a Rho gene or lead to an overactive version of the resulting protein due to a lack of extensive screening or functional redundancy have either escaped detection or simply are lethal. This latter possibility is described by the fact that mouse embryos whose Rac1 or Cdc42 genes have been deleted by gene-targeted mutation die early in development (Sugihara *et al*., 1998; Chen *et al*., 2000). It may also reflect the multifunctional nature of Rho GTPases. Loss-of-function or constitutive gain-of-function mutations in many Rho GTPases thus may interfere with a number of different cellular processes (Boettner and Van Aelst, 2002).

A single Rho GTPase can affect a diverse array of phenomena implicated in a cell's specific biology. In addition, there is also continued speculation that Rho-type GTPases need to cycle between their active and inactive states in order to exert their complete physiological potential (Symons and Settleman, 2000). On the other hand, it is likely that regulators and effectors of Rho GTPases are expressed and act in a more

specific manner. Genetic loss-of-function mutations in these regulators or effectors, even in form of a germline mutation, may result in a weaker impairment than loss of the respective GTPase itself.

1.2 Definition of protein interaction domains

An ever-increasing amount of data suggests that proteins involved in the regulation of cellular events such as signal transduction, the cell cycle, protein trafficking, targeted proteolysis, cytoskeletal organization and gene expression are built in a modular fashion of a combination of interaction and catalytic domains. Interaction domains drive signaling polypeptides into specific multi-protein complexes, and thereby link cell surface receptors to intracellular biochemical pathways that regulate cellular responses to external signals. The pathways and networks that link receptors to their ultimate targets frequently involve a series of protein-protein interactions, which recruit and confine signaling proteins to an appropriate subcellular location, and determine the specificity with which enzymes interact with their targets, such as the association of protein kinases and their substrates. Most of the protein-protein interaction domains are independently folding modules of 35-150 amino acids, which can be expressed in isolation from their host proteins while retaining their intrinsic ability to bind their physiological partners. Their N- and C-termini are usually close together in space, whereas their ligand-binding surface lies on the outer face of the domain. This arrangement allows the domain to be inserted into a host protein while leaving its ligand-binding site to engage another polypeptide.

Protein-protein interaction domains can be divided into different families,

based either by sequence or ligand-binding properties. For example, a large number of cytoplasmic proteins contain one or two SH2 domains that directly recognize phosphotyrosine-containing motifs, such as those found on activated receptors of growth factors, cytokines and antigens. SH2 domains commonly recognize phosphotyrosine, depending on their different preference for the amino acids immediately following the phosphorylated residue, which plays an important role in deciding the specificity in signaling by tyrosine kinases. Interaction domains often appear repeatedly in different proteins to mediate a particular type of molecular recognition, and indeed the human genome is predicted to encode at least 120 SH2 domains. However, phosphotyrosine-containing motifs are also recognized by a quite different class of interaction modules, termed PTB domains, found on docking proteins such as the IRS-1 substrate of the insulin receptor. In addition, a growing family of interactions domains, including 14-3-3 proteins, FHA domains and WD40-repeat domains recognize specific phosphoserine/threonine motifs, and thereby mediate the biological activities of protein-serine/threonine kinases. Recent data suggest that other forms of post-translational protein modification control modular protein-protein interactions. For example, acetylation or methylation of lysine residues on histones creates binding sites for the Bromo and Chromo domains respectively, of proteins involved in chromatin remodeling. Taken together, these findings suggest that the dynamic control of cellular behavior exerted by covalent protein modifications is mediated by interaction domains, regulating the associations of signaling proteins one with another.

There is a large group of interaction domains (SH3, WW, EVH1) that bind proline-rich motifs. Since these complexes are less dependent on post-translational modifications, they seem to be constitutive compared to the phospho-dependent

interactions involving SH2 domains. Similarly, PDZ domains bind the extreme C-termini of other polypeptides, such as ion channels and receptors, in a fashion that appears important for the localization of their targets to particular subcellular sites, as well as for downstream signaling. The interactions discussed above are all related to the ability of a folded interaction domain to recognize a short peptide motif. Furthermore, a lot of modules form homotypic or heterotypic domain-domain interactions. These include PDZ domains, which are rather versatile since they can not only form heterodimers but also bind short C-terminal peptide motifs, as well as SAM domains.

In addition to interaction domains that engage specific peptide motifs, a growing number of modules have been identified that recognize selected phospholipids, such as phosphoinositides (PI). Strikingly, PH domains can bind either PI-4,5-P2 or PI-3,4,5-P3, and thereby mediate the effects of lipid kinases and phosphatases on cellular function. Such phospholipid-binding domains serve both to localize signaling proteins at specific subregions of the plasma membrane, and to regulate the enzymatic activities of their host proteins, either directly or by co-recruitment of another regulatory protein. Modules such as FYVE domains can recognize PI-3-P, and may play an important role in the trafficking of proteins within the cell.

Protein interaction domains have two important features. One is the versatility. For example, although PTB domains were originally discovered through their ability to bind phosphotyrosine in the context of an Asn-Pro-X-Tyr (NPxY) motif which forms a β-turn, it appears that many PTB domains recognize NPxY-related peptide motifs in a phospho-independent manner. Therefore, PTB domains likely evolved to bind unphosphorylated peptides, and have subsequently developed a capacity to

recognize phosphotyrosine in a few specific cases. Furthermore, an individual PTB domain, such as those from the Numb and FRS-2 proteins, can recognize two different peptide ligands. Interestingly, although PTB domains primarily bind peptide motifs and PH domains recognize phosphoinositdes, their structural fold are quite similiar, which is shared by other interaction domains, including EVH1 domains which bind specific proline-rich sequences. It seems that the PH/PTB/EVH1 domain fold provides a framework that can be used for multiple distinct types of intermolecular interactions. Second, different interaction domains are frequently covalently linked within the same polypeptide chain, thus to yield a protein that can mediate multiple protein-protein and protein-phospholipid interactions. This modular organization of signaling proteins can then localize proteins to the appropriate site within the cell, leading to their interactions with cell surface receptors and downstream targets. The reiterated and combinatorial use of interaction domains can in principle provide a wiring plan that controls and integrates the flow of information within the cell (http://www.mshri.on.ca/pawson/domains.html, Tony Pawson research on domain).

Our current study focuses on BPGAP1 (for BNIP-2 and Cdc42GAP Homology (BCH) domain-containing, Proline-rich and Cdc42GAP-like protein subtype-1) which will be described in the subsequent chapters. The various protein domains that BPGAP1 contains include the BCH domain, RhoGAP domain and proline-rich sequences.

1.3 The BCH domain

BCH domain is one of the protein domains that our group first identified and characterized. It is for BNIP-2 and Cdc42GAP Homology. This novel sequence

contains about 145 amino acids and was initially shown to be common to two proteins: BNIP-2 and Cdc42GAP (Low *et al*., 1999; 2000a; 2000b; Figure 1.6).

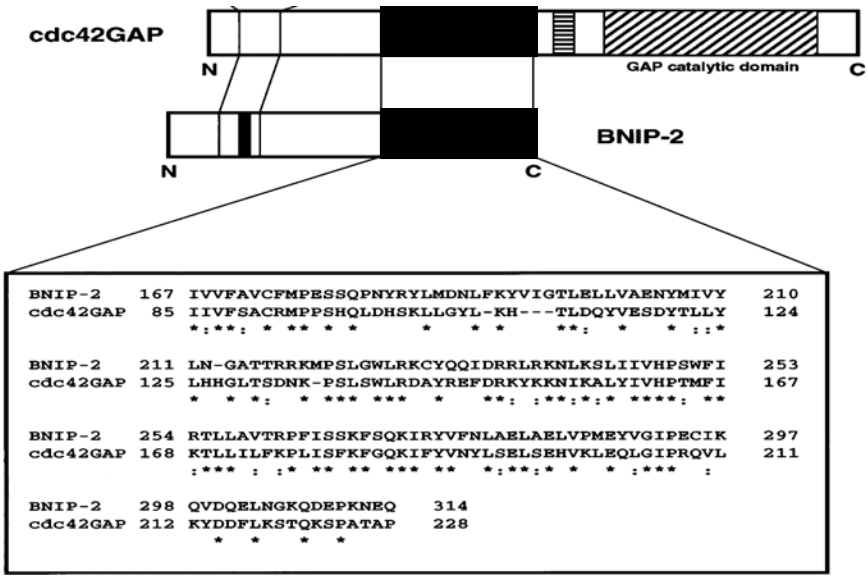

Figure 1.6 Homologous domains in BNIP-2 and Cdc42GAP. Shown are the regions of homology between the BNIP-2 and Cdc42GAP proteins. Identical residues are denoted by *asterisks*, and conserved changes are shown by *colons*. Alignment was done using the Blossum 62 matrix of the BLAST (NCBI server) and SIM programs (ExPASy server). The area *shaded in black* in BNIP-2 is an EF-hand domain (adapted from Low *et al*., 1999).

1.3.1 BNIP-2 and Cdc42GAP

BNIP-2 is originally shown to be an interacting protein for both the viral E1B p19KDa protein and the Bcl-2 anti-apoptotic protein. It is shown that BNIP-2 could be phosphorylated by FGFR-1 *in vivo* and *in vitro* (Low *et al*., 1999). BNIP-2 shares a region of homology with the noncatalytic domain of Cdc42GAP, a GTPase-activating protein for the small GTP-binding molecule, Cdc42. BNIP-2 and Cdc42GAP could directly bind to each other through this homologous region-BCH domain. They also compete for the binding to the same target, Cdc42 (Low *et al*., 2000b). BNIP-2 stimulates the intrinsic GTPase activity of Cdc42 *via* a novel arginine-patch motif.

Tyrosine phosphorylation of BNIP-2 severely impairs its association with Cdc42GAP and its induced GTPase-activating protein–like activity toward Cdc42 (Low *et al*., 2000a).

Cdc42GAP (also known as p50-RhoGAP) is the first identified Rho GTPase-activating protein. *In vitro*, Cdc42GAP strongly stimulates the Rho GTPase activity towards Cdc42 but is much less effective to other Rho GTPases, which indicated that Cdc42GAP is a specific GAP for Cdc42 (Peck *et al*., 2002; Garrett *et al*., 1989). Cdc42GAP contains a proline-rich sequence which could be the potential target for SH3 and WW domain containing proteins. It has been shown that Cdc42GAP binds p85α, the regulatory subunit of phosphatidylinositol 3'kinase (PI3K). This interaction indicates that Cdc42GAP may function as a link between Cdc42 and other signaling pathways (Peck *et al*., 2002; Barfod *et al*., 1993; Ridley *et al*., 1993; Lancaster *et al*., 1994). Cdc42GAP also contains a BCH domain. Although it can bind Cdc42, it is catalytically inactive (Low *et al*., 2000a).

1.3.2 The BCH domain, a novel protein-protein interaction domain

BCH domain was first demonstrated as a novel protein-protein interaction domain when it was found that BNIP-2 and Cdc42GAP could form homophilic and heterophilic complexes *via* their conserved BCH domains. Molecular modeling of the BNIP-2 BCH homodimer complex and subsequent deletion mutagenesis helped to identify the region ^{217}RRKMP^{221} as the major BCH interaction site within BNIP-2 (Low *et al*., 2000b). Recently, it has been found that most of the BCH domains identified could form homophilic and heterophilic complexes with itself or other BCH domain containing proteins *via* the BCH domains. For example: BPGAP1 (reported

here) *vs.* Cdc42GAP, BNIP-2, BNIP-Sα and itself; BNIP-Sα *vs.* Cdc42GAP, BNIP-2 and itself (Low *et al*., 2000b; Zhou *et al*., 2002). Moreover, extensive database searches show that the BCH domain is highly conserved across species from *S. cerevisiae, P. falciparum, A. thaliana, C. elegans, and H. sapiens.* The conservation of BCH domain in various species indicates that BCH domain may have very important physiological functions (Low *et al*., 2000b).

1.3.3 BCH domain, a novel apoptosis-inducing sequence in BNIP-Sα

BNIP-Sα and β (for BNIP-2 Similar) are the two new members of BNIP-2 family. BNIP-Sα contains the conserved complete BCH domain at its C-terminus. And BNIP-Sβ is an isoform of BNIP-Sα lacks the full BCH domain as a result of an alternative RNA splicing that induces a nonsense intron (Zhou *et al*., 2002). It was shown that BNIP-Sα could induce apoptosis through BCH domain, whereas BNIP-Sβ did not have this apoptosis-inducing function because of the truncated BCH domain. Deletion studies in the BCH domain of BNIP-Sα were performed to recognize the motif that is responsible for BNIP-Sα-induced apoptosis. It was discovered that the homophilic interaction of BNIP-Sα *via* the BCH domains coincides with its apoptotic effects, and that the deletion-S mutant (that did not form homophilic complex, motif "ATWYVKA") failed to exert proapoptotic activity whereas deletion mutants of region R (motif "RRLRK") and T (motif "ISLDQVH") were just as potent as the wild type. These reports indicate that BCH domain, at least in BNIP-Sα, represents a novel apoptosis-inducing sequence and may play important roles in regulating cell growth and development (Zhou *et al*., 2002).

1.3.4 Implication of BCH domain in cytoskeletion organization by targeting Rho GTPases

Previous studies of BCH domain have indicated that BCH domains in different proteins could confer versatile functions. Therefore, it should be significant to discover more potential roles of BCH domains in cell signaling and physiology. It has been shown that BCH domain-containing proteins, BNIP-2 and Cdc42GAP target Rho GTPases *via* their BCH domains. BNIP-2 has been found to regulate cell dynamics through targeting Cdc42 (Zhou *et al*., 2004, manuscript in preparation). Therefore, Rho GTPases have emerged as key regulators of the actin cytoskeleton. The implication of the interaction between BCH domain of BPGAP1 and Rho GTPases in the regulation of cell dynamics will be addressed in subsequent chapters.

1.4 Rho GTPase-activating proteins (GAPs)

The RhoGAP family is defined by the presence of a conserved RhoGAP domain in the primary sequences that consists of about 150 amino acids and shares at least 20% sequence identity with other family members. The RhoGAP domain is distinct from the GAP modules that specifically inactivates other classes of GTPases (*e.g.* Ras, Ran or ARF), and it is sufficient for the binding to GTP-bound Rho proteins and accelerating their GTPase activity. So far, over 30 RhoGAPs have been identified in Eukaryotes, from yeast to human. Recent human genome analysis has predicted that as many as 80 RhoGAPs are in *Homo sapiens*, while their cellular substrates, the Rho family GTPases, only contain 20 members. The overabundance of RhoGAPs evidently suggests that each RhoGAP might play a specialized role in regulating

individual Rho GAPase activity and in influencing their specific functions (Moon and Zheng, 2003; Bernards, 2003; Lamarche and Hall, 1994).

1.4.1 Overview of human RhoGAP-containing protein families

A number of RhoGAPs are differentially expressed and contain versatile GAP specificities and cellular functions (Table 1.1).

Name	In vitro specificity	Tissue distribution	Notes
Bcr	Rac and Cdc42	Predominently brain	Bcr-Abr oncoprotein in leukemias
Abr	Rac1, Rac2 and Cdc42	Predominently brain	Deleted in seven of eight informative cases of medulloblastoma
p85α, p85β	No activity	Ubiquitous	An adaptor subunit of PI3K; interacts with Cdc42Hs and Rac
p190RhoGAP	RhoA>Rac1 and Cdc42	Ubiquitous	A substrates of Src; regulate axonal growth and guidance and is required for normal neural development
p190-BRhoGAP	Rac, Rac1 and Cdc42	Ubiquitous	A regulatory molecule of cell and organism size by regulating RhoGTPase, which modulates CREB activity
p122 RhoGAP/ DLC1	RhoA/ND	ND/ubiquitous	Interact with and activate PLC-δ1/A candidate tumor-suppressor gene
ARAP1/ARAP3	Cdc42/RhoA, Rac and Cdc42	Ubiquitous, highly expressed in brain and spleen	PIP3 dependent ArfGAP activity

Table 1.1 Selected mammalian Rho GTPase-activating proteins (adapted from Moon and Zheng, 2003).

Cdc42GAP is the first RhoGAP protein identified (Barfod *et al*., 1993; Lancaster *et al*., 1994). It is expressed ubiquitously and is the smallest member of the RhoGAP family. Cdc42GAP contains an N-terminal BCH (Sec14-like) domain and C-terminal RhoGAP domain. It can stimulate the GTPase activity of Rho, Cdc42 and Rac and appears to have approximately equal affinity for the three Rho GTPases. But

it has been shown that Cdc42 is its preferred substrate in the *in vitro* GAP assay. Cdc42GAP does not show any differential binding affinity towards GDP-bound and GTP-bound forms of the Rho GTPases, while RasGAP shows 100-time greater binding affinity towards the GTP-bound form of Ras than for the GDP-bound form (Peck *et al*., 2002; Barfod *et al*., 1993; Ridley *et al*., 1993; Lancaster *et al*., 1994; Lamarche and Hall, 1994). Phospholipid affinity chromatography studies have recently shown that Cdc42GAP can bind phosphatidylinositol 3,4,5-trisphosphate (PtdIns-(3,4,5)P_3). It is possible that the interaction of Cdc42GAP with PtdIns-(3,4,5)P_3 involves the BCH (Sec14-like) domain. This interaction might lead to its recruitment to the plasma membrane and/or conformational changes that regulate its GAP activity. Another notable feature of Cdc42GAP is the proline-rich region upstream of GAP domain. Such Proline-rich motifs are thought to be the targets of SH3 and WW domains. In facts, Cdc42GAP *in vitro* binds the SH3 domains of c-src tyrosine kinase and p85, the regulatory subunit of phosphatidylinositol 3'-kinase (Lamarche and Hall, 1994). And these interactions may be involved in regulating cell signaling pathways and protein activities.

The p190-A RhoGAP was originally identified as a rat p120 RasGAP-interacting protein (Settleman *et al*., 1992). In humans, two homologs, p190-A RhoGAP and p190-B RhoGAP, contain N-terminal GTPase, and a C-terminal RhoGAP domains (Tikoo *et al*., 2000; Burbelo *et al*., 1995). *In vitro* GAP assay shows that p190-A has equal GAP activities for Rho, Rac and Cdc42 (Settleman *et al*., 1992), while *in vivo*, it has a preference towards Rho (Ridley *et al*., 1993). Its RhoGAP activity is regulated by phosphorylation. Upon stimulation by growth factors or cell attachment, tyrosine residues in the central portion of p190 are phosphorylated by kinases such as Src (Haskell *et al*., 2001). This phosphorylation induces a

conformational change in the p190 molecule that leads to the activation of its GAP activity. The activation of the GAP activity decreases Rho GTP levels and inhibits signaling to effector proteins, leading to a loss of stress fibers. Mouse knockout studies indicate that p190-A mediates Src-dependent adhesion signals involved in neuritogenesis through its effect on the intrinsic GTPase activity of Rho. These results are consistent with it being the major tyrosine phosphorylated protein in the brain (Brouns *et al*., 2001). Knockout of the mouse p190-B RhoGAP homolog dramatically decreases the size of mice, and the effect that appears to be mediated through a transcription factor, CREB (cAMP-responsive element (CRE) binding protein; Sordella *et al*., 2002). Cells derived from embryos lacking p190-B RhoGAP exhibit excessive Rho activity, are defective for adipogenesis, but undergo myogenesis in response to IGF-1 (Insulin-like growth factor) exposure. *In vitro*, activation or Rho-kinase by Rho inhibits adipogenesis and is required for myogenesis. The activation state of Rho following IGF-1 signaling is determined by the tyrosine-phosphorylation status of p190-B RhoGAP and its resulting subcellular relocalization. Moveover, adjusting Rho activity is sufficient to alter the differentiation program of adipocyte and myocyte precursors (Sordella *et al*., 2003).

The p85-α and p85-β proteins are known to function as regulatory subunits for the 110kDa catalytic subunit of phosphatidylinositol 3' kinase, which phosphorylates the inositol ring at the 3-position (Skolnik *et al*., 1991; Otsu *et al*., 1991). p85-α and p85-β subunits are highly homologous proteins containing an N-terminal SH3 domain, two SH2 domains and a region partially homologous to the consensus RhoGAP domain. Although p85-α does not have catalytic activity towards Rho GTPases (Zheng *et al*., 1993), it can interact with GTP-bound Cdc42 and Rac *in vitro* and co-immunoprecipitates with Cdc42 in a GTP-dependent manner (Zhang *et al*., 1994).

Thus p85 subunits may function as adapter proteins to localize phophatidylinositol 3'kinase activity to sites that active Cdc42 localizes.

α-chimaerin and β-chimaerin both contain a phorbol ester-C1 binding domain and a C-terminal RhoGAP domain, while splice variants of these genes contain additional N-terminal SH2 domains. α-chimaerin only has GAP activity toward Rac (Diekmann *et al*., 1991). The C2 sequences in β2-chimaerin, like those in protein kinase C member, bind phorbol esters and regulate its accumulation both at the plasma membrane and in the perinuclear compartment (Caloca *et al*., 1999).

DLC-1, the human analog of the rat p122 protein (Homma and Emori, 1995), is frequently deleted in hepatocellular carcinoma (Yuan *et al*., 1998). The RhoGAP domain of DLC-1 is located in its C-terminus and studies with the rat homolog reveal RhoGAP activity towards Rho *in vitro* and *in vivo*. DLC-1 can also interact and activate phospholipase C δ1 activity. Transfection studies indicate that the ability of p122 to induce cell detachment is mainly due to its RhoGAP activity (Homma and Emori, 1995; Sekimata *et al*., 1999).

PSGAP, a protein that interacts with PYK2 and FAD and contains multiple domains including a pleckstrin homology (PH) domain, a RhoGAP-activating protein domain and a Src homology 3 (SH3) domain. PYK2 interacts with PSGAP SH3 domain *via* the carboxyl-terminal proline-rich sequence. PSGAP is able to increase GTPase activity of Cdc42 and RhoA *in vitro* and *in vivo*. RYK2 can activate Cdc42 *via* inhibition of PSGAP-mediated GTP hydrolysis of Cdc42. Moreover, PSGAP is localized at cell periphery in fibroblasts in a PH domain-dependent manner. Over-expression of PSGAP in fibroblasts results in reorganization of cytoskeletal structures and changes of cellular morphology, which requires Rho GTPase-activating activity (Ren *et al*., 2001).

There are three other proteins that share high homology with PSGAP, GRAF, GRAF-2 and Oligophrenin-1. All of them contain a PH domain and RhoGAP domain. In addition GRAF and GRAF-2 contain C-terminal SH3 domain. The overall structure of PSGAP resembles that of GRAF, and PSGAP shows high homology to GRAF with 68% identity in amino terminal region, 59% in PH domains, 69% in RhoGAP domains and 68% in SH3 domains. GRAF was originally identified as a chicken cDNA encoding a protein that specifically interacted with focal adhesion kinase and possessed GAP activity for Rho and Cdc42 *in vitro* (Hildebrand *et al*., 1996). Human GRAF-2, was identified in a yeast two-hybrid screen as being able to interact with the Rho effector protein, PKN-β(a novel isoform of PKN; also known as PRK) (Ren *et al*., 2001). *In vitro*, GRAP-2 can inactivate Cdc42 and Rho but not Rac. Current thinking suggests that these molecules may act as adapters to coordinate Rho activity with kinase signaling pathways.

ArhGAP6 was originally identified as a potential cause of microophthalmia with lenear skin defects syndrome (MLS) characterized by eye, skin and central nervous system malformations for it is found be be commonly deleted in MLS. But studies using transgenic mouse suggest that the mutation of ArhGAP6 gene is not responsible for MLS. ArhGAP6 contains a central RhoGAP domain and several sequences that can potentially target SH3 domains. ArhGAP6 has activity for RhoA but not Rac or Cdc42. But the transfection studies show that it can also regulate actin changes independent of the RhoGAP activity (Prakash *et al*., 2000).

RICH-1 was identified in a yeast two-hybrid screen using CIP4, a Cdc42 effector protein as the bait (Richnau *et al*., 2001). RICH-1 contains an N-terminal endopholin homology (EH, EPS15 homology) domain, a RhoGAP domain and several C-terminal proline-rich regions (Farsad *et al*., 2001). RICH interacts through

its proline-rich region with the SH3 domain of CIP4. *In vitro*, RICH-1 has GAP activity for Cdc42 and Rac but not for RhoA. These *in vitro* results are consistent with the *in vivo* over-expression experiments: It inhibits Rac-mediated membrane ruffling, but has no effect on Rho induced stress fiber formation (Richnau *et al*., 2001).

The ARAP subfamily in human is a subclass of Arf GAP-containing proteins, including ARAP1, ARAP2 and ARAP3 (Krugmann *et al*., 2002; Miura *et al*., 2002). All these three ARAP contain five PH domains, an ArfGAP domain and a RhoGAP domain (Miura *et al*., 2002). ARAP1 and ARAP3 have equal GAP activity toward Rho, Rac and Cdc42 *in vitro*. ARAP2 has no GAP activity because it lacks the catalytic arginine. The PH domain in ARAP1 and ARAP3 can interact PtdIns (3,4,5), thus increase Arf GAP activity. The ARAPs may function to regulate protein trafficking with actin cytoskeletal organization (Krugmann *et al*., 2002; Miura *et al*., 2002).

BCR contains multiple protein domains including a Dbl domain, a PH domain and a C-terminal RhoGAP domain. BCR has GAP activity specifically toward Rac (Diekmann *et al*., 1991; Ridley *et al*., 1993). The Dbl region of BCR has RhoGEF activity for Cdc42 and less for Rac and Rho. Since BCR contains both GAP and GEF activities, it may act as regulators of these GTPases (Chuang *et al*., 1995). ABR shares homologies with BCR in its Dbl, PH and RhoGAP domains. It has GAP activity for both Rac and Cdc42 (Tan *et al*., 1993; Heisterkamp *et al*., 1993).

Myosin IX class contain a typical myosin-like head domain, as actin binding region, IQ-calmodulin binding repeats, a protein kinase C regulatory domain and a RhoGAP domain in the C-terminus. Myosin IXb has been identified to have GAP activity specifically toward Rho. This GAP activity may be involved in transiently inhibiting Rho Activity during the early stage of cell spreading (Wirth *et al*., 1996;

Post *et al*., 1998).

MgcRacGAP contains an ERM domain, a protein kinase C-like cysteine-rich motif and a RhoGAP domain. MgcRacGAP has equal GAP activities toward Rac1 and Cdc42, while 30-time less the activity toward RhoA. MgcRacGAP may function to regulate the mitotic spindle formation (Toure *et al*., 1998; Hirose *et al*., 2001).

1.4.2 Function of Rho GTPase-activating proteins—Negative regulators of Rho GTPases

The Rho GTPase-activating proteins (RhoGAPs) are one of the major classes of regulators of Rho GTPases found in all eukaryotes that are crucial in cell cytoskeletal organization, growth, differentiation, neuronal development and synaptic functions (Moon and Zheng, 2003; Figure 1.7). Biochemically the RhoGAP domain binds to the GTP-bound Rho proteins and stimulates their intrinsic GTPase activity. The mechanism of how RhoGAPs to catalyze the hydrolysis of GTP-bound Rho GTPases to GDP-bound Rho GTPases has been well elucidated through the structural studies.

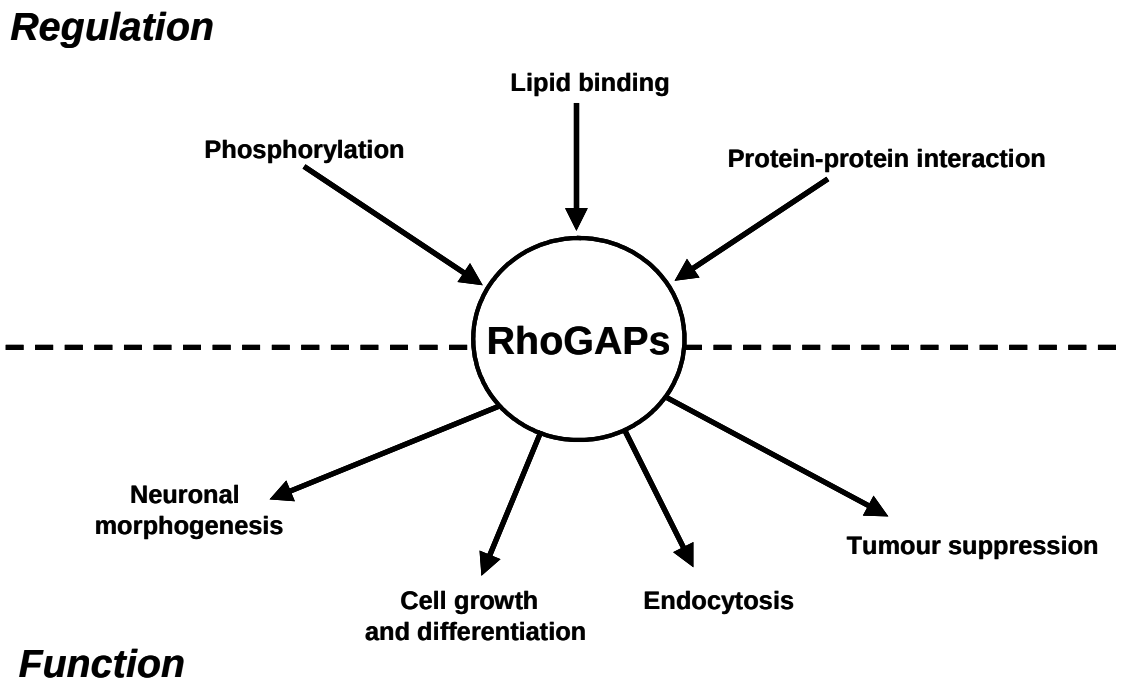

Figure 1.7 Summary for regulation and function of Rho GTPase-activating proteins. Mechanisms of regulation for RhoGAPs include: regulation by phosphorylation, by lipid binding, and by protein-protein interaction. RhoGAPs play multiple roles in regulating neuronal morphogenesis, cell growth and differentiation, endocytosis and tumor suppression (adapted from Moon and Zheng, 2003).

1.4.2.1 Structural basis of Rho GTPase-activating reaction

The sequences of RhoGAP domains are different from those of other classes of GAPs such as Ras GTPase-activating proteins (RasGAPs), while the tertiary folding pattern and the basic GTPase-activating mechanism of the RhoGAP domain are similar to that of RasGAP (Bax *et al*., 1998; Rittinger *et al*., 1998). The RhoGAP domain consists of nine α helices and a highly conserved arginine residue is presented in a loop structure (Gamblin and Smerdon, 1998). The RhoGAP domain interacts with both the switch I and II region and the P-loop of Rho GTPases that constitute the GTP-binding core (Moon and Zheng, 2003). There is a conformational change when RhoGAP interacts with Rho GTPases and forms a transitional state (Nassar *et al*.,

1998). The catalytic arginine residue or RhoGAP is placed into the active site of Rho GTPase and stabilizes charges developed during the formation of this state. The arginine would interact with Gln61 of the GTPase, which is responsible for positioning a hydrolytic water molecule for catalysis (Moon and Zheng, 2003). Stabilization of this glutamine residue restricts the freedom of the water molecule and may reduce the energy barrier for GTP hydrolysis (Li *et al*., 1997; Longenecker *et al*., 2000).

1.4.2.2 Role of RhoGAPs in neuronal morphogenesis

Rho GTPases have important physiological roles on the regulation of the actin cytoskeleton during neuronal migration, axonal growth and guidance, and formation of synapses (Luo *et al*., 2000). Therefore, regulators of Rho GTPases play key roles in neuronal morphogenesis.

Oligophrenin-1, a RhoGAP family member that is highly expressed in human fetal brain was found to be associated with X-linked mental retardation, which implicates that RhoGAP may be involved in the regulation of nervous system development (Billuart *et al*., 1998).

Knockout experiments show that p190 RhoGAP is required for axon outgrowth, guidance and fasciculation, and neuronal morphogenesis. It has been found that p190 plays a negative role in the regulation of Rho-mediated actin assembly within the neuroepithelium; p190 could be an important regulator of Rho-mediated actin reorganization in neuronal growth cones. Furthermore, p190 appears to be one of the major Src kinase substrates in the neuron. These studies implicate p190 in neuronal development and neuritogenesis by mediating Src-dependent adhesion through balancing the Rho GTPase activity (Brouns *et al*., 2000; 2001).

1.4.2.3 Role of RhoGAPs in cell growth and differentiation

The GEFs and effectors of Rho GTPases have been shown to regulate cell growth and differentiation (Bishop *et al*., 2000; Van Aelst *et al*., 2000; Hall, 2000). Recently, RhoGAP are also put into list of the regulators of cell growth and differentiation, possibly through their ability to suppress Rho GTPase function.

Mice lacking p190-B, exhibit a severe reduction in thymus size and axon defects in the brain including a severe reduction in the major midline forebrain crossing tracts as well as a thinner cortex. Theses defects are associated with a failure in cell differentiation (Sordella *et al*., 2002). Interestingly, the knockout of p190-B shows similar effect on cell growth and differentiation as that of CRE-binding factor (CREB). This effect can be attributed to the enhanced Rho activity and its downstream Rho-Rho kinase-insulin receptor substrate-CREB signaling chain induced by the deletion of p190-B (Sordella *et al*., 2002). Therefore, p190-B is indicated as a regulator of cell differentiation in the thymus and brain, and cell size and animal size determination (Moon and Zheng, 2003).

1.4.2.4 Role of RhoGAPs in tumour suppression

Rho family GTPases have been implicated in many aspects of tumorigenesis (Sahai and Marshall, 2002). Increasing Rho GTPases expression or activity has been associated with multiple human tumour types. Therefore, it is logical to deduce that RhoGAPs might be negatively involved in tumour cell growth or progression by downregulating Rho GTPases activity.

DLC1 (also known as p122 RhoGAP) has been found deleted in 44% of primary hepatocellular carcinomas (HCC) and 90% of HCC cell lines (Yuan *et al*., 1998). GRAF, a focal adhesion kinase associated RhoGAP, is related with leukaemia. Deletion, point mutation and insertion of GRAF have been found in patients (Borkhardt *et al*., 2000). Overexpression of p190 RhoGAP in fibroblasts shows that it might act as a tumour suppressor. Either the N-terminal GTP-binding domain or the C-terminal RhoGAP domain of p190 repressed Ras-induced transformation, whereas blocking the expression of endogenous p190 or applying a mutant of the GTP-binding domain induced transformation (Wang *et al*., 1997; Moon and Zheng, 2003).

1.4.2.5 Role of RhoGAPs in endocytosis

There is evidence that RhoGAP could play a role in this process (Ellis and Mellor, 2000). RalBP1, also termed RIP1 or RLIP76, serves as an effector of Ral GTPase and contains a RhoGAP domain that is active towards Cdc42 and Rac1 (Canter *et al*., 1995; Park and Weinberg, 1995). Two EH (Eps15 homology) domain-containing proteins, POB (Partner of RalBP1) and Rel (RalBPa-associated Eps-homology domain), have been found to associate with the C-terminus of RalBP1 (Ikeda *et al*., 1998), whereas the N-terminal region of RalBP1 interacts with the plasma membrane clathrin adaptor AP2 complex (Yamaguchi *et al*., 1997; Jullien-Flores *et al*., 2000). EH domain-containing molecules are often involved in endocytosis. In fact POB has been shown to bind Epsin and Eps15, both of which regulate endocytosis of epidermal growth factor (EGF) and insulin receptors (Nakashima *et al*., 1999). Therefore, RalBP1 might have a role in the endocytosis

process, although how its RhoGAP activity or the relationship with Rho GTPases fits in functionally remains to be seen (Moon and Zheng, 2003).

1.4.3 Regulation of RhoGAPs

1.4.3.1 Regulation by phosphorylation

It is indicated that RhoGAP activities might be modulated by protein kinase. For example, the activity of p190 RhoGAP is regulated by Src family tyrosine kinase. Activation of Src in cells leads to phosphorylation of two tyrosine residues of p190 (Roof *et al*., 1998; Hu and Settleman, 1997). Upon phosphorylation, p190 interacts with p120 RasGAP through an SH2 domain-phosphotyrosine interaction. This interaction could activate its GAP activity towards Rho and induce disruption of actin stress fibers, reduction of focal contacts and impairing the ability of the cell to adhere to fibronectin. This cellular effects is similar with that of inactivation of Rho GTPases (Haskell *et al*., 2001).

Recent studies indicate another mechanism of the regulation of p190-B by phosphorylation. It is found that upon IGF/Insulin stimulation, p190-B could be phosphorylated by the insulin and IGF receptors which are both tyrosine kinases. Although the activity of p190-B remains unchanged after being phosphorylated, the phosphorylation leads to its subcellular translocation to a lipid raft-enriched plasma membrane region, where active GTP bound Rho is localized. Thus the activity of Rho GTPases is greatly decreased after the p190-B is translocated to the membrane (Sordella *et al*., 2003).

1.4.3.2 Regulation by lipid binding

Chimaerin has specific Rho GTPase acitivity towards Rac. It can be regulated *in vitro* by phospholipids through its cysteine-rich (CR) domain. CR domain might also act as a phorbol ester/diacylglycerol (DAG) receptor site (Caloca *et al*., 1997; 1999; 2001). Interestingly, CR domain inhibits the GAP activity of Chimaerin, suggesting that an autoinhibitory mechanism involved which may be through the intramolecular interaction between its N-terminus and C-terminal GAP domain. Studies indicates that Chimaerin interacts lipids and through its noncatalytic motif and this interaction might have regulatory effects on both its intracellular location and biochemical GAP activity (Caloca *et al*., 1997; 1999; 2001).

1.4.3.3 Regulation by protein-protein interaction

CdGAP contains GAP activities towards Cdc42 and Rac1, but not RhoA. There are a RhoGAP domain at its N-terminal end and multiple proline-rich motifs at the C-terminal end. CdGAP can interact through its proline-rich regions with the SH3 domain of intersectin which is an endocytic scaffolding protein. This interaction induces the inhibition of the GAP activity of CdGAP both *in vitro* and *in vivo*. The possible mechanism of this inhibition might be: the interaction between CdGAP and intersectin induces a conformational change in CdGAP, resulting in the inactivation of GAP domain. This case indicates that protein-protein interaction may regulate the GAP activity (Jenna *et al*., 2002).

1.4.4 RhoGAP: A signal convergent or divergent point

Normally RhoGAPs contain other functional domains or motifs besides the RhoGAP domain. These domains and motifs include protein kinase domain, RhoGEF and ArfGAP domains as well as SH2, SH3, PH and CR domains. Therefore the activity of RhoGAP domain may be regulated, sometimes be inhibited by the other domains or motifs located at the same protein (Moon and Zheng *et al*., 2003).

For example, BCR and ABR both contain a Dbl domain, a PH domain and a C-terminal RhoGAP domain. Dbl and PH domain form a combination that may activate Rho proteins as a GEF. Thus BCR and ABR potentially contain both GEF and GAP activities (Chuang *et al*., 1995; Voncken *et al*., 1995). Another example is the ARAP subfamily of RhoGAPs which contain an ArfGAP domain that may interact with Ras, while also contain an RhoGAP domain. These two domains cooperate in mediating cytoskeleton reorganization and cell morphological changes upon interacting with the PI3K product, PI(3,4,5)P3. Therefore, ARAPs can potentially initiate multiple signals and may regulate the activities of three classes of small GTPases (Krugmann *et al*., 2002; Miura *et al*., 2002).

1.5 Proline-rich sequence, a potential target for SH3 and WW domains

1.5.1 Proline-rich sequences

Proline-rich sequences are often found in many proteins that function as docking proteins for signaling modules (MacArthur *et al*., 1991; Kay *et al*., 2000). Proline is involved in the recognition with many important protein-protein interaction

modules. It contains several features that distinguish it from the other 19 amino acids including: it has an unusual shape of pyrrolidine ring; the conformation of its dihedral angles is constrained imposed by the cyclic side chain; it has specific secondary structural preferences; its amide nitrogen can be substituted; and it has a relative stability of the *cis* isomer in a peptide bond. The recognition domains of proline-rich sequences take use of some combination of these distinctive features of proline, thus achieve specific binding to them (Zarrinpar *et al*., 2003).

To bind to signaling domains, proline-rich motifs have some specific properties: One property is it can form polyproline type II (PPII) helix (MacArthur *et al*., 1991; Williamson *et al*., 1994; Siligardi *et al*., 1995). The second unique property is that it is the only naturally occurring N-substituted amino acid. The third property is that proline also distinguishes from other natural amino acids in its ability to exist stably as a *cis* isomer about the peptide bond (Kay *et al*., 2000; Petrella *et al*., 1996). Therefore, a lot of chemical properties of proline make it stand out from the other 19 naturally occurring amino acids, which are exploited by the proline recognition domains. If the property of proline involved in a recognition event is sufficient to distinct among the natural 20 amino acids, the interaction doesn't have to have high affinity to be selective. There are plenty of proline-based recognition motifs there for the weak but specific interaction in intracellular signaling pathways (Zarrinpar *et al*., 2003).

1.5.2 Proline recognition domains

Domains that bind proline-rich sequences are important to regulate many intracellular signaling complexes and pathways. Proline-rich sequences are very important in biology. It has been found that proline-rich sequences are the most

common sequence motif in the *Drosophila* genome and the second most common in the *Caenorhabditis elegans* genome. Domains that have been defined as proline recognition domains are mainly including: Src homology (SH3), WW (named for a conserved Trp-Trp motif), and Enabled/VASP homology (EVH1) domain (Whisstock and Lesk, 1999).

Proline recognition domains are usually found in some multidomain signaling proteins. Through the proline targeted interaction, those proteins are often involved in cell growth (Rozakis-Adcock *et al*., 1993; Lowenstein *et al*., 1992; Buday *et al*., 1993), cytoskeletal rearrangements (Renfranz *et al*., 2002; Holt *et al*., 2001), transcription (Sudol *et al*., 2001), postsynaptic signaling (Ball *et al*., 2002; Tu *et al*., 1998) and other important cellular processes (Mcpherson *et al*., 1999). And these interactions can also have some regulation functions. These functions are often through autoinhibitory interactions by competing binding events (Nguyen and Lim, 1997).

1.5.2.1 SH3 domain

SH3 domain is the first and best characterized proline recognition modules (Mayer *et al*., 2001). It contains about 60 amino acids and normally plays assembly or regulatory function in cell signaling pathways. Grb2, which contains SH3 domains, plays an assembly role in cell signaling. It is involved in the p21 Ras-dependent growth factor signaling pathway (Lowenstein *et al*., 1992). Grb2 contains a Src homology (SH2) domain, flanked with two SH3 domains. Upon growth factor stimulation, receptor tyrosine kinases are phosphorylated, which cause the phosphorylation of other membrane-associated proteins. Therefore Grb2 is recruited to the membrane by the interaction through its SH2 domain to the phosphorylated membrane proteins. The

Grb2 SH3 domains bind to proline-rich sequence in SOS, which is a guanine nucleotide exchange factor (GEF) for Ras, thus recruiting SOS to the membrane. Since Ras is membrane-localized, the translocation of SOS helps to catalyze the cycle from GDP-bound Ras to GTP-bound Ras. The activation of Ras will stimulate a mitogen-activated protein kinase (MAPK) cascade, regulating cell growth and differentiation (Rozakis-Adcock *et al*., 1993; Buday *et al*., 1993). SH3 domain containing proteins play similar recruitment roles in a variety of biological processes such as endocytosis (McPherson *et al*., 1999) and cytoskeletal dynamics (Buday *et al*., 2002).

SH3 also has regulatory functions. One example is the Src family of tyrosine kinase. Src kinases contain an SH2 domain, an SH3 domain and a kinase domain (Nguyen *et al*., 1997; Moarefi *et al*., 1997). In basal conditions, the kinase domain is kept at an inactive conformation through the SH2 and SH3 involved intramolecular interaction. When the SH2 or SH3 domain interacts with other partners, the autoinhibitory interaction will be disrupted, which induces the activation of the kinase domain. The mechanism of this regulatory role is that interactions of SH2 or SH3 domain with external interacting partners is related with the activation of the kinase, which leads to the precise regulatory function (Zarrinpar *et al*., 2003).

The basic fold of SH3 domains contains five anti-parallel beta-strands to form two perpendicular beta-sheets. The ligand-binding site consists of a hydrophobic patch that contains a cluster of conserved aromatic residues and is surrounded by two charged and variable loops (Zarrinpar *et al*., 2003).

SH3 domains generally bind to proline-rich peptides that form a left-handed poly-proline type II helix, with the minimal consensus Pro-x-x-Pro (Aasland *et al*., 2003). Each proline is usually preceded by an aliphatic residue. Each of these aliphatic-Pro pairs binds to a hydrophobic pocket on the SH3 domain. The detailed requirements of SH3

domain binding to its ligand have been examined by numerous approaches including phage display combinatorial peptide chemistry, nuclear magnetic resonance and crystal structure analysis. From this, two classes of SH3 domains have been defined (Class I and Class 2) which recognize RKxxPxxP and PxxPxR motifs respectively (Table 1.2). The ligand can, in principle, bind in either orientation (Feng *et al*., 1994; Lim *et al*., 1994; Lim *et al*., 1994; Yu *et al*.,1994). Directionality is conferred by the interaction of the Arginine or Lysine residue with the charged outer face of the SH3 domain while the tandem prolines bind within two hydrophobic pockets of the SH3 domain. An additional non-Pro residue, frequently Arginine, can form part of the binding core and contacts the SH3 domain. Such peptides usually bind to the SH3 domain with Kds in the mM range. The binding affinity and specificity can be markedly increased by tertiary interactions involving loops on the SH3 domain. In a few proteins, SH3 domains have been observed to bind in an unconventional non-PxxP manner. In these cases, either an alpha helical element or a tandem tyrosine motif interacts with a site on the SH3 domain that is either distinct or overlapping with the classical PxxP binding cleft (Wollacott *et al*., 2001; Brannetti *et al*., 2000; Musacchio *et al*., 1992; Ren *et al*., 1993).

Proteins with SH3 domains	**Peptide ligand motif**
Src	RPLPPLP or PPVPPR
Yes	RxLPxLP
Lyn	RxxRPLPPLPxP
Abl	PPPψPPPPψP
PI3K	RxxRPLPPLPP
PLCγ	PPVPPRP
Cortactin	+PPψPψKP
p53BP2	RPxψPψR
Grb2A	PLPxLP
CrkA	PxLPx(K/R)
Amphiphysin I	PxRPx(R/H) (R/H)
Nck, SH3B	PxPPxRxxSL
CAP, SH3C	PxPPxRxSSL

Table 1.2 SH3 domain-containing proteins and their ligand binding motifs (adapted from Kay *et al*., 2000). Residues labeled ψ, +, and x correspond to aliphatic (A, I, L, V, and P), positively charged (R, K, or H), and any residues, respectively.
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1.5.2.2 WW domain

WW domains are small 38 to 40 amino acid residue modules that have been implicated in binding to proline-rich sequences. WW domain is folded into a three-stranded β-sheet structure. A flat binding surface for the proline-rich ligand is formed by conserved hydrophobic residues. The domain name is derived from two conserved tryptophan residues spaced 20 to 22 residues apart within the consensus sequence (Sudol and Hunter, 2000).

WW domains bind protein with the specificity of containing short linear sequence motifs. There are mainly four groups of WW domain targeting sequences (Table 1.3). Group I binds polypeptides with the minimal core consensus Pro-Pro-X-Try; Group II is Pro-Pro-Leu-Pro; Group III target polyproline sequences flanked by Arg or Lys; Group IV is represented by WW domains which preferentially target phosphor-Ser-Pro or phosphor-Thr-Pro containing ligands (Sudol and Hunter, 2000).

Representative Proteins of the Group	**Consensus Sequence of the Recognized Peptide**	**Abbreviation of the Sequence Motif**	**Representative Ligands (Cognate or Putative)**
Group I YAP65, Nedd4/Rsp5p, Dystrophin	PPxY	"PY" or "PPxY"	PEBP2 transcriptional coactivator, ENac sodium channels, β-Dystroglycan
Group II Formin Binding Proteins, FE65	PPLP usually within long polyproline sequences	"PPLP"	Formin, Mena
Group III Formin Binding Proteins, Npw38/PQBP-1	[R]-R/K/x-PP or PP-R/x-[R]	"PPR"	Splicing factors: SmB, SmB', U1C, NpwBP
Group IV Pin1/Ess1p, (Nedd4/Rsp5p)b (Prp40p)	(phospho-S/T)P	"(p-S)P or (p-T)P"	RNA Polymerase II Cdc25C phosphatase

Table 1.3 Classification of WW domains based on their ligand specificity (adapted from Sudol and Hunter, 2003).

1.5.3 Nedd4, a WW domain-containing protein

1.5.3.1 Ubiquitin system

The selective turnover of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin system. In this system, proteins are targeted for degradation by covalent ligation to ubiquitin, a highly conserved small protein (Sakamoto, 2002; Hershko and Ciechanover, 1998). Ubiquitin-mediated protein modification regulates numerous cellular processes including protein turnover and trafficking, cell-cycle progression, signal transduction, transcriptional regulation, receptor down-regulation, endocytosis and virus budding (Hershko and Ciechanover, 1998; Harvey *et al*., 2002). The conjugation of ubiquitin to a protein substrate contains multisteps (Hochstrasser *et al*., 1996; Weissman *et al*., 2001). In the first step that is ATP-dependent, a thioester is formed between the carboxyl terminus of ubiquitin and an internal cysteine residue of a ubiquitin-activating enzyme (E1). In the second step, the activated ubiquitin is transferred to a specific cysteine of one of several ubiquitin-conjugating enzymes (E2). In the third step, E2 enzymes may donate ubiquitin directly to protein substrates, leading to branched protein conjugates. Through this conjugates, the carboxyl terminus of ubiquitin is linked by an isopeptide bond to specific internal lysine residues of target proteins. Substrates can also be recognized by associated substrate-recognition proteins named E3 proteins or ubiquitin-protein ligases. E3 proteins play a major role in defining the substrate specificity of the ubiquitin system. Hect (homologous to E6-Ap Carboxyl terminus) domain containing proteins are a major class of E3 protein. For example, Nedd4 family proteins belong to this class (Huibregtse *et al*., 1995; Scheffner *et al*., 1995).

1.5.3.2 Nedd4, a ubiquitin ligase

Nedd4 belongs to a family of ubiquitin-protein ligases. Our current study of BPGAP1 indicated that Nedd4 is its interacting partner, which will be described later. It was originally identified as a developmentally regulated gene and was highly expressed in the mouse embryonic central nervous system. Further studies showed that Nedd4 was widely expressed at varying levels in several embryonic and adult tissues while not restricted to the embryonic central nervous system. It contains 2-4 WW domains, a carboxyl-terminal Hect domain and in most cases an amino-terminal C2 domain (Sakamoto, 2002; Hershko and Ciechanover, 1998). It has been found that the second WW domain of mouse Nedd4 can bind Pro-Pro-X-Tyr and p-Ser-Pro/p-Thr-Pro containing peptides.

The Nedd4 family proteins have been implicated in a variety of cellular processes including endocytosis, TGF-β (transforming growth factor β) signaling, virus budding, transcription and protein trafficking (Huibregtse *et al*., 1995; Scheffner *et al*., 1995). Nedd4 participates in the ubiquitination of several phosphoporteins (Figure 1.8), for example, the Cdc25 phosphatase and the Fur4p uracil permease (Marchal *et al*., 2000). Nedd4 can also induce ubiquitination of the amiloride-sensitive sodium channel, ENaC, in a phosphorylation-independent manner (Chang *et al*., 2000).

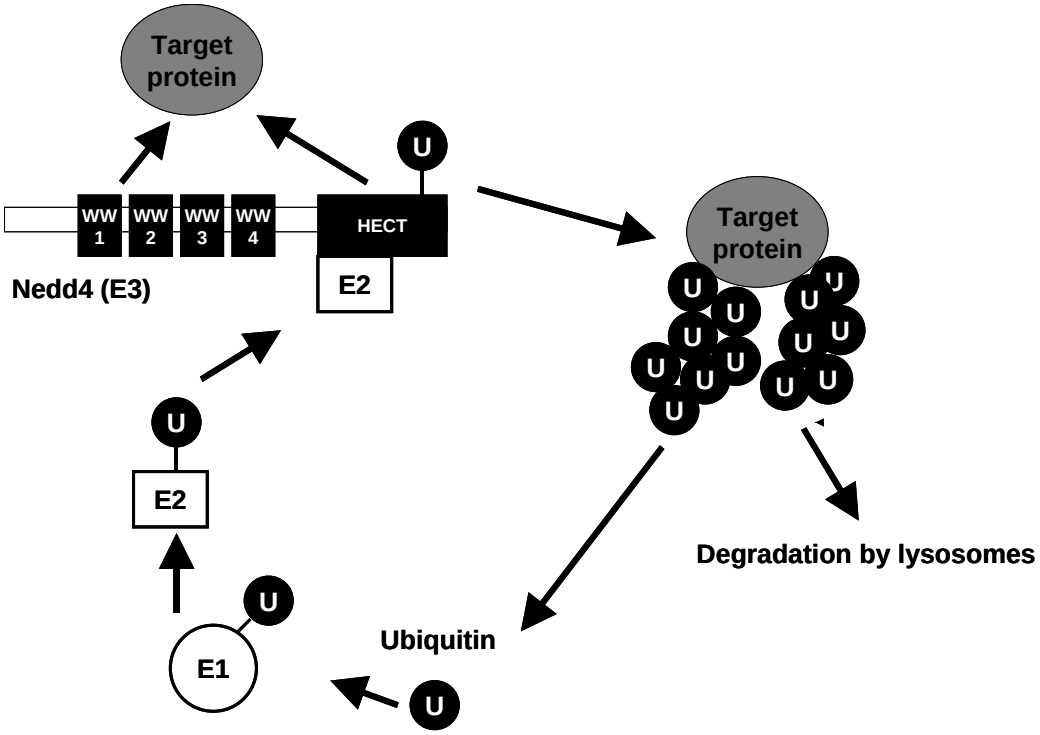

Figure 1.8 Protein degradation by Nedd4 dependent ubiquitination. Ubiquitin is transferred *via* E1 and E2 to a cysteine of Nedd4, which binds the target protein. The target protein is then ubiquitinated, leading to a rapid degradation by the lysosome.

1.6 Cell culture system was used to study the cellular and physiological functions of BPGAP1

Appropriate research model must be well-chosen in order to study gene functions. Over the last three decades, cell and tissue culture methods have been refined and have now become an essential tool in biomedical research. This system is widely used because it allows the study of single cellular functions under controlled environmental conditions, and it can exclude the influence of other organs and of the circulatory and immune system, thus providing the possibility to study direct effects on a cell population. Cell line system also has some advantages such as: it is

homogeneous and accessible; it is easy to be transfected; it is convenient to be applied for microscope observation for cell morphology and cell migration studies.

Currently, 293T cell line is widely used in cell signaling and protein function studies. Transient transfection into 293T cells is a convenient way to overexpress and obtain both cellular and extracellular (secreted or membrane) proteins. 293 is a human renal epithelial cell line which is transformed by adenovirus E1A gene product. 293T is a derivative which also express SV40 large T antigen, allowing episomal replication of plasmids containing the SV40 origin and early promoter region (http://cbr.med.harvard.edu/investigators/springer/lab/protocols/jun_293T.html). MCF7 is human breast adenocarcinoma cell line obtained from a plural effusion described as retaining many characteristics of a differentiated mammary epithelium. These cells are often used in models of human breast cancer and oestrogen receptor studies. Because of their flat cytoplasm, MCF7 cells are often used in various imaging and immunofluorescence studies, for example, apoptosis and cell morphology studies (http://home.t-online.de/home/I-A-Z/albino.htm).

1.7 Objectives of this study

The BCH domain has been proved, at least now, as a protein-protein interaction domain and apoptosis-inducing domain. Furthermore, it targets Rho GTPases (Zhou *et al*., 2004, manuscript in preparation) and may have significant implications as regulatory module in different cell signaling pathways and might be involved in different cellular functions.

The Rho GTPase-activating proteins (RhoGAPs) are one of the major classes of regulators of Rho GTPases found in all eukaryotes that are crucial in cell

cytoskeletal organization, growth, differentiation, neuronal development and synaptic functions.

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. Bioinformatics 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 diversed sequences in other regions. This family is named BPGAP family and one of its member BPGAP1 (for BNIP-2 and Cdc42GAP Homology (BCH) domain-containing, Proline-rich and Cdc42GAP-like protein subtype-1) is isolated and identified. Our current research focuses on BPGAP1. The overall objective of our research is to characterize the roles of different domains of BPGAP1 in regulating its cellular functions including to**:**

- clone the full-length BPGAP1 from mammalian cell lines.
- identify if there exist other isoforms of BPGAP1.
- characterize the expression profiles of BPGAP family proteins in different cell lines and organs.
- express BPGAP1 and study its properties of protein interactions and biochemical activities.
- investigate the cellular and physiological functions of BPGAP1 in regulating cell dynamics such as cell morphology and cell migration, and
- elucidate potential mechanisms underlying the observed cellular effects.

Chapter 2

Materials and Methods

2.1 Blast search for BPGAP1

To identify novel proteins containing GAP domains, 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 interactive BLAST" against the current non-redundant sequence as well as human and mouse EST databases (http://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.) while genome organization of BPGAP1 was analysed using the Genomatix online tool, *ElDorado* (http://www.genomatix.de/).

2.2 RT-PCR cloning of BPGAP1 isoforms and plasmid constructions

RT-PCR (reverse transcription PCR) and normal PCR were used for isolation of BPGAP1 and BPGAP2, BPGAP5 cDNAs. Several expression vectors including pXJ40 and pGEX4T1 were used for the cloning of BPGAP family members. Automatic DNA sequencing was then used for confirming the sequences.

2.2.1 RNA isolation and RT-PCR

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. Spectrophotometric method was used to determine the concentration and purity of the RNA. The RNA was stored at -80ºC. 5 µg of this RNA was subjected to the first-strand cDNA synthesis with Expand Reverse Transcriptase Kit (Roche

Molecular Biochemicals) primed with oligo(dT) (Operon) for 60 mins 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 *Taq* polymerase enzyme (Roche Molecular Biochemicals) using specific primers corresponding to the putative sequence BAA91614. The two primers used for PCR in two directions were list at Table 2.1. These PCR primers contained *Hind*III and *Xho*I restriction sites on the forward and reverse primers, respectively, to facilitate their subsequent cloning in the pXJ40 vectors. PCR conditions were as following: 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. The forward PCR primers for the cloning to pGEX4T1 vectors contained *EcoR*I restriction site instead of *Hind*III, which was listed at Table 2.1.

2.2.2 Cloning of the BPGAP1 constructs

2.2.2.1 Cloning of BPGAP1 deletion fragments

The full-length PCR products were gel-purified (Qiagen) and cloned into a FLAG epitope-tagged, GFP-tagged or HA-tagged expression vector, pXJ40 (Dr. E. Manser, Institute of Molecular and Cell Biology, Singapore) and GST-tagged expression vector pGEX4T1 respectively. The resulting bacteria colonies were screened by double restriction digestion of *Hind*III/*EcoR*I and *Xho*I respectively. 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. The PCR primers used for domain fragment amplification for pXJ40 vector cloning were listed

at Table 2.1. Of the primers used, BPGAP1 full-length, PC and C fragments share the same reverse primers whereas its full-length, NP, NNP fragments share the same forward primers.

2.2.2.2 Cloning of BPGAP1 deletion mutants by inverse-PCR

For generation of deletion mutant P1, inverse-PCR was carried out as previously described (Low *et al*., 2000a) to exclude region of interest proline-rich sequence. Primers used for this PCR were listed at Table 2.1. The resulting bacteria colonies were screened by single restriction digestion with *EcoR*V.

2.2.2.3 Point mutation by site-directed mutagenesis

Point mutation R232A was performed by site-directed mutagenesis as previously decribed (Low *et al*., 2000a). The primers used were listed at Table 2.1.

The PCR conditions for temperature cycling were: initial denaturation 95°C, 30sec; subsequent cycling (12 cycles) at 94 °C, 30 s; annealing at 55 °C, 1 min; extension at 68 °C, 2 min; and final extension at 68 °C, 7 min.

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 (Sambrook J and Russell DW, 2001). All plasmids were purified using Qiagen miniprep kit for subsequent use in transfection experiments. All the GST-tagged BPGAP1 constructs were got by subcloned from FLAG epitope-tagged constructs.

2.2.3 Expression vectors

2.2.3.1 pXJ 40 FlAG-tagged and GFP-tagged expression vectors

The pXJ40 FLAG epitope-tagged expression vector is a 4.3 Kb plasmid containing a multiple cloning site (MCS), e.g. *Hind*III, *Bam*HI and *Xho*I restriction sites. The MCS is a flanked by the CMV enhancer and promoter, β-globin intron and FLAG epitope-tagged sequence (DYKDDDDK) at the 5' end, and the SV 40 polyadenylation signal sequence on the 3' end. As for the GFP-tagged vector, the DNA sequence of green fluorescent protein was fused with the insert sequence at the 5' end. This recombinant DNA is then introduced into cells, which expresses the protein of interest as well as the tagged protein. To localize the protein of interest, the tag, which is now part of that protein, is localized. These two pXJ40 vectors also contain ampicillin resistance genes. T7 primer can be used to sequence the insert DNA in the direction from 5' to 3'.

2.2.3.2 pGEX4T1

The pGEX4T1 vector (Amersham Pharmacia Biotech) is a 4.95 Kb plasmid containing a MCS, e.g. *Eco*RI, *Bam*HI and *Xho*I. This vector contains the glutathione S-transferase (GST) gene from *Schistosoma japonicum* upstream of the MCS, as well as an ampicillin resistance gene. GST fusion proteins can be obtained by cloning the gene encoding the protein of interest into the MCS, and purified by conjugated to glutathione-sepharose beads. In addition, the pGEX 5' and 3' primers can be used to sequence the insert DNA.

2.2.4 Sequencing the cloned BPGAP1 constructs

For each construct, several clones were chosen and sequenced entirely in both directions. Automatic DNA cycle sequencings were performed using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystem). The PCR reaction for sequencing was set with 4 μL of Terminator Ready Reaction mix, 100ng of plasmid DNA template, 2 pmole of primer and H_2O to a final volume of 20 μL. Primers used for sequencing are T7, pGEX 5', and specific cloning primers of BPGAP1 indicated before. The sequence of T7 and pGEX 5' were listed at Table 2.1.

PCR was run for 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C for 25 cycles. The products were precipitated and washed by ethanol and analysed on a Applied Biosystems Inc. PRISM ^{TM} 377 automated DNA sequencer (PE Applied Biosystems, USA).

2.3 Semi-quantitative RT-PCR for gene expression analysis

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 products 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 house-keeping 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.

Primer name	**Primer sequence**
BCH-F	5’ CCGGGATCCGTTGTCACGTTCAGCTGCTGCCGGATG 3’
BCH-R	5’ CCGCTCGAGTCAGAGCTTCTCATCGTACCGCAAAACTTCG 3’
GAP-F	5’ CGGGGATCCCAAGGCGAACTCATCCCCCCTGTGCTGAGG 3’
GAP-R	5’ CCGCTCGAGTCACCCAGGTGCCTCCGGGGTGCTGAAG 3’
NP-R	5’ GCGCTCGAGTCAATTTTTGTCTTTGAGGTATTGCAG 3’
PC-F	5’ GCAGGATCCCAGAGCCTGCACGAGGGCCGGACG 3’
ΔP1,2-F	5’ CGCGATATCCACACAGCAGTTTGGCGTCAGTC 3’
ΔP1,3-R	5’ CGCGATATCCGTCCGGCCCTCGTGCAGGC 3’
ΔP2-R	5’ CGCGATATCGGTGGGAGGCGGCGTC 3’
ΔP3-F	5’ CGCGATATCAAGACACCACCGCCGC 3’
R232A-F	5’ CGAGGGCCTGTTCGCGAGATCCGCCAGC 3’
R232A-R	5’ GCTCCCGGACAAGCGCTCTAGGCGGTCG 3’
T7	5’ TAATACGACTCACTATAGGG 3’
pGEX 5’	5’ GGGCTGGCAAGCCACGTTTGGTG 3’
BPGAP1 FL-F(*Hind*III)	5’ GCCAAGCTTATGGCTGGCCAGGATCCTGCG 3’
BPGAP1 FL-F(E*coR*I)	5’ GCCGAATTCATGGCTGGCCAGGATCCTGCG 3’
BPGAP1 FL-R	5’ CCGCTCGAGCTAGAGGACGTCTTCTGGCTGCC 3’

Table 2.1 Primers used for the cloning of BPGAP1 full length, domain and mutant constructs.

2.4 Cell Culture and transfection

2.4.1 Cell Culture

Human breast cancer cells MCF7, human embryonic kidney epithelial cells 293T, human stomach cancer lines MCN45 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% CO_2 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).

2.4.2 Spectrophotometric quantitation of plasmid DNA for transfection

The concentration and purity of the plasmid DNA were determined before they were used for transfection. Plasmid DNA were isolated from *E. coli* by using the plasmid miniprep kit (QIAGEN). The absorbances of DNA solution at wavelengths of 260 nm (OD_{260}) and 280 nm (OD_{280}) were measured in quartz cuvettes using a spectrophotometer (Spectronic Genesys™ 5 Spectrophotometer). The DNA sample was diluted 1:100 with ddH_2O so that OD_{260} fell within 0.2 and 1.0 to ensure that the absorbance reading is within the linear range. Taking an absorbance of 1 unit of OD_{260} to be equivalent to 50 μg/ml of double-stranded DNA, the concentration of each DNA sample was calculated accordingly, taking into account the dilution factor:

DNA concentration (μg/ml) = OD_{260} ×dilution factor (100) ×50 μg/ml

The ratio of OD_{260} to OD_{280} indicated the quality of the DNA sample which could be applied for transfection. DNA of good quality should have a reading of 1.7 to 1.9. Any ratio below 1.7 indicated contamination with proteins and above 1.9 indicated RNA contamination.

2.4.3 Transfection

Cells at 90% confluence in 100-mm plates or 6-well plates were transfected with 5 μg or 2 μg of the indicated plasmids using FuGENE 6 Transfection Reagent, according to the manufacturer's instructions (Roche Molecular Biochemicals). The steps of transfection for 6-well scale were as following:

1. Preparation of cells for transfection. Cells were plated one-day before the transfection experiment. The appropriate plating density will depend on the growth rate and the condition of the cells. Cells that are 50–80% confluency were used on the day of the experiment. Most cell lines that are plated at 1–3 x 10^5 cells in 2 ml in a 35 mm culture dish (or 6-well plate) will achieve this density after overnight incubation.

2. Preparation of FuGENE 6 Reagent:DNA complex. In a small sterile tube, sufficient serum-free medium was added as diluent for FuGENE 6 Transfection Reagent, to a total volume of 100 μL. 3 μL of FuGENE 6 Reagent was added directly into this medium. The order of addition is critical. The serum-free medium must be pipetted into the tube first and be tapped gently to mix. 2 μg DNA solution (0.02–2.0 μg/μL) was added to the prediluted FuGENE 6 Reagent. The total volume of DNA solution was between 0.5–50 μL. The tube was gently tappted to mix the contents and then was incubate for a minimum of 15 minutes at room temperature.

3. Transfection of cells. The medium of cells were changed with 2 mL serum-free medium. And then the complex mixture from Step 2 was added to the cells. The wells were swirled to ensure even dispersal. After the cells were incubated at incubator for 3-6 hours, 2 mL 10% serum medium should be added. Cells were incubated at incubator for 24-48 hours and then could be lysed.

2.5 Precipitation/"pull-down" studies and Western blot analyses

Control 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 ethylenediamine tetraacetic acid (EDTA), 1% (w/v) sodium deoxycholate, 1% (v/v) Trition X-100, 50 mM sodium fluoride, 5 mM sodium orthovanadate, and a mixture of protease inhibitors (Roche Molecular Biochemicals). 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 (Low *et al*., 2000a). Samples were run in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and analyzed by Western blotting with FLAG monoclonal antibody (Sigma), HA monoclonal antibody (Sigma) and anti-Nedd4 polyclonal antibody (BD Biosciences Pharmingen) at 1:1000 to 1:3000 dilution, followed by anti-mouse, anti-rabbit horseradish peroxidase secondary antibodies and ECL detection (Amersham Pharmacia Biotech).

Other plasmids used in the "pull-down" studies were constructs of SH3 domains (gifts of Dr David Schlaepfer, *via* Dr Peter Sims, The Scripps Research Institute, La Jolla) and WW domains (courtesy of Dr Marius Sudol, Mount Sinai School of Medicine, New York) of various proteins as indicated.

2.5.1 Preparation of GST-fusion proteins for "Pull-down" experiments

The genes encoding the protein of interest were cloned into the pGEX4T1 vector for the production of GST fusion proteins. The constructs were subsequently transformed into *E. coli.* DH5α cells. 5 mL overnight cultures of the transformants

obtained were diluted in 250 mL of LB medium containing ampicillin and grown at 37°C with shaking to 0.3-0.6 at OD_{600}. 0.1 mM IPTG then was added to the culture for overnight induction, incubated at room temperature with shaking at 150 rpm. The induced *E. coli.* cells were harvested by centrifugation at 4 °C, frozen at –80 °C for at least 30 mins and then thawed on ice. The cells were resuspended in 10 mL of cold lysis buffer (10 mL PBS, 1% Triton-X, 1.52 % DTT (w/v), and multiple protein inhibitors (Roche Molecular Biochemicals)), and then applied the suspension to sonication. Sonication was carried out using intensity input at 3 on a MISONIX Sonicator XL 2020 for a total time 3 mins (15 sec for "pulse on" and 30 sec for "pause" for each cycle, 12 cycles totally). The sonicated cell lysates were centrifuged at 10,000x rpm for 1 h at 4 °C and the supernatant were removed to a new tube. To obtain purified GST fusion protein bound to the beads, the supernatant was incubated with glutathione-sepharose beads (Amersham Pharmacia Biotech) overnight at 4 °C The quality of the GST fusion proteins was verified by Comassie blue staining (0.2 % Comassie blue R-250, 40 % Methanol, 10% Acetic acid).

2.6 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 were separated using SDS-PAGE. The anti-Cdc42, RhoA, Rac1, HA, and Nedd4 antibodies were probed to reveal the binding of targets respectively.

2.7 Preparations of GST-fusion proteins for in vitro GTPase assay

2.7.1 Approach for the 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 [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 150 mM $NaCl_2$, 1 mM EDTA, multiple protein inhibitors (Roche Molecular Biochemicals), 0.1% (w/v) β-mercaptoethanol, and 0.1% (w/v) Triton-100]. Following centrifugation (10,000 rpm, 30 min, 4°C), the supernatants of lysates were mixed with glutathione-agarose beads (Amersham Pharmacia Biotech) and incubated at 4°C for overnight. Beads were washed 3 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 (Biorad).

2.7.2 Bradford assay for protein concentration measurement

2.7.2.1 Standard curves

To make the standard curve, dilute the 1mg/ml stock BSA solution in H_2O to 2, 4, 6, 8, 10, 12 μg/mL at the final volume of 800 μL. Add 200 μL concentrated Biorad Bradford reagent and incubate at room temperature for 5 minutes, then apply for spectrophotometer at A_{595}.

2.7.2.2 Determination of protein concentrations

Volume of 1, 2, 3, or 4 μL of concentrated unknown was added to water to bring volume up to 800 μL. 200 μL concentrated Biorad Bradford reagent was added to the solution made and was incubated at room temperature for 5 minutes, then was applied for spectrophotometer at A_{595}. Finally the concentration of the protein can be got using the BSA stand curve.

2.8 *In vitro* GTPase activity assay

GTPase activity assays were performed by using Enz-check™ Phosphate Assay Kit. Molecular Probes EnzChek® Phosphate Assay Kit (E-6646, Molecular Probes) provides a fast and sensitive spectrophotometric method for the quantitation of inorganic phosphate (Pi) in solution, including Pi released from enzymatic reactions. This kit enables continuous assay of reactions that generate Pi such as those catalyzed by ATPases and GTPases. The EnzChek phosphate assay is based on a method originally described by Webb (Webb, 1992). In the presence of Pi, the substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG) is converted enzymatically by purine nucleoside phosphorylase (PNP) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine (Figure 2.1). Enzymatic conversion of MESG results in a spectrophotometric shift in maximum absorbance from 330 nm for the substrate to 360 nm for the product. Sensitivity of the assay is in the range of 2 to 150 μM Pi (2 to 150 nanomoles Pi in a 1 mL volume), and the reaction can be performed over a pH range of 6.5 to 8.5, with the proper controls.

To monitor the rate of the phosphate release from GTP hydrolysis by recombinant Cdc42, RhoA or Rac1 (pGEX plasmids of these and Cdc42GAP are gifts of 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 (Wu *et al*., 2000) with some modifications. In brief, 0.5 nmol of purified GST-BPGAP1 full-length, domains or mutant proteins (in a volume of 15 µL), was mixed with 10 µL of 0.2mM GTP, 0.2 mL of 2-amino-6-mercapto-7-methylpurine ribonucleoside, 10 µL (1 unit) of purine nucleotide phosphorylase, and 0.78 mL of HEPES buffer (pH 7.5). The cuvette was immediately placed in the spectrophotometer to monitor absorbance at 360 nm, A_{360}. Meanwhile, 10 µL of 1 M $MgCl_2$ solution was added to 0.25 nmol of eluted GST-Cdc42, GST-RhoA and GST-Rac1 fusion proteins and incubated for 10 minutes at room temperature. When the first multiple turnover reached an equilibrium at A_{360}, the second mixture of small GTPase solution was added to initiate the reaction. The reading at A_{360} was recorded every 10 seconds.

2.9 *In vivo* GTPase activity and binding assay

GTP-bound Cdc42, Rac1, or RhoA was determined by specific binding to the p21-binding domain of PAK1 (GST-PBD; Bagrodia *et al*., 1995) or rhotekin (GST-RBD; Ren *et al*., 1999) (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 ethylene glycol-bis-(2-aminoethyl)-tetraacetic acid, 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 Molecular Biochemicals). Whole-cell lysates were also analyzed for the presence of expressed Cdc42, Rac1, RhoA, and BPGAP1 for normalization. For detecting binding of endogeneous Rho GTPases, the following antibodies were used: polyclonal anti-Cdc42 (Santa Cruz), polyclonal anti-RhoA and monoclonal anti-Rac1 (both from Upstate Biotechnology).

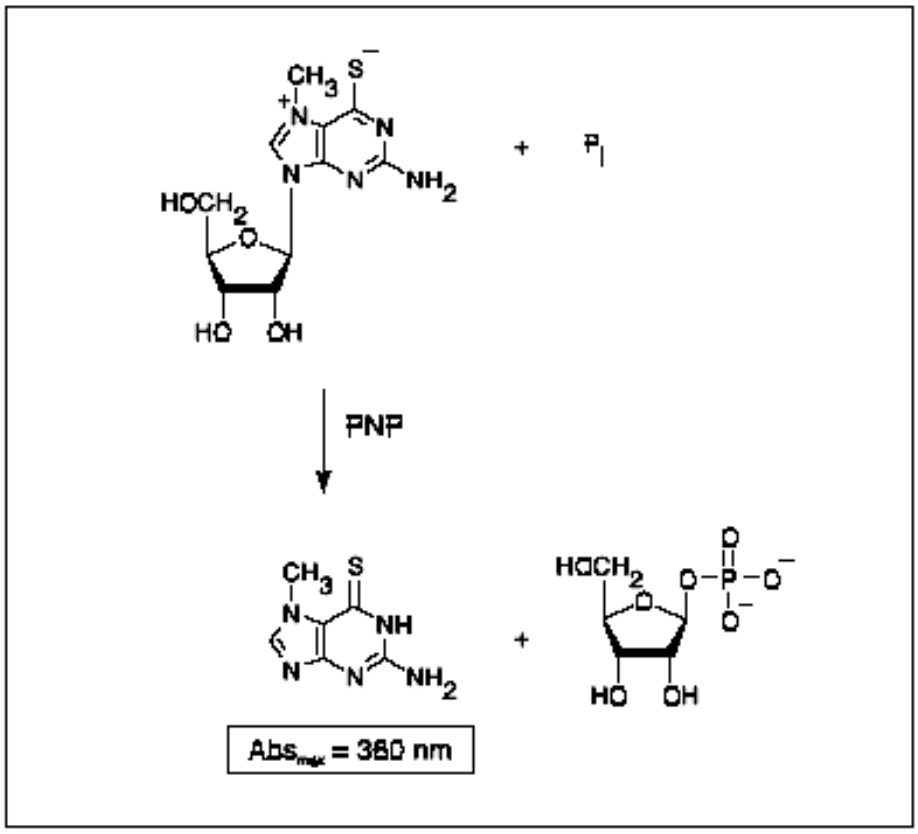

Figure 2.1 Principle of GTPase activity assays. The assays were performed using Enz-check^{TM} Phosphate Assay Kit. Enzymatic conversion of 2-amino-6-mercapto-7-methylpurine riboside (MESG) to ribose 1-phosphate and 2-amino-6-mercapto-7-methylpurine by purine nucleoside phosphorylase (PNP).The accompanying change in absorption at 360 nm allows quantitation of inorganic phosphate (Pi) consumed in the reaction (Adapted from Molecular probes products information).

2.10 Immunofluorescence

2.10.1 Indirect immunofluorescence by confocal microscope

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 (Lim *et al*., 2000). Briefly, the cells were washed with cold phosphate-buffered saline supplemented with 10 mM calcium chloride and 10 mM magnesium chloride (PBSCM) and fixed with cold 3% paraformaldehyde in PBSCM for 30 min at 4 °C. The fixed cells were washed twice with PBSCM, twice with PBSCM containing 50 mM NH_4Cl, and twice again with PBSCM. For cell permeabilization, cells on the coverslips were incubated with 0.1% saponin (Sigma) in PBSCM at room temperature for 15 min. The primary antibody for single was diluted to 1 mg/100 ml in FDB (7% (v/v) fetal bovine serum, 2% (w/v) bovine serum albumin in PBSCM), and each coverslip was incubated with 100 ml of diluted antibody for 1 h at room temperature. The coverslip was then washed three times for 2 min in 0.1% saponin-containing PBSCM before incubation with secondary antibodies. After the final wash (five times in 0.1% saponin containing PBSCM), each coverslip was prepared for microscopic examination by applying mounting medium (Crystal Mount, Biomeda). Fluorescent images were taken with a confocal laser microscopy system (Fluoview, FV300, Olympus). FLAG-tagged or HA-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.

2.10.2 Direct fluorescence by the expression of GFP-tagged contructs

For direct fluorescence, cells were seeded on coverslips in 6-well plates, transfected with various GFP-tagged expression constructs for 16-20 h. The morphology of cells was examined directly under a fluorescent microscope as previously described (Zhou *et al*., 2002). The 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.

2.11 Cell measurement

MCF7 cells were transfected with GFP control or GFP-tagged BPGAP1 fulllength, NP, and PC domains. After 20 h, the longest diameter (LD) and shortest diameter (SD) that bisected the center of cells and perpendicular to each were measured (Maddox and Burridge, 2003). The total cell areas and the length of the cell protrusion (PT) were also measured after image capturing as previously described and analysed using the Leica IM 1000 software. Measurements were means and standard deviations 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.

2.12 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 previous described (Koo *et al*., 2002). The lower surface of the filters was coated with 0.5 μg fibronectin (Sigma) as a chemoattractant. MCF7 cells transiently transfected with GFP vector, GFP-BPGAP1 full-length, different fragments or mutants were seeded at a density of 3 x 10^5 cells in 100 μL RPMI with 0.2 % BSA. The lower compartment was added with 600 μl of RPMI containing 10 % FBS. After incubation for 1 day at 37 °C in 5 % CO_2, 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 (Figure 2.2). Three independent experiments were performed for each experimental condition. The data were represented as the means of three independent experiments with standard deviations indicated. Statistical comparison was made using ANOVA (StatsDirect). *P* values of <0.01 indicate significant difference compared with the vector control.

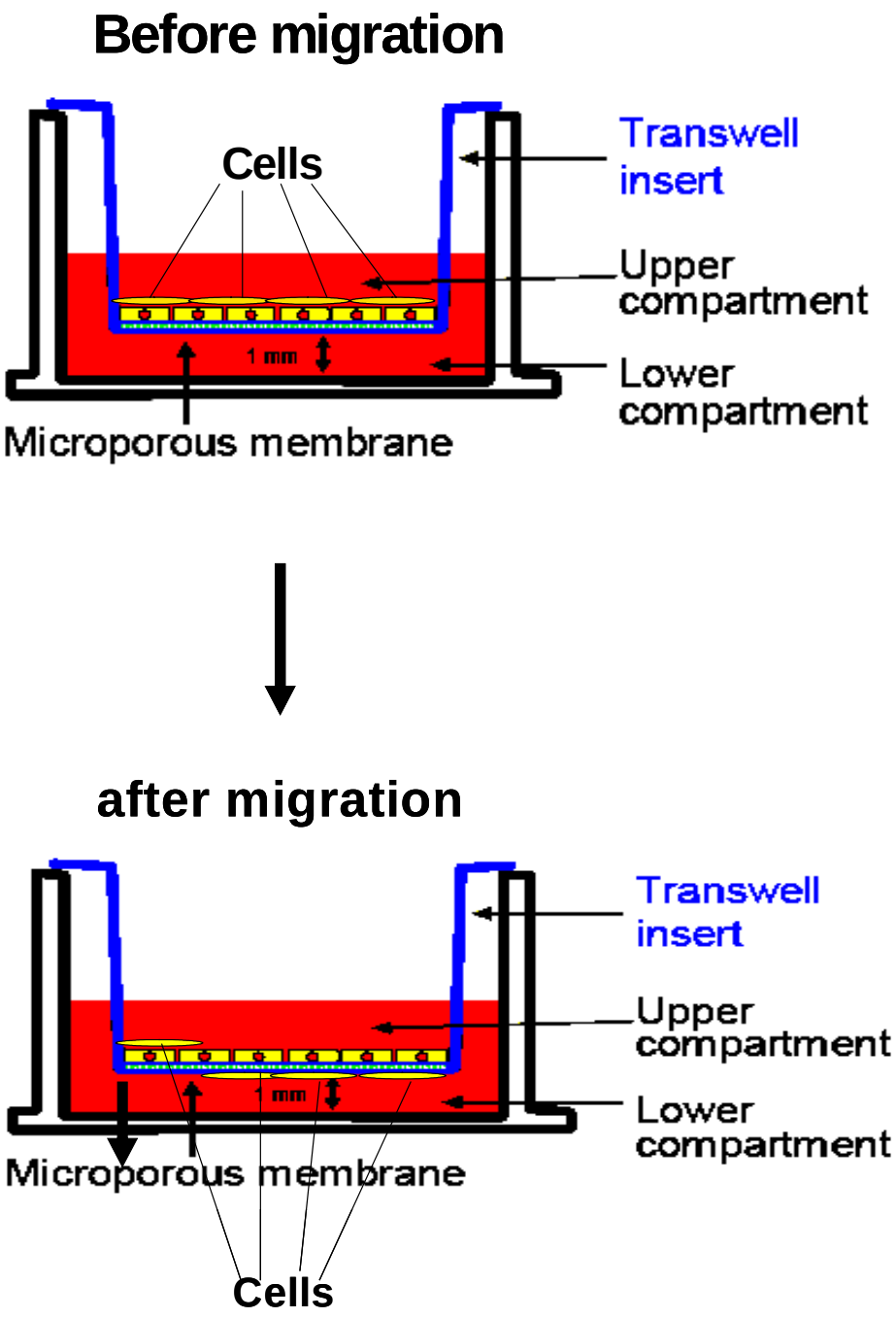

Figure 2.2 Cells migrate from the upper compartment to the lower compartment through a microporous membrane (Adapted from Products introduction of Corning and Transwell, New York). The ability of cells to migrate through coated filters was measured with a modified Boyden chamber. The lower surface of the filters was coated with 0.5 μg fibronectin (Sigma) as a chemoattractant. Cells were seeded at a density of 3 x 10^5 cells in 100 μL RPMI with 0.2 % BSA at the upper compartment. The lower compartment was added with 600 μl of RPMI containing 10 % FBS. After incubation for 1 day at 37 ºC in 5 % CO_2, some cells at the upper compartment migrate to the lower compartment.

2.13 Ubiquitination assay

293T cells were transfected with FLAG-BPGAP1, HA-Ub, T7-Nedd4 (either wild-typed (*Wt*) or catalytically inactive (*CS*) mutant) as previously described (Pham and Rotin, 2001). This Nedd4 CS mutant carries a point mutation of the conserved Cys (Cys to Ser) of the HECT domain and is unable to transfer ubiquitin and hence catalytically inactive. Lysate from the transfected cells (WCL) were immunoprecipitated with anti-FLAG M2 beads to precipitate BPGAP1 and immunoblotted with anti-HA antibodies to detect ubiquitinated BPGAP1 (BPGAP1-UB). Corresponding lysates were immunoblotted with either anti-T7 antibodies (Novagen) to verify expression of Nedd4 or anti-FLAG antibodies to verify expression of BPGAP1. Expression of ubiquitin were examined with anti-HA antibody. The T7 tagged Nedd4 (*Wt*) and Nedd4 (*CS*) plasmids were gifts of Dr. Daniela Rotin, Hospital for Sick Children, University of Toronto, Toronto).

Chapter 3

Results

3.1 Identifying novel GTPase-activating proteins

3.1.1 Bioinformatics was used to identify novel GTPase-activating proteins from database

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) (http://www.ncbi.nlm.nih.gov/BLAST/) with the well characterised 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 (ASAP-1), Src Homology-3 domain (p85α), Fes/CIP4 Homology domain (srGAP2), Rho guanine nucleotide exchange factor domain(BCR), and the p21 Rho-binding domain (Myosin IXb).(Figure 3.1) One of these classes is represented by several putative members that resemble the organisation of the Cdc42GAP protein. They are typified by the presence, at the proximal N-terminus, of the newly identified BNIP-2 and Cdc42GAP Homology (BCH) / Sec14p-like domain that we first described in the BNIP-2 family (Low *et al*., 1999; 2000a; 2000b) and a well conserved GAP domain at its distal C-terminus. Present in between these two domains is a stretch of proline-rich moiety (Figure 3.2).

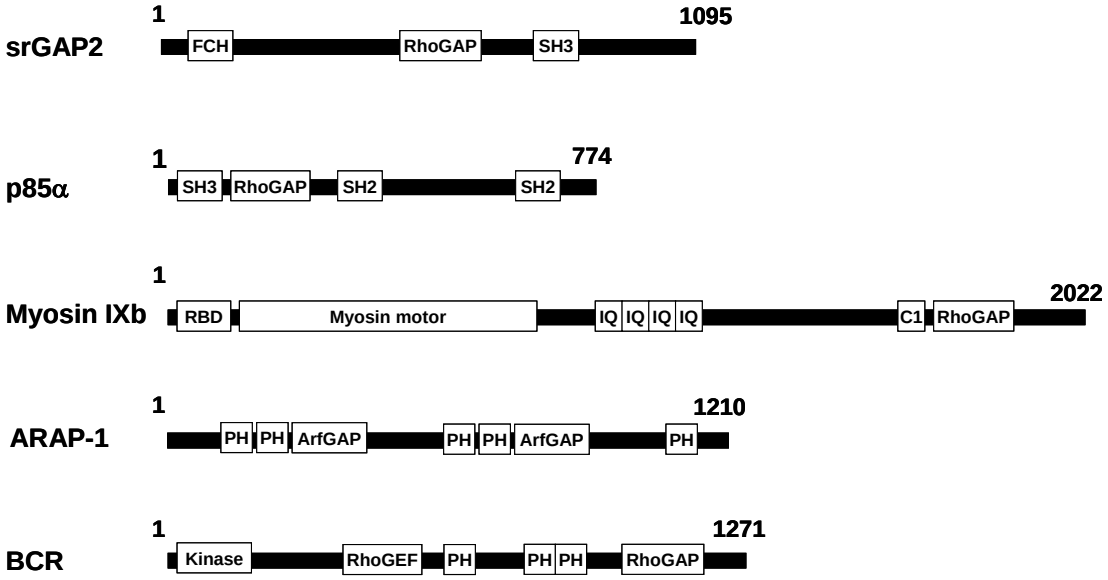

Figure 3.1 Schematic representation of selected human RhoGAP domain-containing preoteins. Domain name abbreviations: FCH, Fes/CIP4 homology; SH3, Src homology 3; SH2, Src homology 2; RBD, Ras binding domain; IQ, Calmodulin-dependent motif; C1, cysteine-rich phorbol ester binding; PH, pleckstrin homology; C2, calcium-dependent lipid binding.

Figure3.2 Domain organization of Cdc42GAP-like proteins. From its proximal N-terminus to the distal C-terminus, it contains BCH domain, proline-rich region(P) and RhoGAP domain (GAP).

3.1.2 Cloning of BPGAP family members

A putative RhoGAP-like protein (Database accession number: BAA91614) which contains BCH domain, GAP domain and Proline-rich sequence in between was blasted out. Based on the predicted open reading frame of this putative protein, several conserved primers were designed and used in reverse-transcription based PCR to isolate the full length cDNA from human MCF7 cells. The PCR products were used for molecular cloning. When clones were screened using restriction digest, three deferent clones of different sizes of inserts were obtained (Figure 3.3). When they were applied for sequencing, we found one unique sequence of cDNA identified (1.3 Kb) (Figure 3.4) which codes for a protein that differs from BAA91614 by lacking 31 amino acids (Figure 3.5; *upperline*). The protein also differs at the N-terminus, from two putative proteins encoded from the same human ARHGAP8 locus (Genbank accession numbers: Q9NSGO 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 organisation, 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 named this novel protein BPGAP1 (for BCH domain-containing, Proline-rich and Cdc42GAP-like protein, subtype 1; GenBank accession number: AF544240)

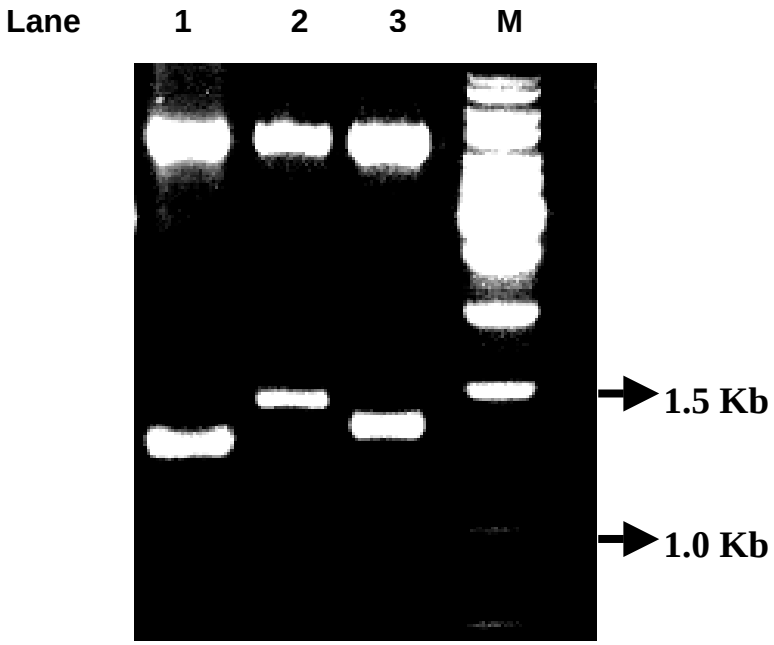

Figure 3.3 Molecular cloning of different isoforms of BPGAP family. Three isoforms of BPGAP family, BPGAP5, BPGAP2 and BPGAP1 (*lane1 to lane3*) respectively, were double-digested with *Hind*III and *Xho*I. *M*, maker.

```
1         ATGGCTGGCCAGGATCCTGCGCTGAGCACGAGTCACCCGTTCTACGACGTGGCCAGACAT
1          M  A  G  Q  D  P  A  L  S  T  S  H  P  F  Y  D  V  A  R  H

61        GGCATTCTGCAGGTGGCAGGGGATGACCGCTTTGGAAGACGTGTTGTCACGTTCAGCTGC
21         G  I  L  Q  V  A  G  D  D  R  F  G  R  R  V  V  T  F  S  C

121       TGCCGGATGCCACCCTCCCACGAGCTGGACCACCAGCGGCTGCTGGAGTATTTGAAGTAC
41         C  R  M  P  P  S  H  E  L  D  H  Q  R  L  L  E  Y  L  K  Y

181       ACACTGGACCAATACGTTGAGAACGATTATACCATCGTCTATTTCCACTACGGGCTGAAC
61         T  L  D  Q  Y  V  E  N  D  Y  T  I  V  Y  F  H  Y  G  L  N

241       AGCCGGAACAAGCCTTCCCTGGGCTGGCTCCAGAGCGCATACAAGGAGTTCGATAGGAAA
81         S  R  N  K  P  S  L  G  W  L  Q  S  A  Y  K  E  F  D  R  K

301       TACAAGAAGAACTTGAAGGCCCTCTACGTGGTGCACCCCACCAGCTTCATCAAGGTCCTG
101        Y  K  K  N  L  K  A  L  Y  V  V  H  P  T  S  F  I  K  V  L

361       TGGAACATCTTGAAGCCCCTCATCAGTCACAAGTTTGGGAAGAAAGTCATCTATTTCAAC
121        W  N  I  L  K  P  L  I  S  H  K  F  G  K  K  V  I  Y  F  N

421       TACCTGAGTGAGCTCCACGAACACCTTAAATACGACCAGCTGGTCATCCCTCCCGAAGTT
141        Y  L  S  E  L  H  E  H  L  K  Y  D  Q  L  V  I  P  P  E  V

481       TTGCGGTACGATGAGAAGCTCCAGAGCCTGCACGAGGGCCGGACGCCGCCTCCCACCAAG
161        L  R  Y  D  E  K  L  Q  S  L  H  E  G  R  T  P  P  P  T  K

541       ACACCACCGCCGCGGCCCCCGCTGCCCACACAGCAGTTTGGCGTCAGTCTGCAATACCTC
181        T  P  P  P  R  P  P  L  P  T  Q  Q  F  G  V  S  L  Q  Y  L

601       AAAGACAAAAATCAAGGCGAACTCATCCCCCCTGTGCTGAGGTTCACAGTGACGTACCTG
201        K  D  K  N  Q  G  E  L  I  P  P  V  L  R  F  T  V  T  Y  L

661       AGAGAGAAAGGCCTGCGCACCGAGGGCCTGTTCCGGAGATCCGCCAGCGTGCAGACCGTC
221        R  E  K  G  L  R  T  E  G  L  F  R  R  S  A  S  V  Q  T  V

721       CGCGAGATCCAGAGGCTCTACAACCAAGGGAAGCCCGTGAACTTTGACGACTACGGGGAC
241        R  E  I  Q  R  L  Y  N  Q  G  K  P  V  N  F  D  D  Y  G  D

781       ATTCACATCCCTGCCGTGATCCTGAAGACCTTCCTGCGAGAGCTGCCCCAGCCGCTTCTG
261        I  H  I  P  A  V  I  L  K  T  F  L  R  E  L  P  Q  P  L  L

841       ACCTTCCAGGCCTACGAGCAGATTCTCGGGATCACCTGTGTGGAGAGCAGCCTGCGTGTC
281        T  F  Q  A  Y  E  Q  I  L  G  I  T  C  V  E  S  S  L  R  V

901       ACTGGCTGCCGCCAGATCTTACGGAGCCTCCCAGAGCACAACTACGTCGTCCTCCGCTAC
301        T  G  C  R  Q  I  L  R  S  L  P  E  H  N  Y  V  V  L  R  Y

961       CTCATGGGCTCTCTGCATGCGGTGTCCCGGGAGAGCATCTTCAACAAAATGAACAGCTCT
321        L  M  G  S  L  H  A  V  S  R  E  S  I  F  N  K  M  N  S  S

1021      AACCTGGCCTGTGTCTTCGGGCTGAATTTGATCTGGCCATCCCAGGGGGTCTCCTCCCTG
341        N  L  A  C  V  F  G  L  N  L  I  W  P  S  Q  G  V  S  S  L

1081      AGTGCCCTTGTGCCCCTGAACATGTTCACTGAACTGCTGATCGAGTACTATGAAAAGATC
361        S  A  L  V  P  L  N  M  F  T  E  L  L  I  E  Y  Y  E  K  I

1141      TTCAGCACCCCGGAGGCACCTGGGGAGCACGGCCTGGCACCATGGGAACAGGGGAGCAGG
381        F  S  T  P  E  A  P  G  E  H  G  L  A  P  W  E  Q  G  S  R

1201      GCAGCCCCTTTGCAGGAGGCTGTGCCACGGACACAAGCCACGGGCCTCACCAAGCCTACC
401        A  A  P  L  Q  E  A  V  P  R  T  Q  A  T  G  L  T  K  P  T

1261      CTACCTCCGAGTCCCCTGATGGCAGCCAGAAGACGTCTCTAG
421        L  P  P  S  P  L  M  A  A  R  R  R  L  *
```

Figure 3.4 cDNA and protein sequences of BPGAP1. Depicted is the unique coding region and the translated protein sequence for BPGAP1 cDNA (*accession number: AF544240*) isolated by RT-PCR from MCF7 cells.

```
BPGAP1    (1) --------------------------------------------------
BPGAP3    (1) --------------------------------------------------
BPGAP2    (1) --------------------------------------------------
BPGAP4    (1) MRTLRRLKFMSSPSLSDLGKREPAAAADERGTQQRRACANATWNSIHNGV

BPGAP1    (1) --------------------------------------------------
BPGAP3    (1) ------------------------------------------MVILR
BPGAP2    (1) --------------------------------------------------
BPGAP4   (51) IAVFQRKGLPDQELFSLNEGVRQLLKTELGSFFTEYLQNQLLTKGMVILR

BPGAP1    (1) --------------------------------------------------
BPGAP3    (1) DKIRFYE-------------------------------------------
BPGAP2    (1) --------------------------------------------------
BPGAP4  (101) DKIRFYEGQKLLDSLAETWDFFFSDVLPMLQAIFYPVQGKEPSVRQLALL

BPGAP1    (1) --------------------------------------------------
BPGAP3    (1) --------------------------------------------------
BPGAP2    (1) --------------------------------------------------
BPGAP4  (151) HFRNAITLSVKLEDALARAHARVPPAIVQMLLVLQGVHESRGVTEDYLRL

BPGAP1    (1) --------------------------------------------------
BPGAP3    (1) ----------------------------ELQRDKAAAAAVLGAVRKRP
BPGAP2    (1) --------------------------------------------------
BPGAP4  (201) ETLVQKVVSPYLGTYGLHSSEGPFTHSCILELQRDKAAAAAVLGAVRKRP

BPGAP1    (1) ----MAGQDPALSTSHPFYDVARHGILQVAGDDRFGRRVVTFSCCRMPPS
BPGAP3   (33) SVVPMAGQDPALSTSHPFYDVARHGILQVAGDDRFGRRVVTFSCCRMPPS
BPGAP2    (1) ----MAGQDPALSTSHPFYDVARHGILQVAGDDRFGRRVVTFSCCRMPPS
BPGAP4  (251) SVVPMAGQDPALSTSHPFYDVARHGILQVAGDDRFGRRVVTFSCCRMPPS

BPGAP1   (47) HELDHQRLLEYLKYTLDQYVENDYTIVYFHYGLNSRNKPSLGWLQSAYKE
BPGAP3   (83) HELDHQRLLEYLKYTLDQYVENDYTIVYFHYGLNSRNKPSLGWLQSAYKE
BPGAP2   (47) HELDHQRLLEYLKYTLDQYVENDYTIVYFHYGLNSRNKPSLGWLQSAYKE
BPGAP4  (301) HELDHQRLLEYLKYTLDQYVENDYTIVYFHYGLNSRNKPSLGWLQSAYKE

BPGAP1   (97) FDR------------------------------KYKKNLKALYVVHPTS
BPGAP3  (133) FDR------------------------------KYKKNLKALYVVHPTS
BPGAP2   (97) FDRKDGDLTMWPRLVSNSKLKRSSHLSLPKYWDYRYKKNLKALYVVHPTS
BPGAP4  (351) FDRKDGDLTMWPRLVSNSKLKRSSHLSLPKYWDYRYKKNLKALYVVHPTS

BPGAP1  (116) FIKVLWNILKPLISHKFGKKVIYFNYLSELHEHLKYDQLVIPPEVLRYDE
BPGAP3  (152) FIKVLWNILKPLISHKFGKKVIYFNYLSELHEHLKYDQLVIPPEVLRYDE
BPGAP2  (147) FIKVLWNILKPLISHKFGKKVIYFNYLSELHEHLKYDQLVIPPEVLRYDE
BPGAP4  (401) FIKVLWNILKPLISHKFGKKVIYFNYLSELHEHLKYDQLVIPPEVLRYDE

BPGAP1  (166) KLQSLHEGRTPPPTKTPPPRPPLPTQQFGVSLQYLKDKNQGELIPPVLRF
BPGAP3  (202) KLQSLHEGRTPPPTKTPPPRPPLPTQQFGVSLQYLKDKNQGELIPPVLRF
BPGAP2  (197) KLQSLHEGRTPPPTKTPPPRPPLPTQQFGVSLQYLKDKNQGELIPPVLRF
BPGAP4  (451) KLQSLHEGRTPPPTKTPPPRPPLPTQQFGVSLQYLKDKNQGELIPPVLRF

BPGAP1  (216) TVTYLREKGLRTEGLFRRSASVQTVREIQRLYNQGKPVNFDDYGDIHIPA
BPGAP3  (252) TVTYLREKGLRTEGLFRRSASVQTVREIQRLYNQGKPVNFDDYGDIHIPA
BPGAP2  (247) TVTYLREKGLRTEGLFRRSASVQTVREIQRLYNQGKPVNFDDYGDIHIPA
BPGAP4  (501) TVTYLREKGLRTEGLFRRSASVQTVREIQRLYNQGKPVNFDDYGDIHIPA

BPGAP1  (266) VILKTFLRELPQPLLTFQAYEQILGITCVESSLRVTGCRQILRSLPEHNY
BPGAP3  (302) VILKTFLRELPQPLLTFQAYEQILGITCVESSLRVTGCRQILRSLPEHNY
BPGAP2  (297) VILKTFLRELPQPLLTFQAYEQILGITCVESSLRVTGCRQILRSLPEHNY
BPGAP4  (551) VILKTFLRELPQPLLTFQAYEQILGITCVESSLRVTGCRQILRSLPEHNY

BPGAP1  (316) VVLRYLMGSLHAVSRESIFNKMNSSNLACVFGLNLIWPSQGVSSLSALVP
BPGAP3  (352) VVLRYLMGFLHAVSRESIFNKMNSSNLACVFGLNLIWPSQGVSSLSALVP
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BPGAP2  (347) VVLRYLMGFLHAVSRESIFNKMNSSNLACVFGLNLIWPSQGVSSLSALVP
BPGAP4  (601) VVLRYLMGFLHAVSRESIFNKMNSSNLACVFGLNLIWPSQGVSSLSALVP

BPGAP1  (366) LNMFTELLIEYYEKIFSTPEAPGEHGLAPWEQGSRAAPLQEAVPRTQATG
BPGAP3  (402) LNMFTELLIEYYEKIFSTPEAPGEHGLAPWEQGSRAAPLQEAVPRTQATG
BPGAP2  (397) LNMFTELLIEYYEKIFSTPEAPGEHGLAPWEQGSRAAPLQEAVPRTQATG
BPGAP4  (651) LNMFTELLIEYYEKIFSTPEAPGEHGLAPWEQGSRAAPLQEAVPRTQATG

BPGAP1  (416) LTKPTLPPSPLMAARRRL
BPGAP3  (452) LTKPTLPPSPLMAARRRL
BPGAP2  (447) LTKPTLPPSPLMAARRRL
BPGAP4  (701) LTKPTLPPSPLMAARRRL
```

Figure 3.5 Comparison of BPGAP1 with three other putative isoforms derived from sequences deposited in GenBank. Regions predicted to be encoded by alternative RNA splicing are highlighted in *black* and *gray*. The *upperline* represents unique difference among the BCH domain of BPGAP isoforms. BPGAP2, BPGAP3 and BPGAP4 are derived from BAA91614, AF195968 and Q9NSG0 respectively. Mutation F324S present in BPGAP1 could be due to polymorphism.

We also found one sequence of the clones, which is 1.4 Kb in length, encodes the protein sequence of BAA91614 except several point mutations. It was named BPGAP2.

The cDNA sequence of the third clone was found to be the smallest compared with BPGAP1 and BPGAP2. Although its cDNA sequence encodes a larger GAP domain than BPGAP1 and BPGAP2, its BCH domain appeared to be of smaller size than that of BPGAP1. When we compared its cDNA sequence with that of BPGAP1 using Pairwise BLAST, an 88 bp region of BPGAP1 from the 81-168nt, 19 bp ahead of the N-terminal section of the BCH domain was found to be missing (Figure 3.6). Analysis of the translated sequence showed that this has led to the introduction of a stop codon at the 85nt. The pairwise BLAST results also revealed a 51 bp sequence that is unique to its GAP domain and absent in BPGAP1. It was named BPGAP5.

```
1   ATG GCT GGC CAG GAT CCT GCG CTG AGC ACG
1     M   A   G   Q   D   P   A   L   S   T

31  AGT CAC CCG TTC TAC GAC GTG GCC AGA CAT
11    S   H   P   F   Y   D   V   A   R   H


61  GGC ATT CTG CAG GTG GCA GGG GAT GAC CGC
21    G   I   L   Q   V   A   G   D   D   R

91  TTT GGA AGA CGT GTT GTC ACG TTC AGC TGC
31    F   G   R   R   V   V   T   F   S   C


121 TGC CGG ATG CCA CCC TCC CAC GAG CTG GAC
41    C   R   M   P   P   S   H   E   L   D

151 CAC CAG CGG CTG CTG GAG TA TTT GAA GTA C
51    H   Q   R   L   L   E   V  F   D   V


181 AC ACT GGA CCA ATA CGT TGA
61  Q   T   G   P   I   R   *
```

Figure 3.6 cDNA and protein sequence of BPGAP5. Compared with BPGAP1, its cDNA sequence has a deletion (red color) of an 88 bp region (from the 81-168nt), which leads to an movement of the open reading frame (*under line*) and a stop codon (*).

Genomic sequence analysis revealed that BPGAP1 is encoded by 11 exons from chromosome 22q13.3 (Table 3.1). Efforts are underway to isolate the full contigs for other putative isoforms, BPGAP3 (AF195968) and the longest subtype, BPGAP4 (Q9NSGO). 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 database (encoded by accession NP_082731 or AI430858).

Exon	mRNA/cDNA			genomic sequence		
	Begin	End	Length	Begin	End	Length
Exon 1	1	79	79 bps	38971	39049	79 bps
Exon 2	80	167	88 bps	54520	54607	88 bps
Exon 3	168	299	132 bps	60750	60881	132 bps
Exon 4	300	386	87 bps	67115	67201	87 bps
Exon 5	387	485	99 bps	74814	74912	99 bps
Exon 6	486	596	111 bps	77926	78036	111 bps
Exon 7	597	670	74 bps	97712	97785	74 bps
Exon 8	671	748	78 bps	100416	100493	78 bps
Exon 9	749	877	129 bps	101375	101503	129 bps
Exon 10	878	981	104 bps	112174	112277	104 bps
Exon 11	982	1302	321 bps	114718	115038	321 bps

Table 3.1 Structure of BPGAP1 gene locus. BPGAP1 sequence was mapped to ARHGAP8 gene locus (NT_011522) using the Genomatix online tool, *ElDorado* (http://www.genomatix.de/) with its exon and intron positions indicated. *bps*, basepairs.

Preliminary morphological studies were performed and we found expression of BPGAP1 full-length in MCF7 cells induced drastic morphological changes while expression of BPGAP2 could not (Figure 3.7). Further work was then focused on BPGAP1. We sought to understand how BPGAP1 regulates cellular processes *via* these protein domains.

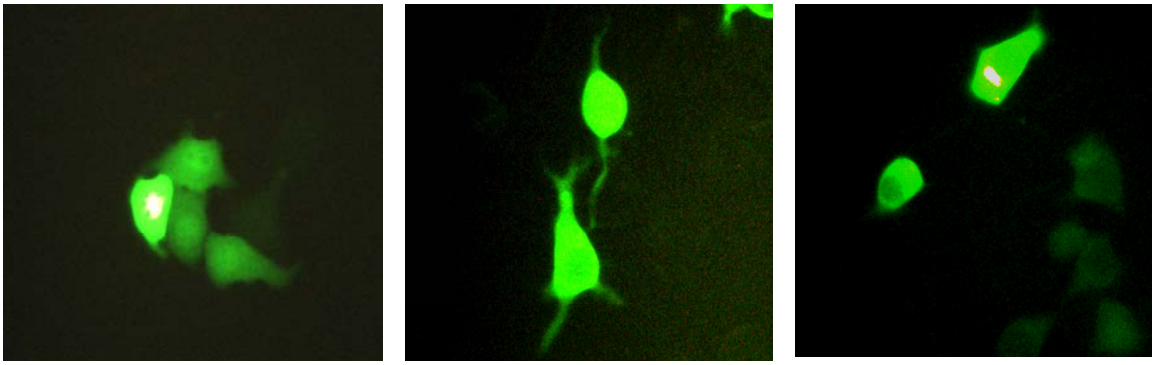

Figure 3.7 BPGAP1 induced cell morphogical changes while BPGAP2 could not. MCF7 cells transfected for 16h with GFP expression plasmids for BPGAP1 full-length, BPGAP2 full-length or GFP vector alone were visualized for GFP expression by direct fluorescence detection.

3.1.3 Sequence comparison between BPGAP1 and Cdc42GAP

Comparing to Cdc42GAP, BPGAP1 displays unique divergence at various regions. Notably, the BPGAP1 has a much shorter sequence at the N-terminus but a much longer carboxyl tail than Cdc42GAP (Figure 3.8). 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.

Firstly, the BCH domain of BPGAP1 is more closely related to that of Cdc42GAP (84% similarity) and notably it lacks the 4-amino acid residues (E/RSSQ/I) (amino acid 43-44 of BPGAP1) found in between two highly conserved prolines of the corresponding BCH domains in BNIP-2 and BNIP-S (Figure 3.9).

Secondly, the GAP domain of the BPGAP1 shares highest degree of homology with that of Cdc42GAP (Figure 3.10), and by lesser extents to the other GAPs. More importantly, BPGAP1 contains an invariant arginine at residue 232. This

residue in other functional GAPs is known as “arginine finger” and shown to be critical for acting as a catalytic residue *in-trans* (Nassar *et al*., 1998; Gamblin *et al*., 1998; Fidyk and Cerione, 2002).

Lastly, BPGAP1 possesses several more proline residues in the proline-rich sequence as well as an additional short stretch of proline-rich sequence at the distal carboxyl end (Figure 3.10, 3.11). The longer polyproline region of BPGAP1 is very similar to those identified in RNB6, extensin-like, ena-VASP-like and cdc-related proteins, which could imply some common regulatory mechanism among all these proteins. The two proline-rich regions of BPGAP1 could comprise more than one putative binding sites for either SH3 or WW domains (Macias *et al*., 2002; Sudol and Hunter, 2000; Figure 3.11). The sequence of amino acids ^{183} PPRP ^{186}, ^{186} PPLP ^{189}, ^{419} PTLP ^{422}, ^{422} PPSP ^{425} tally with the consensus SH3 domain binding sites “PxxP”, while the sequence of amino acids ^{182} PPPRPPLP ^{189}, ^{182} PPPR ^{185}, ^{181} TP^{182}, ^{424} SP ^{425} contain the features of group 2,3,4 WW domain bind sites respectively. Therefore, BPGAP1 might have multiple interacting partners though these binding sites.

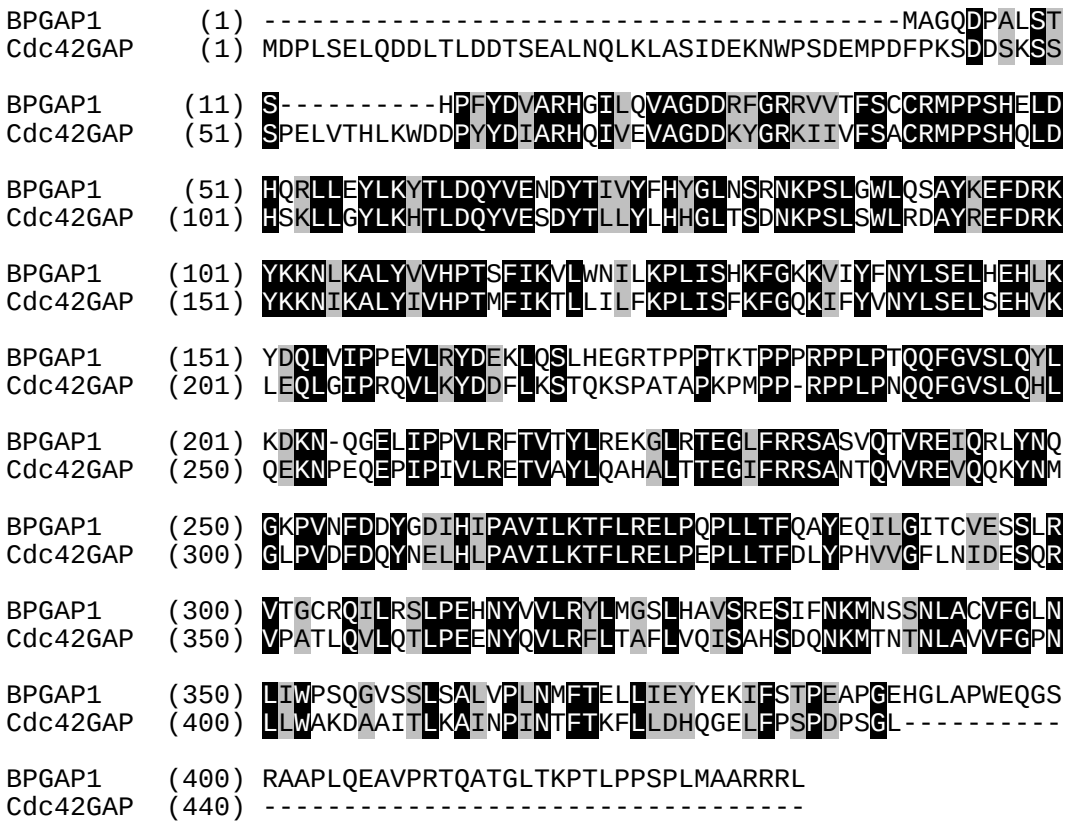

Figure 3.8 Alignment of BPGAP1 with Cdc42GAP protein sequences reveals regions of homology and divergence. The sequence alignments were performed using Vector NTI Suite. Residues that are totally conserved in all members are shaded *black*, those that are conserved in most of the members are in *drak gray* while the significant but least conserved ones are in *light gray* shading. GenBank accession numbers used for alignments above are human BPGAP1 (AF544240), human Cdc42GAP (Q07960).

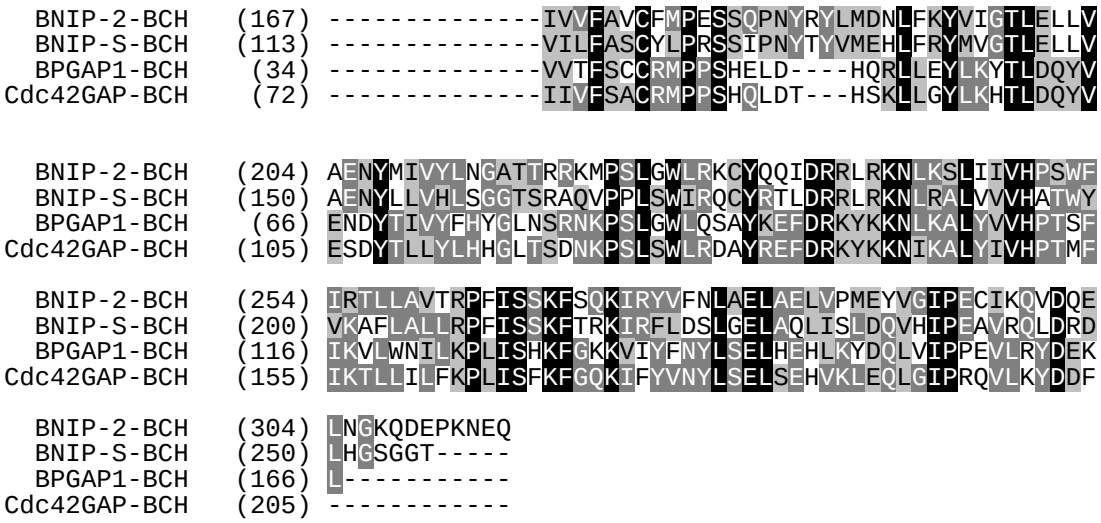

Figure 3.9 Alignment of BCH domains among BPGAP1, Cdc42GAP, BNIP-2 and BNIP-Sα. The sequence alignments were performed using Vector NTI Suite. Residues that are totally conserved in all members are shaded *black*, those that are conserved in most of the members are in *drak gray* while the significant but least conserved ones are in *light gray* shading. GenBank accession numbers used for alignments above are BPGAP1 (AF544240), human Cdc42GAP (Q07960), human BNIP-2 (U15173), human BNIP-Sα (NM_138278).

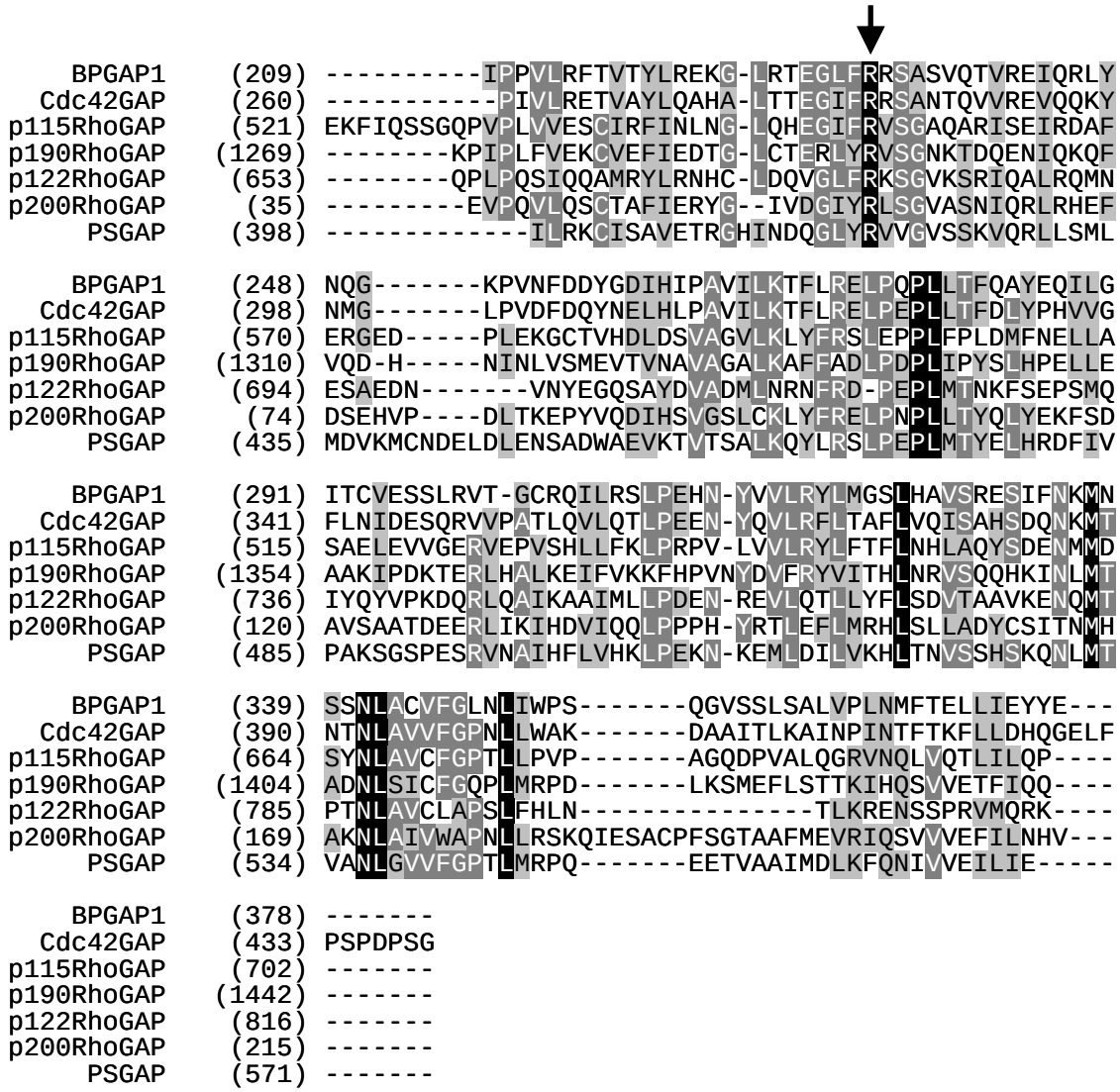

Figure 3.10 Alignment of GAP domains among BPGAP1, Cdc42GAP, PSGAP, p115RhoGAP, p122RhoGAP, p190RhoGAP and p200RhoGAP. *Arrow* indicates the invariant "arginine-finger" necessary for its GAP function. The sequence alignments were performed using Vector NTI Suite. Residues that are totally conserved in all members are shaded *black*, those that are conserved in most of the members are in *drak gray* while the significant but least conserved ones are in *light gray* shading. GenBank accession numbers used for alignments above are BPGAP1 (AF544240), human Cdc42GAP (Q07960), mouse PSGAP (NP_084389), human p190RhoGAP (NP_001164), mouse p115 RhoGAP (NP_619536), mouse p122RhoGAP/DLC1 (NP_056617), human p200RhoGAP (NP_055530).

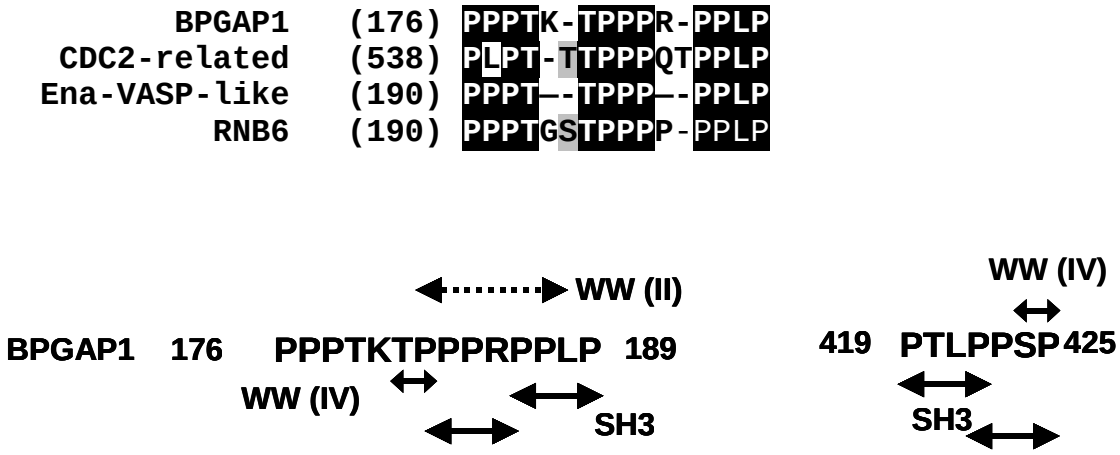

Figure 3.11 Alignment of the proline-rich regions of BPGAP1, RNB6, Extensin-like protein PRP5, Ena-VASP-like protein and Cdc2-related kinase 7. Indicated are putative targets for SH3 and WW domains based on consensus binding motifs. The sequence alignments were performed using Vector NTI Suite. Residues that are totally conserved in all members are shaded *black*, those that are conserved in most of the members are in *drak gray* while the significant but least conserved ones are in *light gray* shading. GenBank accession numbers used for alignments above are rat RNB6 (NM_024147), mouse Ena-VASP-like isoform (AF279662), human CDC2-related protein kinase 7 (NM_016507).

3.2 Expression profile of BPGAP1

To gain an insight into the potential cellular function(s) of BPGAP1, we examined the general expression profile of BPGAP1. 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 most cells of epithelial origin such as breast cancer MCF7, servical cancer HeLa, and kidney 293T, the expression of BPGAP1 appeared more ubiquitous (Figure 3.12). 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. Two bands were amplified with the sizes of around 400bp and 500bp respectively, indicating there were at least two

different isoforms of BPGAP1 containing different BCH domains in the full-length PCR products (Figure 3.12). BPGAP isoforms are also expressed ubiquitously in various mouse organs as shown in Figure 3.13. Similarly, BPGAP1 expression can be detected in all mouse tissues/organs tested, including the lung, liver, heart, kidney, brain and testis. Interestingly, two bands were amplified in the mouse organs whereas only one band appeared in the PCR product from Swiss 3T3 cell line, indicating that there were at least two isoforms of BPGAP family members in different mouse tissues.

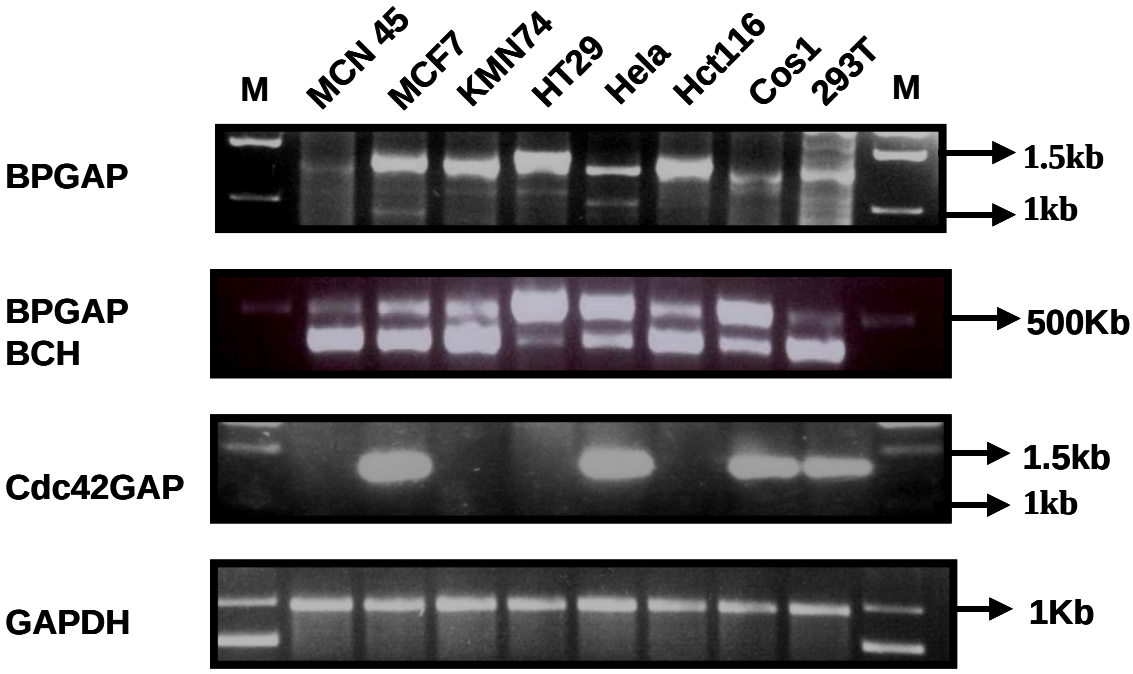

Figure 3.12 Expression profiles of BPGAP family cDNAs in various cell lines.Cells were grown in appropriate media containing 10% serum and total RNA isolated for semi-quantitative RT-PCR to detect the expression of BPGAP isoforms as described in "Materials and Methods". Expression of Cdc42GAP and GAPDH were analysed for comparison and normalization of samples respectively. *M*, markers

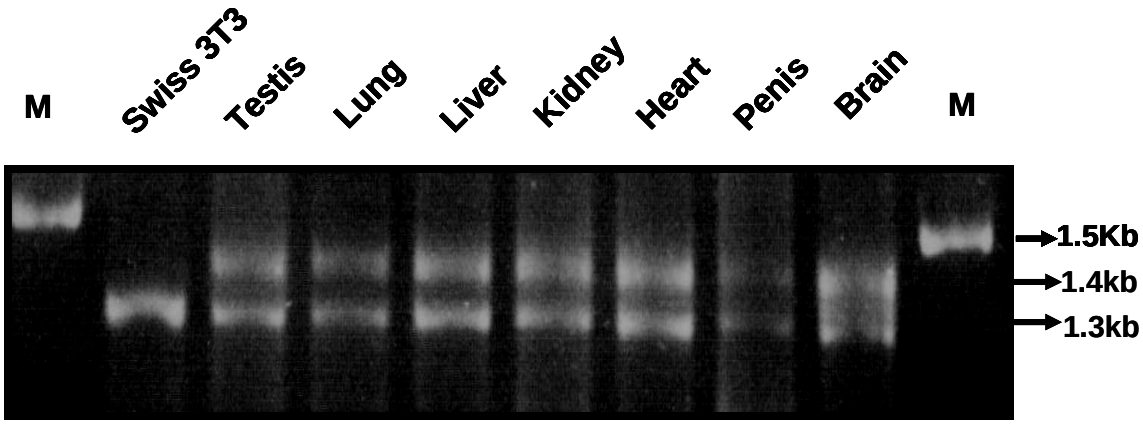

Figure 3.13 Expression profiles of BPGAP family cDNAs in various mouse organs. Total RNA was isolated from various organs as indicated from a two-week-old mouse for semi-quantitative RT-PCR to detect the expression of BPGAP isoforms as described in "Materials and Methods". Primers for the fulllength BPGAP1 were used for the PCR. *M*, markers

3.3 Multiple interacting partners of BPGAP1

In order to understand the specificity versus redundancy nature of the BPGAP1 as well as the roles of their various signaling modules, the binding repertoire of the various protein domains it harbors was studied.

3.3.1 Protein expression of the domains of BPGAP1 in mammalian cells

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: NNP fragment contains BCH domain; NP fragment contains both BCH domain and proline-rich sequence; PC fragment contains both proline-rich sequence and GAP domain; C fragment contains GAP domain (Figure 3.14).

Cells were transfected with these constructs, lysed and analysed for their expression by Western blot as described in "Materials and Methods". Figure 3.14 shows that full-length and deletion mutants all expressed equally well and were suitable for subsequent GST "pull-down" experiments or cellular studies. It also showed that the relative molecular masses of BPGAP1 full length (FL), NNP, NP, PC and C were around 48, 18, 23,29,25 KD respectively as predicted.

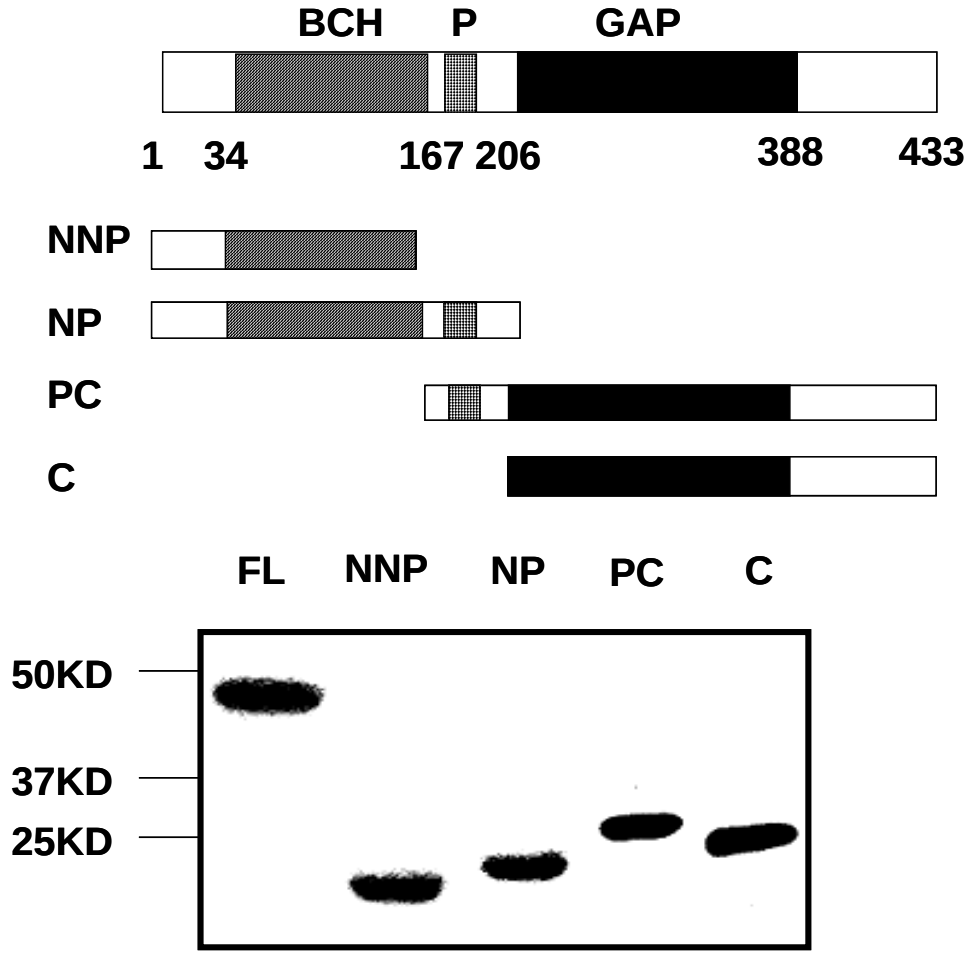

Figure 3.14 Expression constructs of BPGAP1 and its protein expression profiles in mammalian cells. Various expression constructs were tagged with FLAG epitope as indicated: NNP(N-terminus, non-proline region; amino acid 1-167), NP (N-terminus, with proline; amino acid 1-206), PC (Proline-containing carboxyl end; amino acid 168- 433) and C (Carboxyl end without proline; amino acid 207-433). Cells were transfected with plasmids expressing these domains and analysed for their expression by anti-FLAG Western blot analyses. Same regions were also tagged with either GST or GFP for use in subsequent experiments.

3.3.2 BPGAP1 forms homophilic/heterophilic interactions *via* BCH domain

3.3.2.1 *In vitro* "Pull Down"

We recently showed that BCH domain confers a novel protein interaction domain (Low *et al*., 1999; 2000a; 2000b). To test whether BPGAP1 could indeed interact with other BCH domain-containing proteins, the lysates of 293T cells transfected with FLAG-BPGAP1 and its fragments plasmids 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. Figure 3.15 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 could now form a heterophilic partner with this fragment. Furthermore, the interaction with Cdc42GAP or its BCH domain was also enhanced. Under all this experimental condition, the fragment C that carried the GAP domain (but without BCH domain) did not bind to any of the GST recombinants at all.

To further confirm these interactions, the reciprocal "pull-down"s were performed. Cdc42GAP and BNIP-2 were expressed as FLAG-tagged proteins in the mammalian cells and subjected to GST-BPGAP1 binding. Figure 3.16 shows that, as expected, FLAG-Cdc42GAP was precipitated by GST-BPGAP1. In contrary to the inert status of FLAG-BPGAP1 towards GST-BNIP-2 (Figure 3.16), the GST-BPGAP1 was reactive towards FLAG-BNIP-2. Likewise, full-length BPGAP1 and its BCH domain could also be precipitated with GST-BPGAP1.

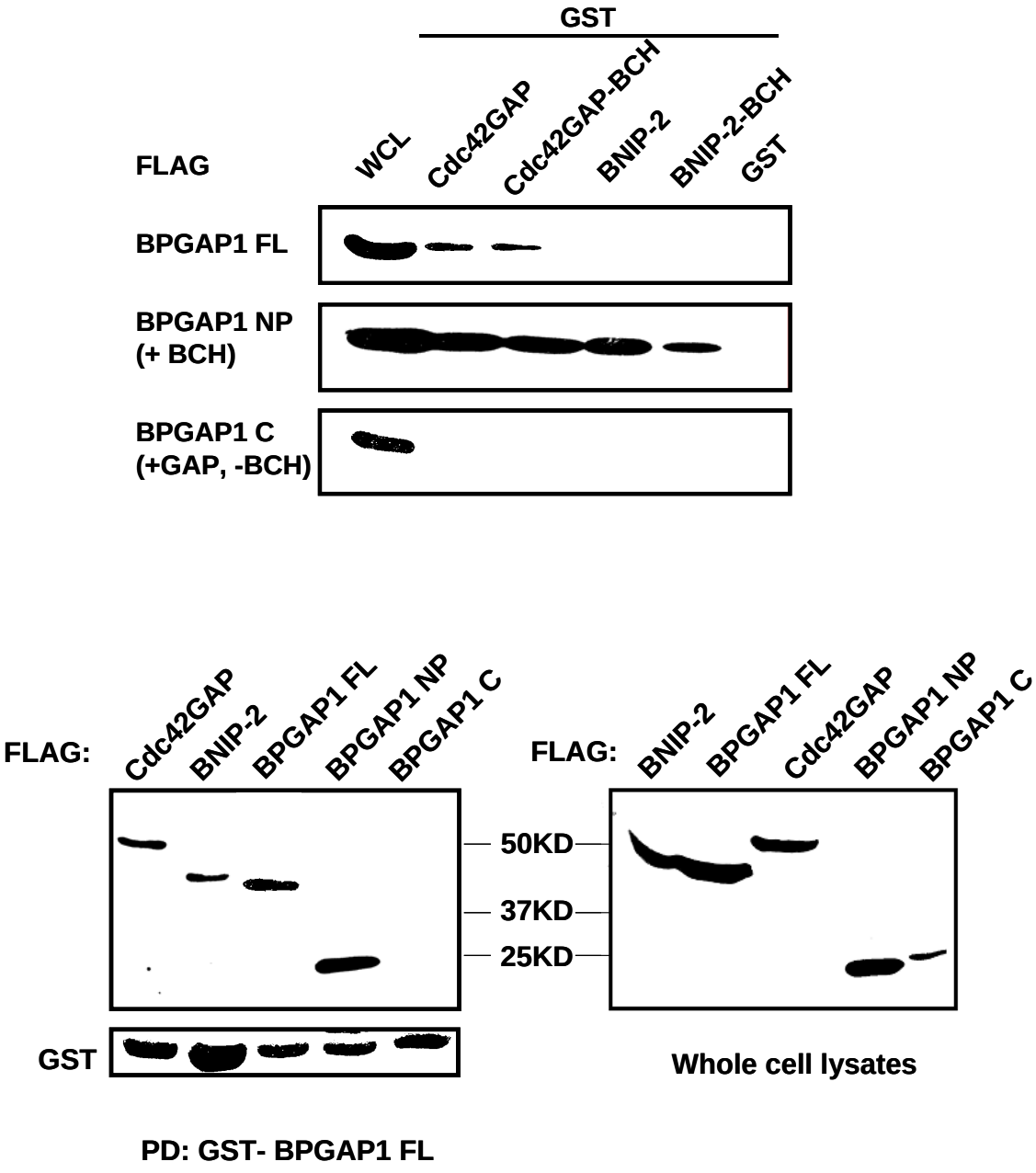

Figure 3.15, 3.16 *In vitro* "Pull-down" of BPGAP1 with other BCH domain containing proteins. GST-recombinants of various proteins were prepared as agarose beads and used for "pull-down" assays using cell lysates expressing FLAG-tagged proteins as indicated in separate experiments. Beads were then washed and processed for Western blot analyses using FLAG antibodies as described in "Materials and Methods". BPGAP1 is shown to be involved in either homophilic or heterophilic binding to other BCH domain-containing proteins.Blots were stripped and stained with amido black to reveal equal loading of GST-recombinants. *WCL*, whole-cell lystaes.

These results indicate that BPGAP1 can form heterophilic complex with Cdc42GAP more readily than it can with BNIP-2. The lack of binding of full-length BPGAP1 to BNIP-2 was probably due to the fact that when expressed in mammalian host, BPGAP1 could assume some modifications or conformational changes that selectively reduce its binding capacity to BNIP-2. One such mechanism is *via* the intramolecular interaction.

To test this hypothesis, full-length, the NP fragment and C fragment of BPGAP1 were subjected to their respective GST-recombinants to cross-examine their ability to bind to adjacent domains in the same protein. Figure 3.17 shows that the GST-BPGAP1 fragment C bound weakly to the full-length molecule but strongly to the FLAG-tagged NP fragment that contained the BCH domain. However, it did not bind to itself at all. Likewise, GST-NP fragment failed to interact with the full-length, but it bound very strongly to itself or to the FLAG-tagged C fragment. Interestingly, the BPGAP1-C fragment did not bind to Cdc42GAP or BNIP-2 at all (compare with Figure 3.15).

Taken together, these results are consistent with the notion that BPGAP1 could assume at least three different conformations for binding- the homophilic interaction *via* its own BCH domain, heterophilic interaction between its BCH domain and other similar BCH domains, or it could exist in an intramolecular interaction manner between its N-terminus that contains the BCH domain and the C-terminus that harbors the GAP domains.

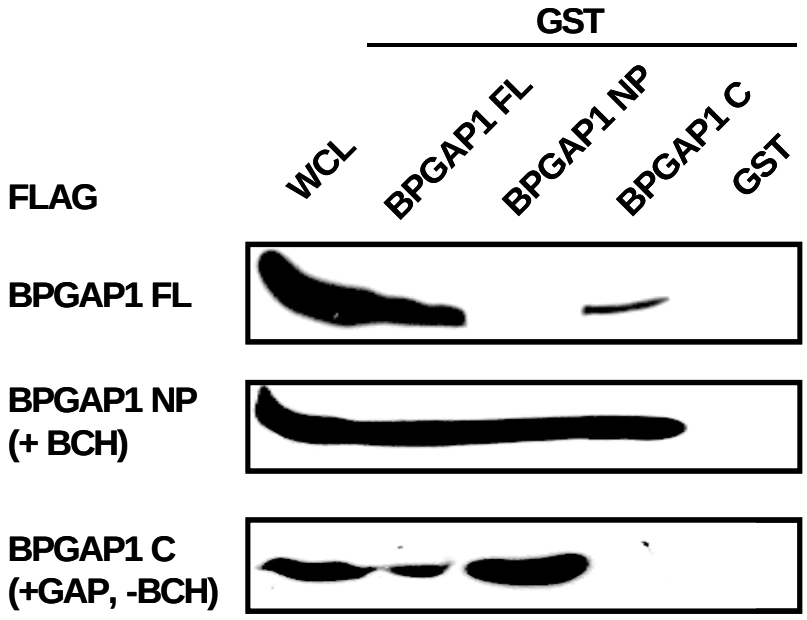

Figure 3.17 Intramolecular interaction of BPGAP1. GST-recombinants of various proteins were prepared as agarose beads and used for "pull-down" assays using cell lysates expressing FLAG-tagged proteins as indicated. Beads were then washed and processed for Western blot analyses using FLAG antibodies as described in "Materials and Methods". BPGAP1 is shown to be involved in intramolecular interaction. Blots were stripped and stained with amido black to reveal equal loading of GST-recombinants. *WCL*, whole-cell lystaes.

3.3.2.2 *In vivo* Co-immunoprecipitation

To further study the interactions between BPGAP1 with itself and the other BCH domain-containing proteins Cdc42GAP and BNIP-2 *in vivo*, co-immunuprecipitation was performed. Consistent with our observation *in vitro*, HA-tagged BPGAP1, Cdc42GAP and BNIP-2 could also be co-immunoprecipitated with FLAG-tagged BPGAP1 when co-expressed *in vivo* (Figure 3.18). These results confirmed that BPGAP1 could interact with other BCH domain-containing proteins not only *in vitro* but also *in vivo*.

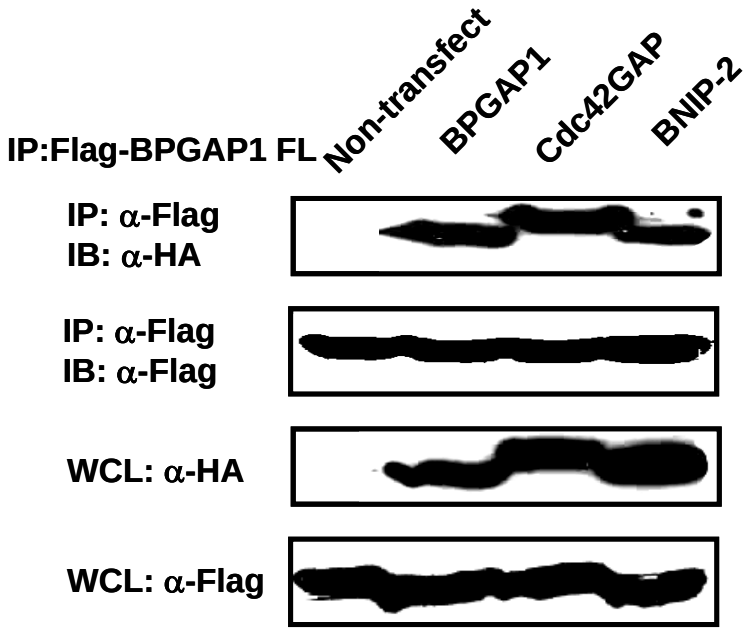

Figure 3.18 *In vivo* binding of BPGAP1 with itself and other BCH domain-containing proteins. Cells were cotransfected with plasmids expressing FLAG-tagged full-length BPGAP1 and Hatagged full-length BPGAP1, Cdc42GAP or BNIP-2. Lysates were immunoprecipitated (IP) with anti-FLAG M2 beads and the associated proteins were separated on SDS-PAGE, and probed with HA antibody as described in "Materials and Methods". Expression of HA-tagged BPGAP1, Cdc42GAP and BNIP-2, and FLAG-tagged BPGAP1 were verified by anti-HA (*third panel*) or anti-FLAG (*fourth panel*) Western analyses of the whole cell lysates (WCL). Equal loading of anti-FLAG M2 beads is shown by applying the IP blot with anti-FLAG antibody *(second panel).*

3.4 BPGAP1 targeted Cdc42, RhoA and Rac1 differentially via their BCH and GAP domains

With the conserved GAP domain that includes the invariant arginine finger motif (Figure 3.10), BPGAP1 was predicted to bind and confer catalysis towards the GTP hydrolysis of certain Rho GTPases. To examine this, *in vitro* and *in vivo* GTPase activity assays were performed.

3.4.1 GAP activity *in vitro* and *in vivo*

3.4.1.1 *In vitro* GAP activity assay

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, its deletion mutants NP, PC or the site-directed mutagenesis mutant R232A, using the enzyme-coupled spectrophotometric assays (Wu *et al*., 2000) as described in "Materials and Methods". The release of Pi catalyzed by the GAP activity was measured by the product concentration after it reacted with the substrate MESG. And the product concentration could be measured by spectrophotometric assay at A_{360}. We found that after the initiation of the reaction, the increasing value of A_{360} is linear and proportional to the time in one minute, whereas the value will not increase one minute later. It was demonstrated that the reaction occurred in one minute. The GAP activities were indicated by the rate of A_{360} inclination in one minute compared with that of the control. Figure 3.19 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 activation towards Rac1. 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, but devoid of the GAP domain. Cdc42GAP was used as the positive control. Furthermore, such activation was abrogated after introducing a mutation R232A at the invariant arginine residue (see Figure 3.10).

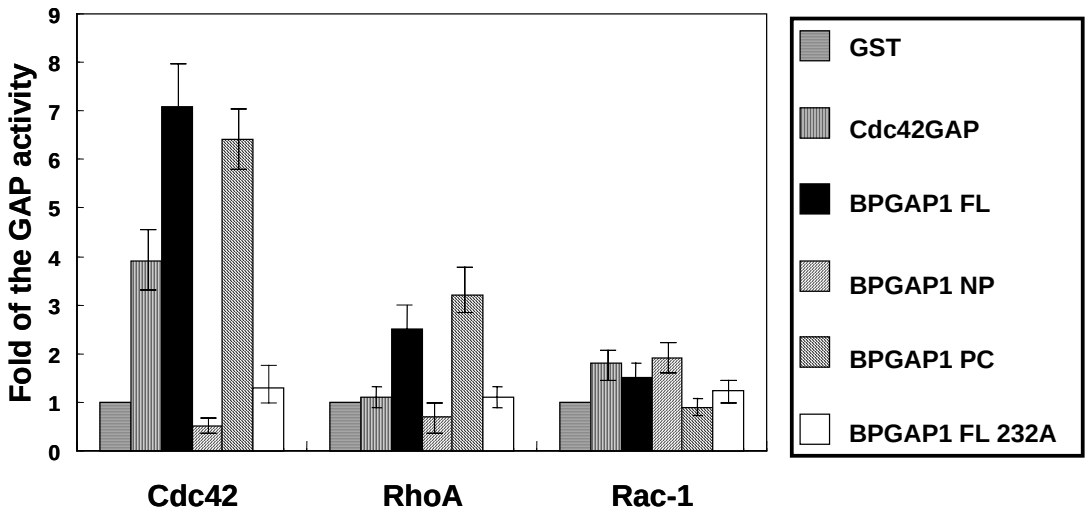

Figure 3.19 *In vitro* GAP assays. Purified Cdc42, RhoA or Rac1 were loaded with GTP and their GTPases activity determined in the absence or presence of Cdc42GAP, BPGAP1 full-length or mutants using an enzyme-coupled assay as described in "Materials and Methods". The activity was expressed as *fold* over the control using GST alone. Results are means ± S.D. of three replicate determinations.

3.4.1.2 *In vivo* GAP activity assay

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 vivo*. 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 in "Materials and Methods". Figure 3.20 shows that *in vivo*, RhoA binding to its effector domain, hence its activity was reduced in the presence of BPGAP1. In contrast, the existing activity of Cdc42 and Rac1 were not affected, and at times they seemed to be modestly increased instead.

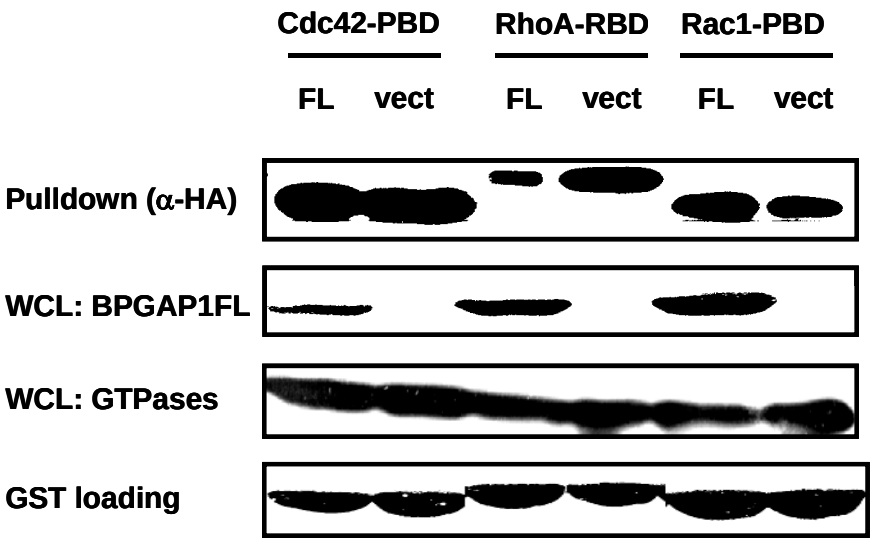

Figure 3.20 *In vivo* GTPase binding assays. Cells were transfected with HA-tagged wildtype Cdc42, RhoA or Rac1 in the presence of either the vector control or FLAG-tagged BPGAP1. Cell were lysed and incubated with GST-PBD or GST-RBD immobilized on beads to assess the ability of BPGAP1 in down-regulating GTPase pathway as described in "Materials and Methods". Bound GTPases were resolved on SDS-PAGE and detected by immunoblotting with HA-antibody (*top panel*). Expression of BPGAP1 and GTPases were verified by anti-FLAG (*second panel*) or anti-HA (*third panel*) Western analyses of the whole cell lysates (WCL) respectively. Equal loading of GST fusion proteins is shown in the *bottom panel*.

Such discrepancies between *in vitro* and *in vivo* activities could be due to one or more of the several reasons, including (i) the presence of multiple domains in BPGAP1 that only exert its unique properties *in vivo*, and (ii) the likelihood of BPGAP1 being present in different subcellular compartments.

3.4.2 Interactions between BPGAP1 with Rho GTPases

Next, we examined more closely the binding status of both overexpressed and endogenous Rho GTPases to BPGAP1. Figure 3.21 and Figure 3.23 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 both *in vitro* and *in vivo*.

Interestingly, the endogenous RhoA was not readily bound by BPGAP1 unless its level was elevated by overexpression (Figure 3.22). Consistent with this was the observation that endogenous RhoA could be co-immunoprecipitated with overexpressed BPGAP1 (Figure 3.23) and this level of interaction was further increased when RhoA itself was also overexpressed (Figure 3.24).

Similar to the Cdc42, endogenous and overexpressed Rac1 interacted strongly with BPGAP1 in either the "pulldown" or co-immunoprecipitation assays (Figures 3.21-3.24).

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.

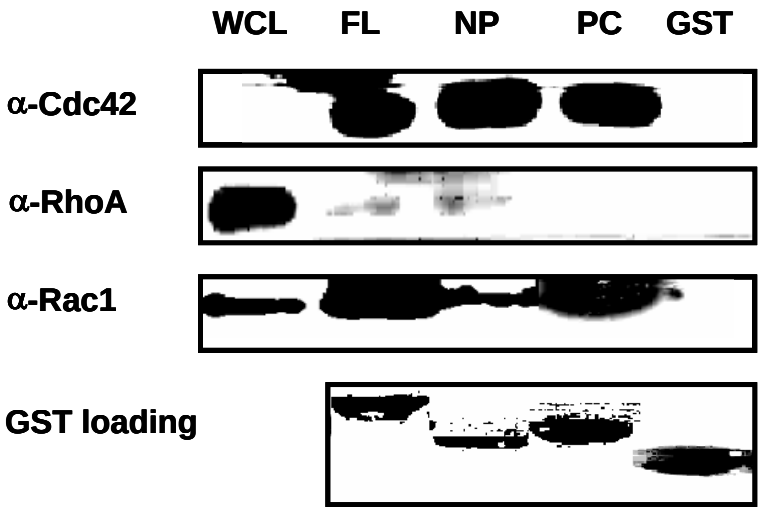

Figure 3.21 *In vitro* binding of BPGAP1 with endogenous Rho GTPases. GST recombinant of BPGAP1, its domains or GST control were used for "pulldown" assays using normal cell lysates as described in 'Materials and Methods". Beads from the "pull-down" experiments were washed and processed for Western analyses using anti-Cdc42, anti- RhoA or anti-Rac1 antibodies. Blots were stripped and stained with amido black to reveal loading of GST-recombinants. Under this level of film exposure, the endogenous level of Cdc42 was too low to be detectable.

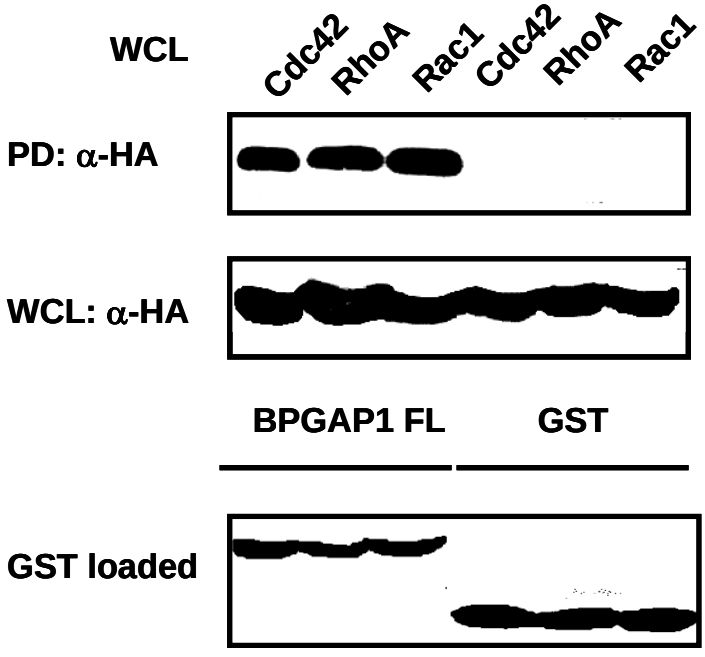

Figure 3.22 *In vitro* binding of BPGAP1 with overexpressed Rho GTPases. Cells were transfected with HA-tagged Cdc42, RhoA or Rac1 and lysed for "pulldown" assays with either the GST control or GST-recombinant of BPGAP1. Bound proteins were detected with anti-HA antibodies while GST staining reveals equal loading of samples.

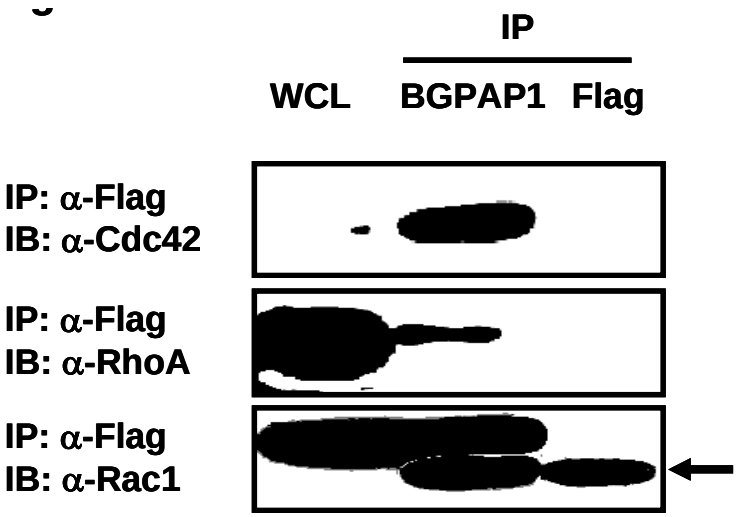

Figure 3.23 *In vivo* binding of BPGAP1 with endogenous Rho GTPases. Cells were transfected with expression vectors for fulllength FLAG-tagged BPGAP1 and FLAG vector control. Lysates were subjected to immunoprecipitation (IP) with anti-FLAG M2 beads and the associated proteins were separated on SDS-PAGE, and probed with anti-Cdc42, anti-RhoA or anti-Rac1 antibodies to reveal the binding of targets. *Arrow* indicates the light chain of the antibody.

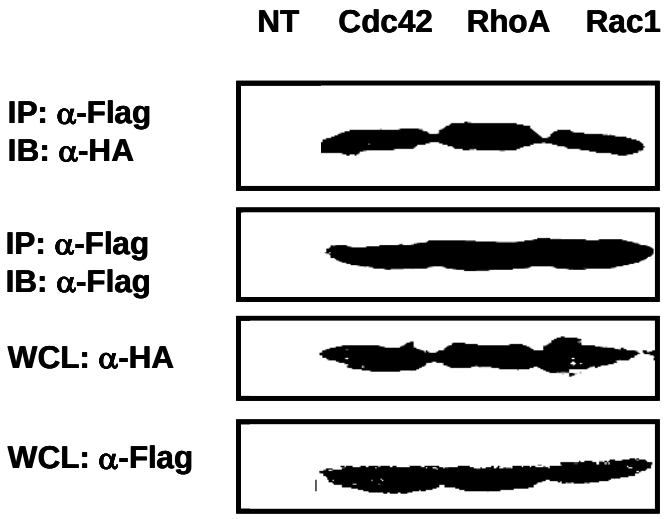

Figure 3.24 *In vivo* binding of BPGAP1 with overexpressed Rho GTPases. Cells were transfected with expression vectors for full-length FLAGtagged BPGAP1 or FLAG vector control, together with either HA-tagged Cdc42, RhoA or Rac1. Lysates were subjected to immunoprecipitation (IP) with anti-FLAG M2 beads and the associated proteins were separated on SDS-PAGE, and probed with anti-HA antibodies to reveal the binding of targets. Expression of HA-tagged GTPases and FLAG-tagged BPGAP1 were verified by anti- HA (*third panel*) or anti-FLAG (*fourth panel*) Western analyses of the whole cell lysates (WCL) respectively. Equal loading of anti-FLAG M2 beads is shown by applying the IP blot with anti-FLAG antibody *(second panel). NT* is for non-transfection control.

3.5 BPGAP1 induced pseudopodia in epithelial cells

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 wished 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.

3.5.1 Indirect immunofluorescence showed that expression of BPGAP1 could induce cell protrusions

We had chosen to use the human breast epithelial cancer cells MCF7 as the model because of its relative ease in transfection, and in monitoring of its cell morphology. MCF7 cells are 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 immunofluoroscence as described in "Materials and Methods". Figure 3.25 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 localised in the cytosol and it induced unique long and short protrusions (collectively referred as pseudopodia) in the transfected cells (*arrows*).

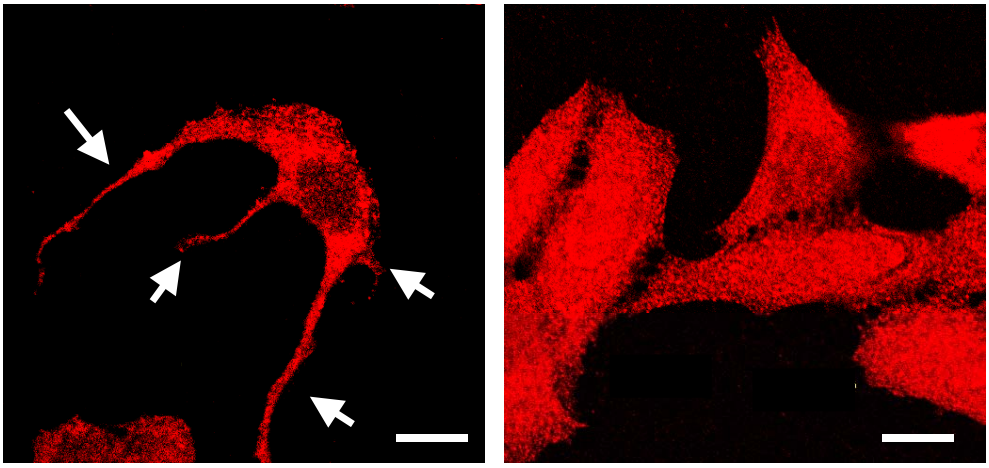

Figure 3.25 BPGAP1 induced pseudopodia. MCF7 cells were transfected with FLAG-tagged expression plasmids for BPGAP1 full-length or control vector. Cells were then fixed, permeabilized and incubated with anti-FLAG monoclonal, followed by Texas Red dye-conjugated rabbit anti-mouse IgG for immunofluorescence detection as described in "Materials and Methods". *Arrows* indicate pseudopodia formation. *Bar*, 10 μm.

3.5.2 Direct fluorescence by GFP expression

To further confirm such observations and to monitor the dynamics directly without resorting to fixing the cells, we opted for direct fluorescence with green-fluorescent protein (GFP) fusion of BPGAP1 and its various deletion mutants (refer to Figure 3.14).

A quantitative measure of the cell dynamics could be performed by identifying certain types of changes in cell morphology and their relative percentages determined (Figures 3.26 and 3.27). 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 again induced pseudopodia in almost 60% of the transfected cells, a unique

feature that was normally typified by long projections of more than 30 micron from the opposing ends of the cell bodies, accompanied by occasional branchings off the main pseudopods (Figure 3.26). 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% to 30% of the transfected cells caused many shorter protrusions (less than 10 micron) with "mircospike-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 Figure 3.11) of BPGAP1 (P1 mutant) did not affect the overall formation of protrusions by the cells.

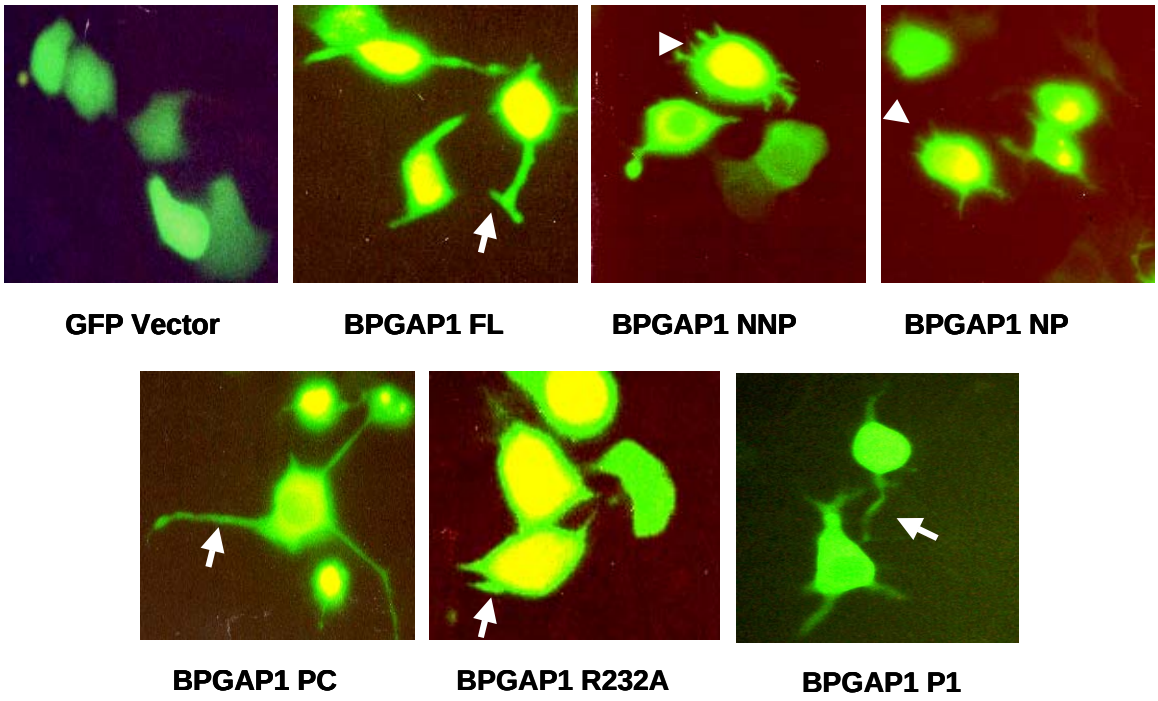

Figure 3.26 BPGAP1 induced pseudopodia *via* BCH and GAP domains. MCF7 cells transfected for 16h with GFP expression plasmids for BPGAP1 full-length, domains, mutants, or GFP vector alone were visualized for GFP expression by direct fluorescence detection. The yellow appearance was due to increased exposure set to allow better detection of pseudopodia. *Arrows* indicate features described in the text.

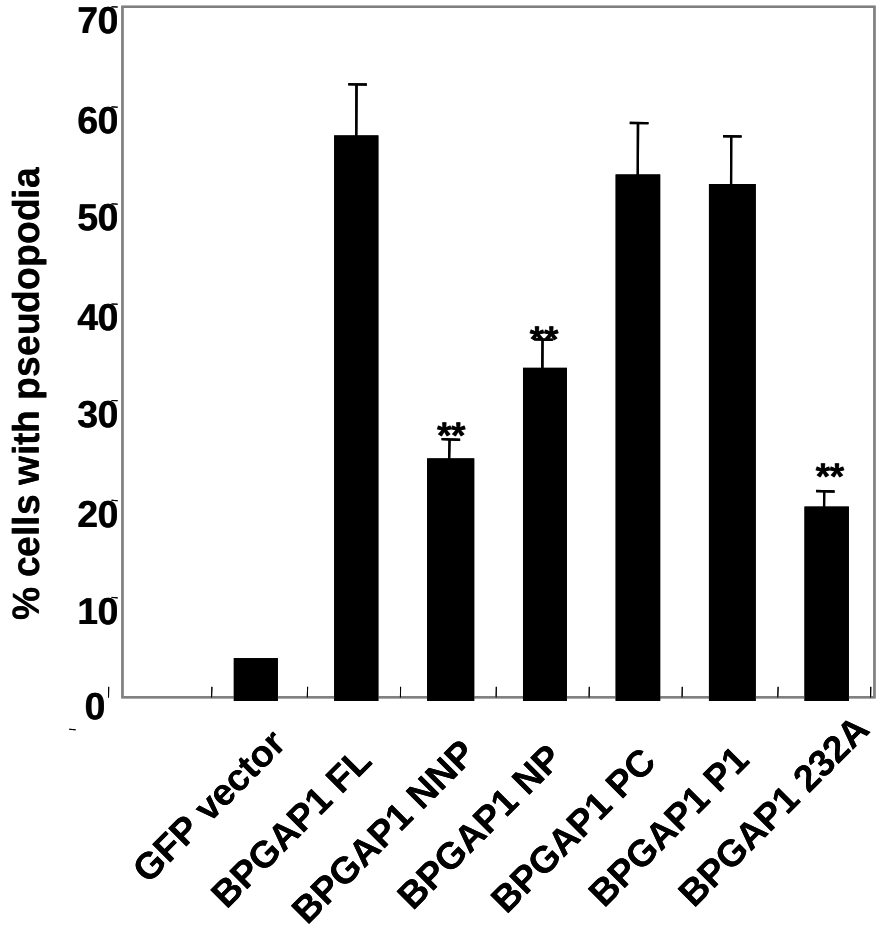

Figure 3.27 BPGAP1 induced pseudopodia *via* BCH and GAP domains. The percentage of cells exhibiting pseudopodia in the presence of various GFP constructs (from experiment *B* above) were determined and represented as a bar graph. Results are averages +/- half the ranges for two determinations that are representative of at least three separate experiments. * indicates only short pseudopodia. All difference are significant at $p<0.01$ *vs* GFP vector control.

3.5.3 BPGAP1-induced cell protrusion was NOT due to cell body retraction

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 in "Materials and Methods". These include the longest diameter (LD) and the shortest diameter (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 (Figure 3.28). The results

show that when compared to 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 suggest that BPGAP1 induces unique pseudopodia formation mainly *via* the GAP domain and BCH domain independently of the proline-rich region. Induction of pseudopodia by BPGAP1 was also observed in 293T and Hela cells.

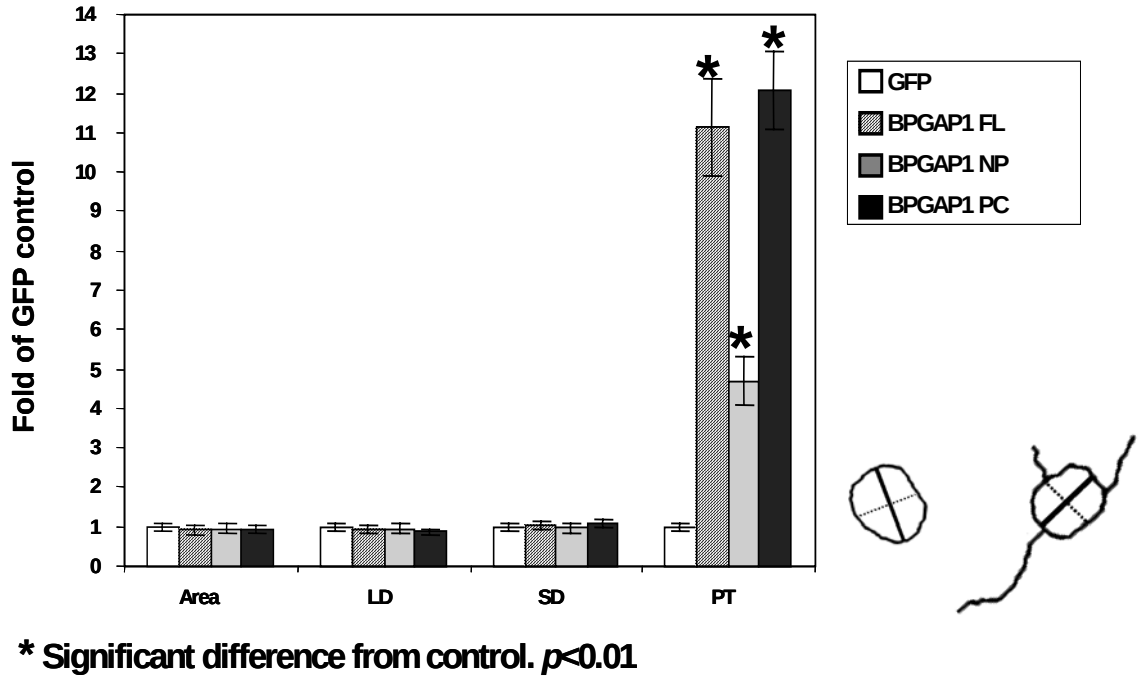

Figure 3.28 BPGAP1-induced morphological changes are protrusions/pseudopodia and not retraction fibers. MCF7 cells were transfected with expression plasmids for GFP tagged BPGAP1 full-length, NP, and PC domains or GFP vector control. After the transfection for 20h, the total cell areas, LD (the longest 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 analysed as described in "Materials and Methods". Measurements are means +/- standard deviations for 30 determinations. Results are representative of three separate experiments. Statistical comparison was made using ANOVA (StatsDirect). * indicates significant difference at $p<0.01$ when compared with the vector control.

3.6 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 (Figure 3.20), 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 wildtype, constitutive active or dominant negative mutants of the RhoA, Cdc42 and Rac1 respectively. Indirect immunofluorescence studies using confocal microscopy shows that expression of wildtype or G14V constitutive active mutant of RhoA could indeed prevent pseudopodia formation whereas its dominant negative mutant T19N did not affect such process (Figure 3.29).

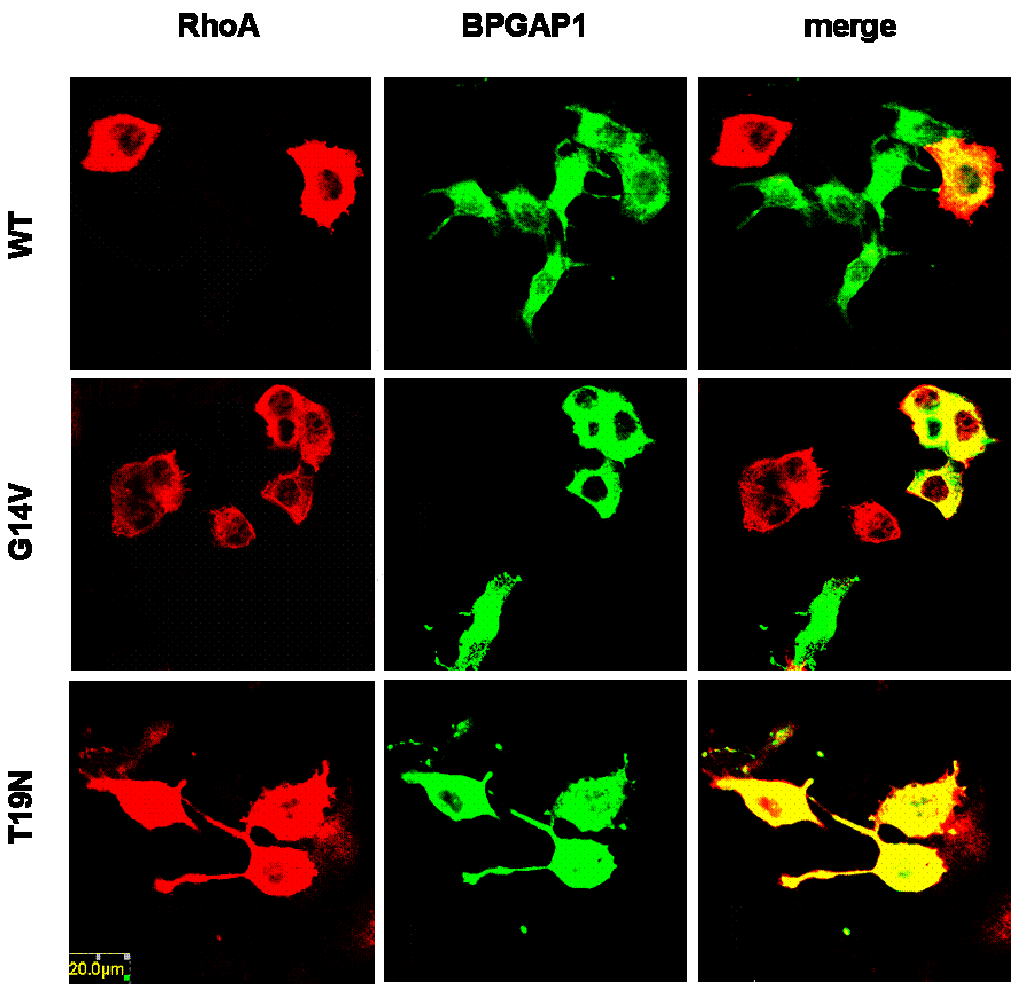

Figure 3.29 BPGAP1-induced pseudopodia involve the regulation of RhoA. MCF7 cells were cotransfected for 16h with GFP expression plasmid for BPGAP1 full-length and HA-tagged expression plasmids for wild-type, constitutive active or dominant negative mutants of RhoA.

In contrast, wildtype or G12V mutant of Cdc42 allowed propagation of cell shapes that saw many more short protrusions with branching formed whereas the T17N negative mutant of Cdc42 completely blocked BPGAP1's effect (Figure 3.30). As for Rac1, expression of the wildtype 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 (Figure 3.31). 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**.** Figure 3.32 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 concerted 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.*

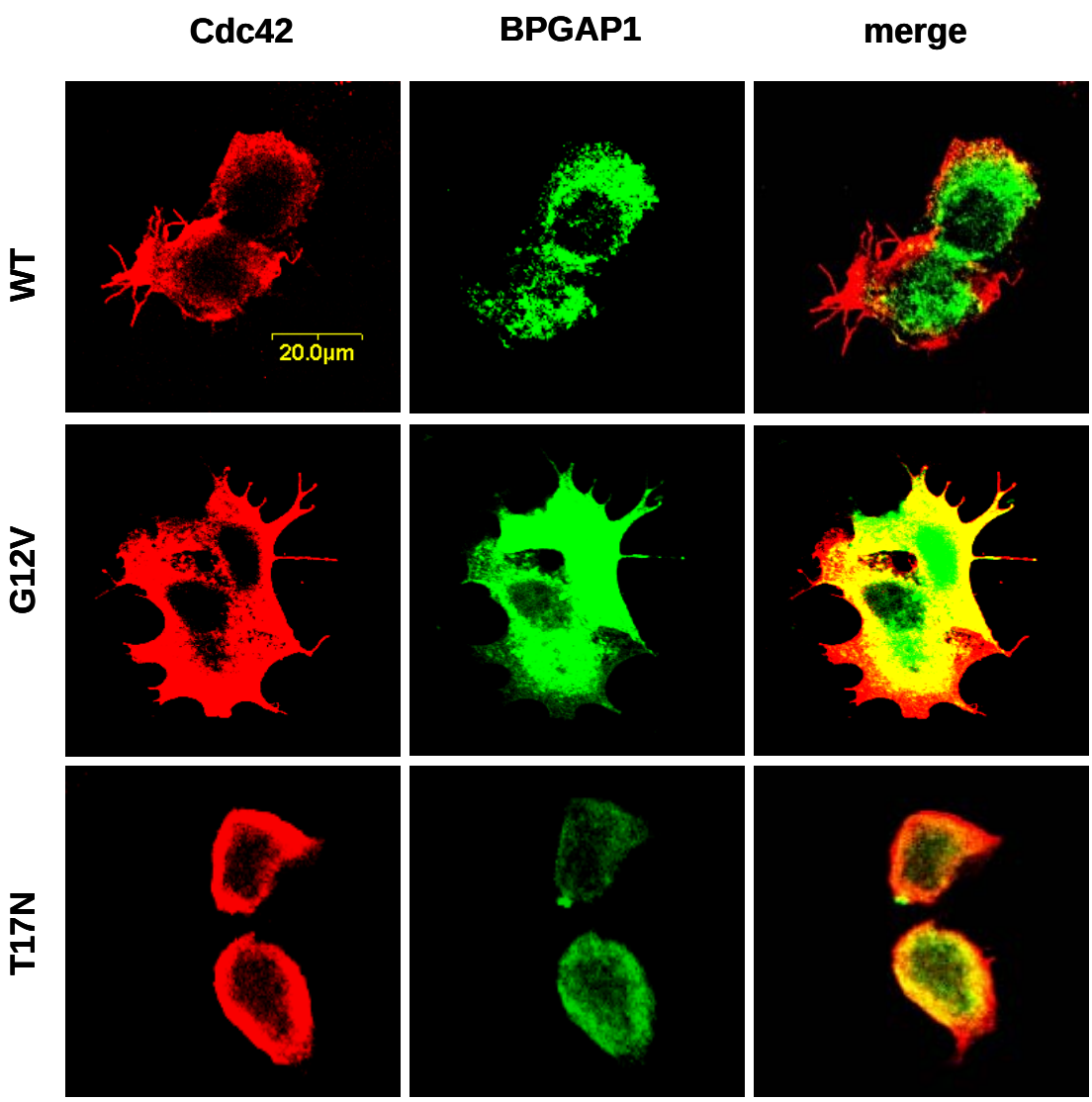

Figure 3.30 BPGAP1-induced pseudopodia involve the regulation of Cdc42. MCF7 cells were cotransfected for 16h with GFP expression plasmid for BPGAP1 full-length and HA-tagged expression plasmids for wild-type, constitutive active or dominant negative mutants of Cdc42.

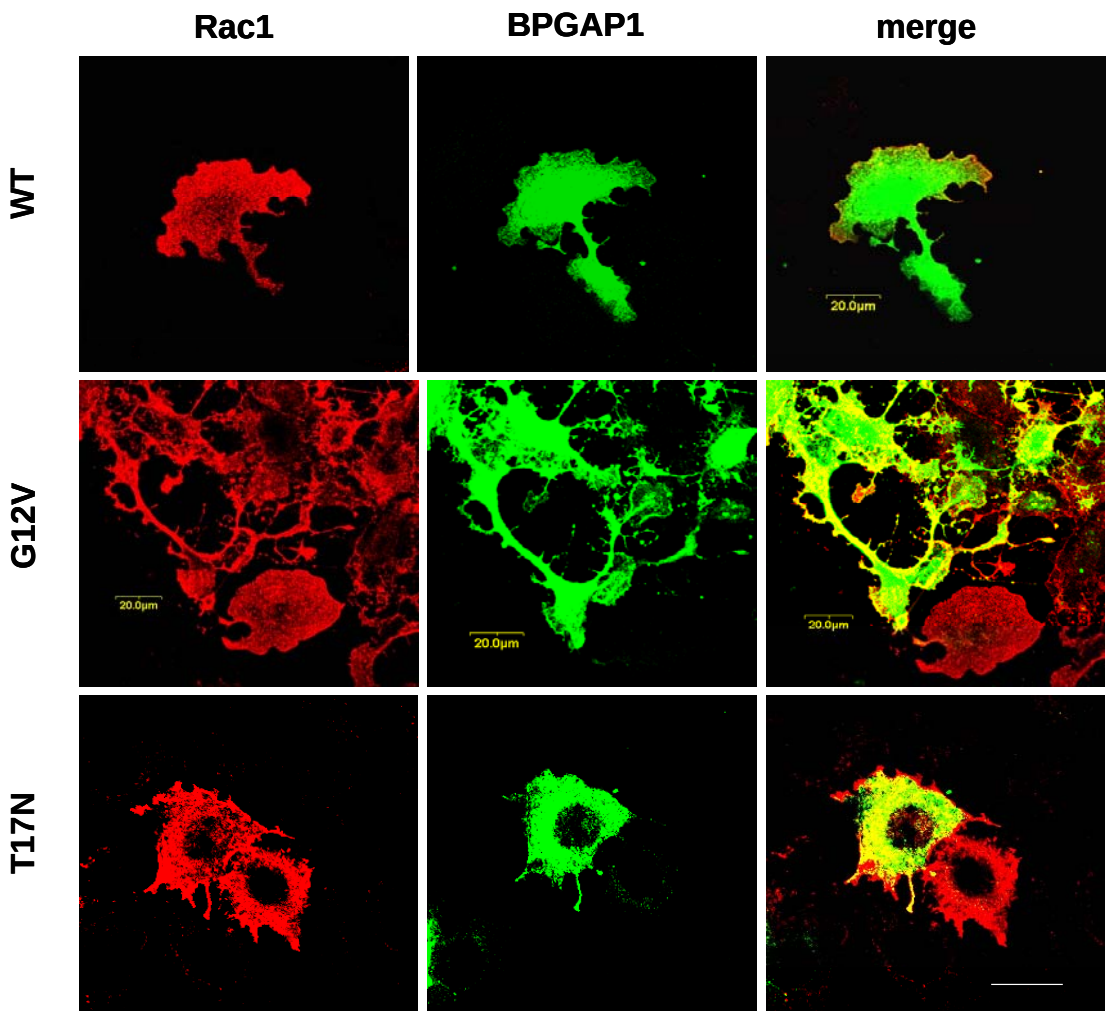

Figure 3.31 BPGAP1-induced pseudopodia involve the regulation of Rac1. MCF7 cells were cotransfected for 16h with GFP expression plasmid for BPGAP1 full-length and HA-tagged expression plasmids for wild-type, constitutive active or dominant negative mutants of Rac1.

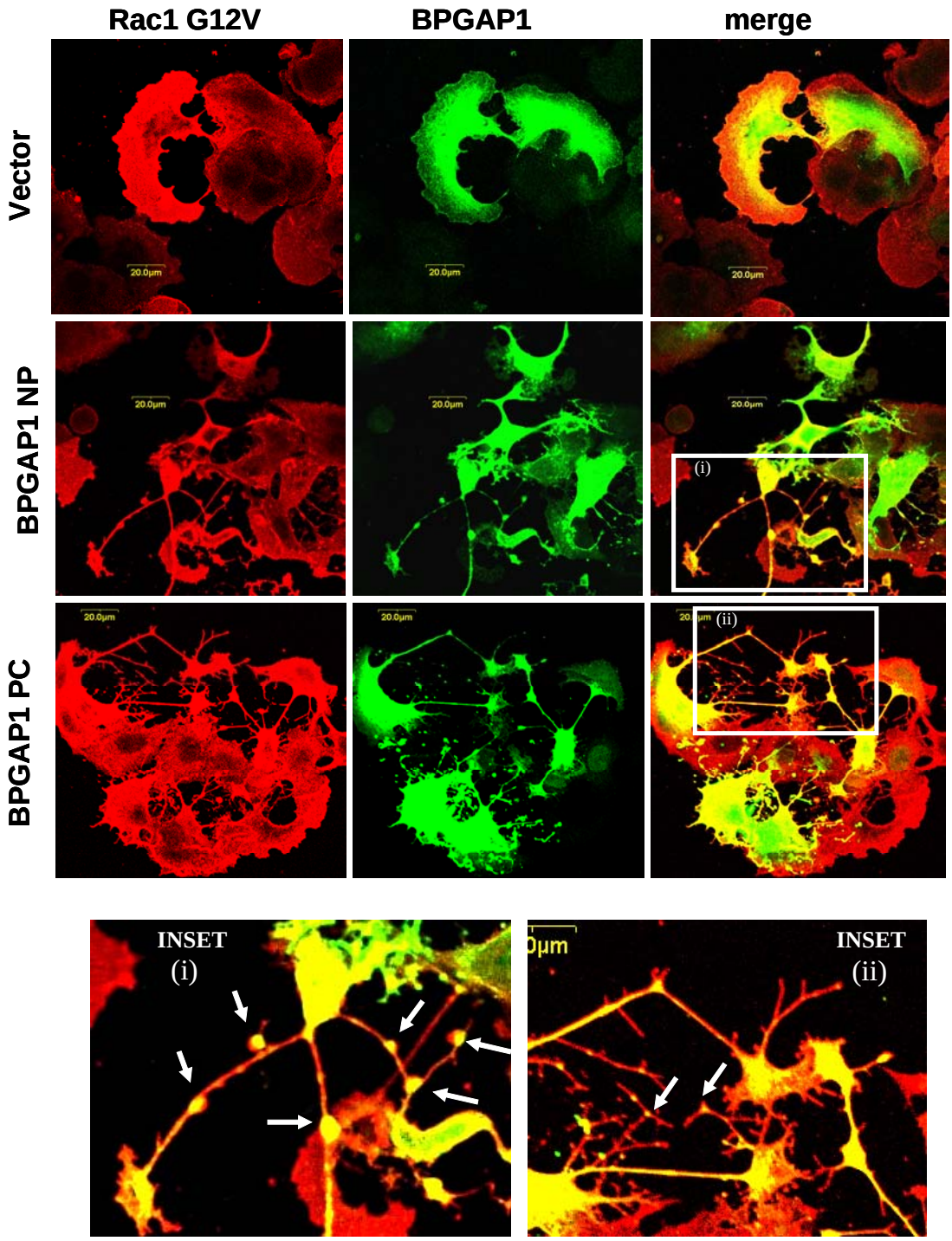

Figure 3.32 Coexpression of BPGAP1 with Rac1 G12V induced "neurite-like" outgrowth of cells. MCF7 cells were cotransfected for 16h with GFP expression plasmid for BPGAP1 FL, NP or PC domains and HA-tagged expression plasmids for constitutive active mutants of Rac1 (G12V) and then monitored for cell dynamics changes as described above. Result for the full-length (FL) is essentially the same as in Fig. 6C. Inset **(i)** and inset **(ii)** are magnified images to highlight distinctive miscroscopic features described in text. *Arrows* indicate "nodule-like" structure.

3.7 BPGAP1 promotes cell migration *via* coupling of BCH and GAP domains with the proline-rich region.

While BPGAP1 induces drastic changes in cell morphology, it remains a key question as to what are the physiological outcomes that might accompany such effects. Changes in cell morphology are often associated with cell motility as exemplified in macrophages and in numerous metastatic cancer cells (Evers *et al*., 2000; Wittmann and Waterman-Storer, 2001). 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 towards fibronectin-coated surfaces, as described in the "Materials and Methods". Fig. 3.33 shows that around 45% of the cells transfected with the full-length BPGAP1 had migrated to the fibronectin-coated surfaces, two-fold more than the control cells, whereas NP or PC domains did not elicit any significant increase in their migration potential despite the induction of pseudopodia (compare to Figure 3.26 and 3.27). This implies 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 will be discussed later.

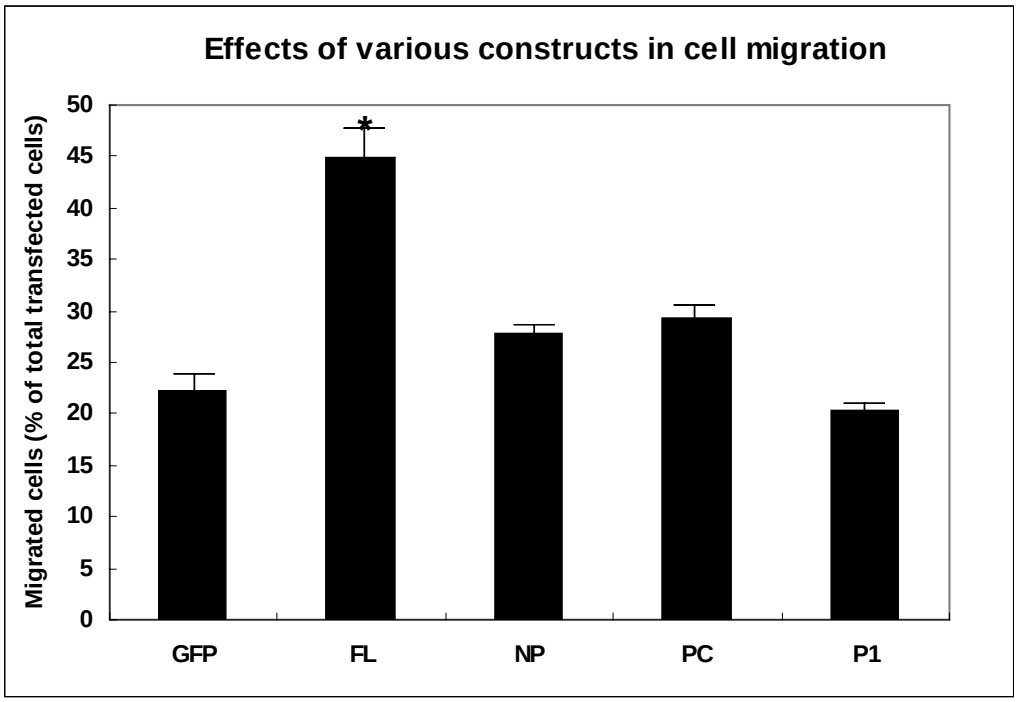

Figure 3.33 Effects of BPGAP1 on cell migration. MCF7 cells were transfected with either GFP vector control or GFP fusion of BPGAP1 full-length (FL), NP, PC or proline-deletion mutant (P1), and seeded on special chamber for 24 h to monitor their effects on cell migration, as described in "Materials and Methods". Cells that had migrated through the pores in the filter were scored by microscopy visualisation and expressed as percentage over the total transfected cells. Results are means ± S.D. of three independent experiments performed in duplicates. * indicates $p<0.01$ *vs* GFP control.

3.8 Interaction of BPGAP1 with Nedd4, a ubiquitin ligase, indicates the possible turnover of BPGAP1-induced cell signaling

3.8.1 BPGAP1 has multiple interacting partners *via* its proline-rich region

BPGAP1 contains two polyproline sequences. One is between the BCH domain and GAP domain, the other is at the C-terminus. When these two proline-rich sequences were analyzed, we found several consensus binding sites of SH3 and WW

domains inside the two polyproline sequences (Figure 3.11). Therefore, we examined how the proline-rich region of BPGAP1 could serve as a ligand for some selective SH3 or WW domains *in vitro*. Lysates expressing FLAG-tagged BPGAP1 full-length were subjected to GST "pull-down" with the SH3 domains (Figure 3.34) or WW domains (Figure 3.35) of various signaling proteins. The binding profile reveals that SH3 domain of p85 regulatory subunit of phosphatidylinosotol 3-Kinase, c-Src and phospholipase C-γ (PLC-γ) as well as the WW domains of FE65 (a nuclear protein that regulate transcription), Yes-associated protein (YAP, a transcriptional co-activator) and NEDD4-III, all exhibited strong binding to BPGAP1 whereas the NEDD4-I and NEDD4-II fragments interacted less strongly. Based on our studies, BPGAP1 was mainly expressed in cytosol (Figures 3.25 and 3.26), which implied that the possibilities of the interactions between BPGAP1 with FE65 and YAP in the cells are very low.

Nedd4, as a ubiqutin ligase, facilites the degradation of target proteins, thus induces the turn over of the cell signaling of the target proteins. Our studies have addressed the cellular and physiological functions of BPGAP1. However, there must exist "on/off" switches for every signaling pathway which will take the protein functions under control. Protein degradation thus may play an important role in turning off these signaling pathways in which BPGAP1-induced cell signaling might also be included. We next focused on the interaction of Nedd4 and BPGAP1 and the implication of this interaction on the BPGAP1 degradation.

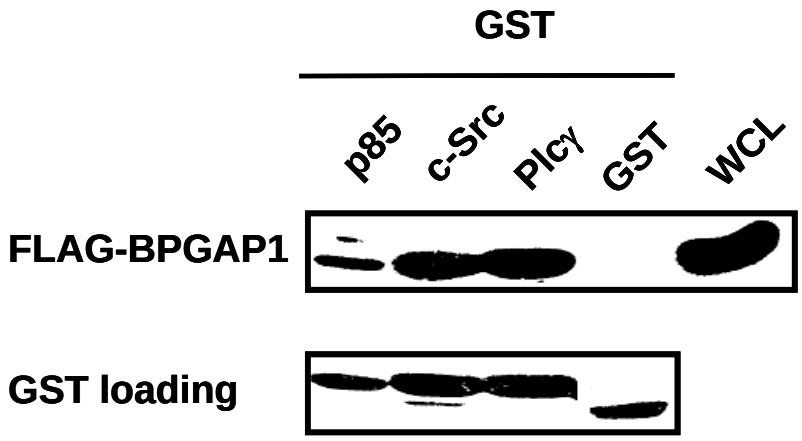

Figure 3.34 *In vitro* binding between BPGAP1 and various SH3 domains. GST-recombinants of various proteins were prepared as agarose beads and used for "pull-down" assays using cell lysates expressing FLAG-tagged BPGAP1. Beads were then washed and processed for Western blot analyses using FLAG antibodies as described in "Materials and Methods". BPGAP1 is shown to be involved in binding to selective SH3 domains. Blots were stripped and stained with amido black to reveal equal loading of GST-recombinants. p85, regulatory subunit of phosphatidylinositol 3-Kinase; PLCγ, phospholipase Cγ. *WCL*, whole-cell lystaes.

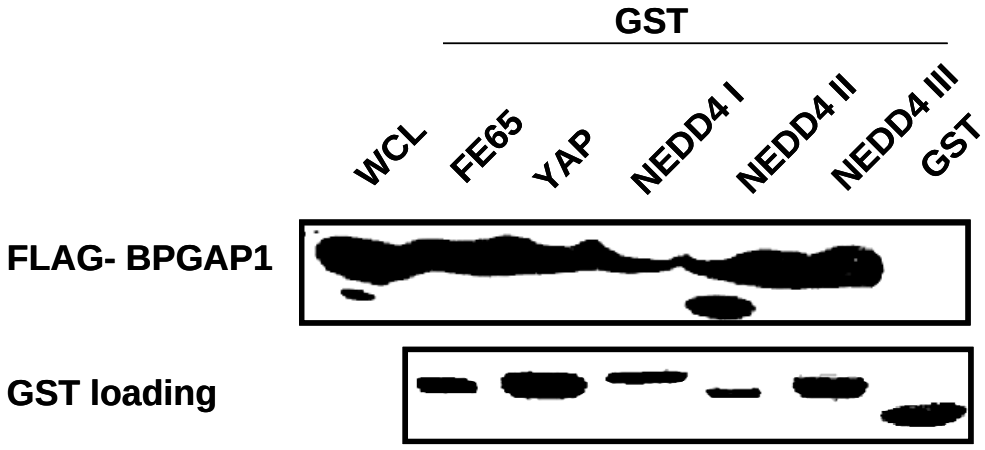

Figure 3.35 *In vitro* binding between BPGAP1 and various WW domains. GST-recombinants of various proteins were prepared as agarose beads and used for "pull-down" assays using cell lysates expressing FLAG-tagged BPGAP1. Beads were then washed and processed for Western blot analyses using FLAG antibodies as described in "Materials and Methods". BPGAP1 is shown to be involved in binding to selective WW domains. Blots were stripped and stained with amido black to reveal equal loading of GST-recombinants. YAP,Yes-associated protein; NEDD4I, WW domain-1 of NEDD4 (amino acid 218-252); NEDD4II, WW domain-2 of NEDD4 (amino acid 375-408); NEDD4III, WW domain-3 of NEDD4 (amino acid 448-481). *WCL*, whole-cell lystaes.

3.8.2 BPGAP1 interacted with Nedd4

To investigate the possible interaction between BPGAP1 and Nedd4 *in vitro* and *in vivo*, both "Pull-down" and co-immunoprecipitation were performed.

Untransfected 293T cells lysates were subjected to "pull-down" with GST recombinants of the full-length BPGAP1. Endogenous Nedd4 bound by GST-BPGAP1 was applied for SDS-PAGE and the anti-Nedd4 antibody was used to recognize the Nedd4. Figure 3.37 shows that BPGAP1 interacted with endogenous Nedd4 *in vitro*. To confirm this interaction in cells, co-immunoprecipitation was also performed. Consistent with our observation *in vitro*, BPGAP1 also interacted with endogenous Nedd4 *in vivo* (Figure 3.38). Interestingly, the interaction between BPGAP1 and endogenous Nedd4 *in vivo* is much stronger than that *in vitro*, indicating some post-translational modification may be involved to facilitate this interaction.

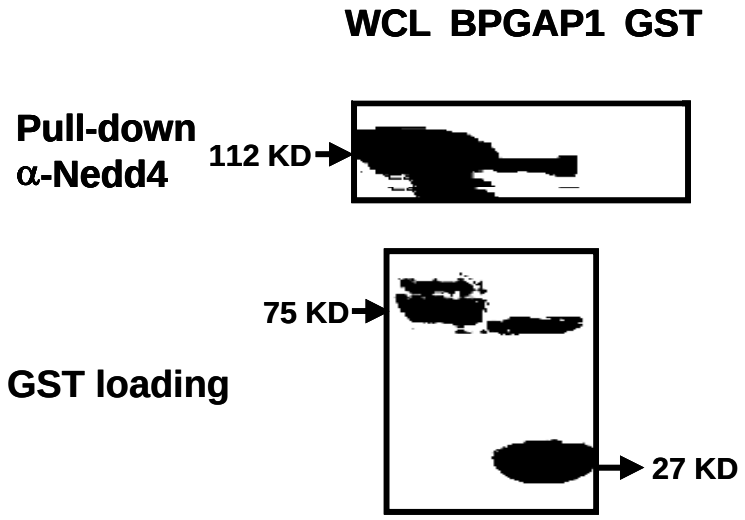

Figure 3.37 *In vitro* binding of BPGAP1 with endogenous Nedd4. GST recombinants of BPGAP1 or GST control were used for "pulldown" assays using normal cell lysates as described in 'Materials and Methods". Beads from the "pull-down" experiments were washed and processed for Western analyses using anti-Nedd4 antibodies. Blots were stripped and stained with amido black to reveal loading of GST-recombinants.

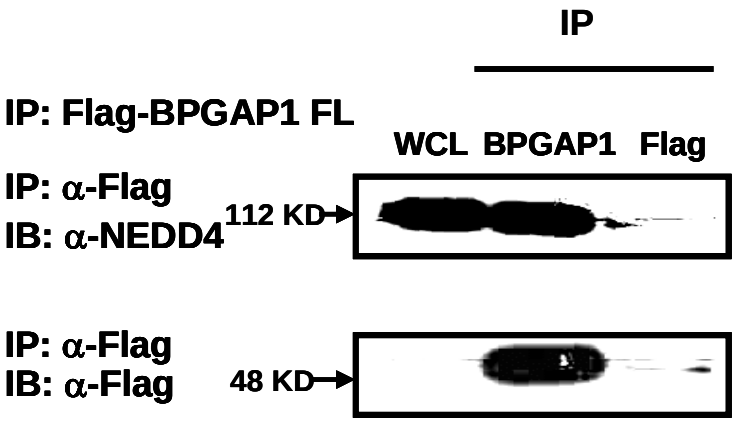

Figure 3.38 *In vivo* binding of BPGAP1 with endogenous Nedd4. Cells were transfected with expression vectors for fulllength FLAG-tagged BPGAP1 and FLAG vector control. Lysates were subjected to immunoprecipitation (IP) with anti-FLAG M2 beads and the associated proteins were separated on SDS-PAGE, and probed with anti-Nedd4 antibodies to reveal the binding of targets.

3.8.3 BPGAP1 was ubiquitinated

To test whether the interaction of Nedd4 and BPGAP1 would promote ubiquitination of BPGAP1, we studied Nedd4-mediated ubiquitination of BPGAP1. We first tested if BPGAP1 becomes ubiquitinated in 293T cells. FLAG-BPGAP1 was co-transfected with HA-ubiquitin (HA-Ub) into 293T cells, BPGAP1 was then immunoprecipitated from transfected cells using anti-FLAG M2 beads and subsequently immunoblotted with anti-HA antibodies to detect conjugation of HA-Ub. As shown in Figure 3.39**,** a high molecular weight smear representing ubiquitinated BPGAP1 (BPGAP1-Ub) is apparent in cells expressing BPGAP1 and HA-UB (*lane 4*), which indicates that BPGAP1 was being targeted for degradation by covalent ligation to ubiquitin.

To test whether Nedd4 is the E3 involved or responsible for the ubiquitination of BPGAP1, we overexpressed T7-Nedd4, either with wild-type Nedd4 (wt) or catalytically inactive Nedd4 (CS) mutant, bearing a Cys to Ser mutation at the Hect

domain, with FLAG-BPGAP1 and HA-Ub and analyzed the extent of BPGAP1 ubiquitination in these cells. As seen in Figure 3.39, overexpression of Nedd4 (wt) (*lane2*) led to a marked increase in BPGAP1 ubiquitination. In contrast, overexpression of Nedd4 (CS) completely abolished ubiquitination of BPGAP1 (*lane3*). All the ubiquitination we mentioned above is poly-ubiquitination which can lead to protein degradation. Interestingly, a band around 55 kDa (*lane 3*) was pulled down when Nedd4 (CS) was overexpressed with BPGAP1 and ubiquitin, indicating the possible mono-ubiquitination.

These results suggest that Nedd4 is the ubiquitin ligase responsible for ubiquitination of BPGAP1 and that the Nedd (CS) mutant is acting in a dominant negative fashion to inhibit ubiquitination of BPGAP1 by blocking endogenous Nedd4.

Interaction between BPGAP1 and Nedd4 leads to the ubiquitination of BPGAP1 in an E3 ligase (Nedd4) dependent manner, which suggests a possible mechanism of the turnover of BPGAP induced cell signaling.

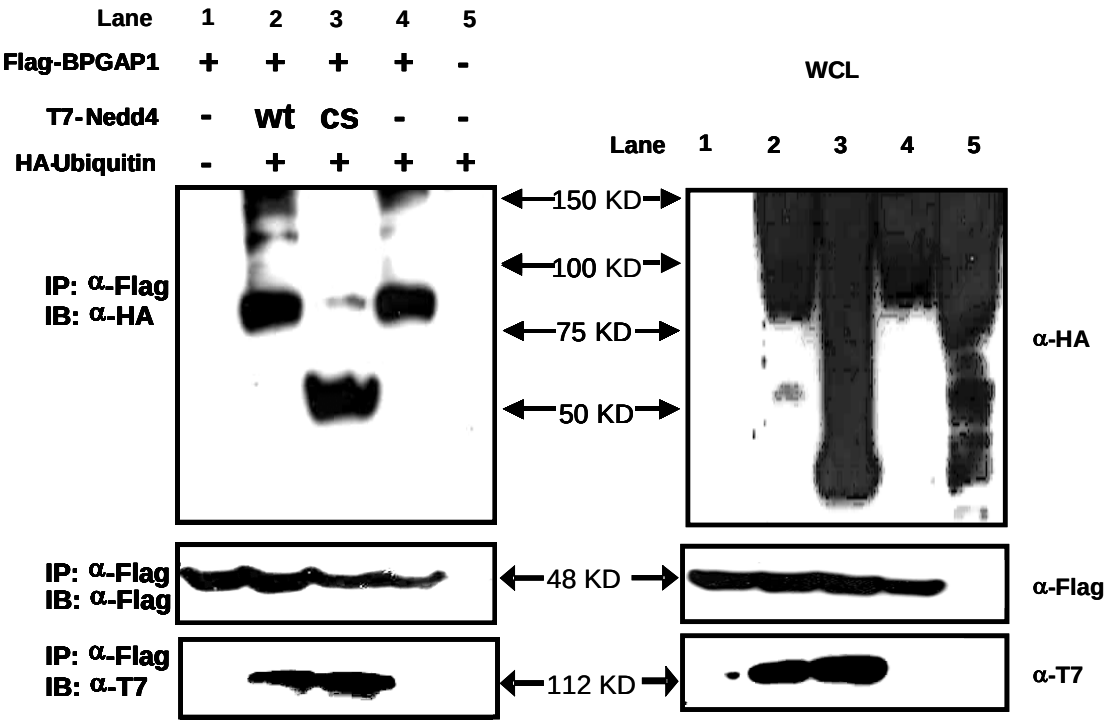

Figure 3.39 Nedd4-mediated ubiquitination of BPGAP1. 293T cells were transfected with the indicated plasmids (FLAG-BPGAP1, HA-Ub, T7-Nedd4, either wild-typed (*Wt*) or catalytically inactive (*CS*) mutant). Lysate from the transfected cells (*WCL*) were immunoprecipitated with anti-FLAG M2 beads to precipitate BPGAP1 and immunoblotted with anti-HA antibodies to detect ubiquitinated BPGAP1(BPGAP1-UB). Corresponding lysates were immunoblotted with either anti-T7 antibodies to verify expression of Nedd4 or anti-FLAG antibodies to verify expression of BPGAP1. Ubiquitinated BPGAP1 as a smear of high molecular weight bands. Expression of ubiquitin, BPGAP1 and Nedd4 were examined with anti-HA, anti-FLAG and anti-T7 antibodies.

Chapter 4

Discussion

4.1 Significance of multi-domain organization

BPGAP1 is a multi-domain containing protein with a BCH domain, proline-rich regions and a RhoGAP domain. Multi-domain organization is common among proteins. Beside the RhoGAP domain, RhoGAP family members typically contain other functional motifs including catalytic domains, protein-protein interaction domains, etc. For example, BCR contains both a kinase domain and a RhoGEF domain aside from the RhoGAP domain (Chuang *et al*., 1995; Diekmann *et al*., 1991); ARAP-1 contains an ArfGAP domain (Krugmann *et al*., 2002; Miura, *et al*., 2002); some other proteins contain protein-protein interaction domains such as SH2, SH3 and protein-lipid adaptor modules such as PH and CR domains (Moon and Zheng, 2003).

The activity of RhoGAP domain may be regulated by the other functional domains located at the same RhoGAPs. Sometimes RhoGAP domain cooperates with other domains for the same cellular function. ARAP subfamily of RhoGAPs is a good example that shows cooperative interplay of multifunctional domains. ARAP contains both a ArfGAP domain that is active towards the Arf GTPases and a RhoGAP domain. These two domains appear to cooperate in mediating cytoskeleton rearrangement and cell morphological changes in response to PI 3-kinase (Krugmann *et al*., 2002; Miura *et al*., 2002). In some other cases, the function of RhoGAP domain is inhibited by other domains of the same protein, thus RhoGAPs might catalyze enzymatic reactions other than the stimulation of GTP hydrolysis of Rho proteins and sometimes even activate Rho protein signaling. BCR is such an example to show us the opposite functions between RhoGAP domain and other domains at the same protein. Both BCR and ABR contain a RhoGEF domain and a RhoGAP domain. Therefore,

potentially they can activate and inactivate the Rho GTPases at the same time. And the RhoGAP activities of BCR and ABR might thus be inhibited under some cellular conditions (Chuang, 1995; Diekmann, 1991).

BPGAP1 can potentially regulate several signaling pathways through interacting with various interacting partners *via* its multiple protein domains. Also, our current study shows that both BCH domain and GAP domain are involved in the regulation of cytoskeleton organization through differentially targeting Rho GTPases. We have yet to see if multi-domain organization of BPGAP1 is also required for other cellular functions in addition to cell migration. Interestingly, we observed that there were intramolecular interaction between BCH domain and GAP domain, homophilic/heterophilic interactions and interactions between the proline-rich regions of BPGAP1 with other interacting partners such as PI3K. Therefore, some signaling pathways might be regulated by BPGAP1 through these protein interactions, which is yet to be investigated.

4.2 Significance of different splicing variants of BPGAP families

To date, we have uncovered and characterized at least one member of the BPGAP family. Its wide distribution in tissues suggests that it could play a very common cellular function such as the control of cell morphology and cell motility as shown in the current study. However, based on our cloning results and bioinformatics analyses of various EST and putative/uncharacterized sequences, there exist at least four more closely-related members, namely BPGAP2 to BPGAP5. Interestingly, when compared to BPGAP1, both BPGAP2 and BPGAP4 were identified to have an insertion of 31 amino acids in their BCH domain whereas BPGAP4 has an even longer extension at the N-terminal sequence (Figure 3.5). BPGAP3 on the other hand,

has the same BCH domain as BPGAP1 but possesses two smaller but distinctive parts of the N-terminus in BPGAP4. BPGAP5 has a 88 bp deletion at its N-terminus, therefore the sequence became out of frame, introducing a stop codon subsequently. It is anticipated that these are alternatively spliced variants of BPGAP1, but we have yet to investigate the cellular functions of each of these members. For example, we might study if the other members of BPGAP family, such as BPGAP2, could form homophilic or heterophilic interactions through their respective BCH domains as did BPGAP1.

It is suggested that different types and proportions of alternatively spliced variants can provide environmental cues to modulate various cellular behaviors (Schwarzbauer, 1991). Our preliminary results show that expression of BPGAP2 did not induce cell protrusions/pseudopodia as did BPGAP1. It is interesting to see what effect the insertion within the BCH domain will have on its interactions with other BCH domains, other small GTPases as well as in regulating its full-length activity.

4.3 Divergent functions of BCH domains in different proteins

We hypothesize that BCH domains from different proteins carry unique biochemical properties, substrate specificity and cellular functions. For examples, we first characterized the BCH domain of BNIP-2 protein being able to confer GAP-like activity towards Cdc42 *via* a novel arginine-finger motif (Low *et al*., 2000a) whereas the homologous BCH domains in Cdc42GAP (Low *et al*., 2000a) as well as BPGAP1 (current study) failed to display this activity due to the lack of this motif in the domain. We have also identified that the BCH domain of BNIP-Sα is a very potent inducer of apoptosis (Zhou *et al*., 2002) that does not interact with Cdc42 but interacting with

other small GTPases, whereas the BCH domain of BNIP-2 induces cell extensive structures *via* targeting Cdc42 (Zhou *et al*., 2004, manuscript in preparation).

The BCH domain of BPGAP1 is more closely related to that of Cdc42GAP (84% similarity) and it lacks the 4-amino acid residues (E/RSSQ/I) uniquely found in the corresponding BCH domains of BNIP-2 and BNIP-S (61% and 54% similarity, respectively). We are now testing to see if this and other regions could potentially serve to confer functional specificity in the subclasses of BCH domain.

We noticed the considerable homology between the BCH domain and the lipid-binding domain Sec14p of the phosphatidylinositol transfer protein (NP_013796) and propose that some of these members could still retain their ability to bind to this non-peptide substrate. Phospholipid affinity chromatography studies indicate that Cdc42GAP can bind phosphatidylinositol 3,4,5-trisphosphate (PtdIns (3,4,5)P_3). The interaction between Cdc42GAP and PtdIns (3,4,5)P_3 likely involves the BCH domain, resulting in its recruitment to the plasma membrane and conformational changes that regulate its GAP activity (Krugmann, 2002). The properties and their functional significance for various BCH or Sec14p-containing proteins in terms of lipid binding remain to be investigated.

4.4 Post-translational modification and intramolecular interaction regulate the conformation and function of BPGAP1

Our results show that BPGAP1 can form homophilic/heterophilic interactions with other BCH domain containing proteins such as BNIP-2 and Cdc42GAP. Interestingly, in the *in vitro* “pull down” assay, GST fusion protein of BNIP-2 did not pull down BPGAP1 expressed in the mammalian cells, whereas in the contrary, the

GST-BPGAP1 was reactive towards FLAG-BNIP-2. The lack of binding of BPGAP1 to BNIP-2 was probably due to the fact that when expressed in mammalian host, BPGAP1 could assume some modifications or conformational changes that selectively reduce its binding capacity to BNIP-2.

One possible mechanism to regulate the interaction between BPGAP1 and BNIP-2 is through post-translational modification such as phosphorylation. *In vitro* binding assay shows that BPGAP1 interacts with the SH3 domains of some protein kinases such as c-Src and PI3K which might phosphorylate BPGAP1, thus further modify its conformation or activity. Studies on other RhoGAPs show similar regulatory mechanism. For example, p250RhoGAP is tyrosine phosphorylated by Fyn , a member of the Src-family protein tyrosine kinases which plays important roles in both neurons and oligodendrocytes. This phosphorylation appears to enhance the interaction between p250GAP and Fyn. Tyrosine phosphorylation of p250GAP by Fyn would regulate its RhoGAP activity, subcellular localization, or interactions with other proteins, leading to morphological and phenotypic changes of oligodendrocytes (Taniguchi *et al*., 2003).

In addition, intramolecular interaction between the BCH domain and GAP domain might play an important role in regulating the binding profile and the activity of BPGAP1. BPGAP1 could form “Close” or “Open” states through intramolecular interation. Like p85, the regulatory subunit of PI3K which can also form intramolecular interaction between its phosphorylated Tyrosine (688) and its N-terminus SH2 domain, leading to relief of the inhibitory activity of p85 on p110, the catalytic subunit of PI3K, thus regulating the signaling pathways of PI3K (Cuevas, 2001; Zhang *et al*., 1994). It will be a challenge to uncover the regulation of intramolecular interaction and its roles in BPGAP functions. To the end, motifs must

be found if this intramolecular interaction is direct binding or indirect which might be though the common interacting partners of both BCH domain and GAP domain.

4.5 BPGAP1 may function as an adapter protein through its interaction with multiple interacting partners

It has been shown that BPGAP1 targets a variety of signaling proteins through its multiple protein domains. Though its BCH domain, it can interact with BNIP-2 and Cdc42GAP; through its proline-rich sequence, it targets several WW domain and SH3 domain containing proteins such as NEDD4, p85α, c-Src, etc. It can also target Rho GTPases through both its BCH domain and GAP domain. Thus, potentially BPGAP1 may function as an adapter protein that co-localizes different signaling proteins together to facilitate their interactions and functions.

A member of RhoGAP-containing protein familiy, p85 has been reported to have such a function as an adapter protein. p85 can interact with GTP-bound Cdc42 and Rac *in vitro* and co-immunoprecipitates with Cdc42 in a GTP-dependent manner. Therefore, p85 may acts as an adapter protein to localize PI3K acitivty to the sites of Cdc42 activation. According to our results, BPGAP1 can interact with both Cdc42 and p85α, and this interaction might lead to these three proteins to form a complex. This may provide a new model for the regulation of the PI3K and Cdc42 activities.

4.6 GTPase activity of BPGAP1

BPGAP1 shows different GTPase activities towards Rho GTPases *in vivo* and *in vitro*. *In vivo*, it only has activity toward RhoA, whereas *in vitro* it has high GTPase activity toward Cdc42, lesser to RhoA, but none to Rac1. Some other Rho GTPase

activating proteins also differentially regulate Rho GTPase activities in *vivo* and in *vitro*. For example, p190-A has equal GAP activity for Rho, Rac and Cdc42 *in vitro*, but demonstrates a preference for Rho *in vivo* (Settleman *et al*., 1992).

This different GTPase activities regulation *in vivo* and *in vitro* might be caused by post-translational modification *in vivo* or the involvement of other protein domains that BPGAP1 harbors, such as BCH domain.

Phosphorylation could play an important role in regulating the GTPase activities of BPGAP1. An example of the role of phosphorylation in GTPase activity is p190RhoGAP. The RhoGAP activity of p190-A RhoGAP is regulated by phosphorylation. Upon stimulation by growth factors or cell attachment, tyrosine residues in the central portion of p190 are phosphorylated by kinases such as Src (Haskell *et al*., 2001). This phosphorylation causes a conformational change in the p190 molecule resulting in the activation of its GAP activity. BPGAP1 contains several consensus phosphorylation motifs that potentially can be recognized by several protein kinases. For example, both ^{379}KIFS^{382} and ^{418}KPT^{421} coincide the consensus recognition motifs of protein kinase C, whereas ^{14}RVVT^{17} and ^{84}KPS^{86} are the potential phosphorylation sites of calmodulin-dependent protein kinase II and cGMP-dependent protein kinase respectively. An example for the consensus tyrosine kinase recognition motif is ^{56}EYL^{58}, which might be phosphorylated by epidermal growth factor receptor kinase. Future investigation will be performed to test if BPGAP1 can be phosphorylated and if the phosphorylation induces any conformational or functional changes.

It has been known that BCH domain also targets Rho GTPases. Both the BCH domains in BNIP-2 and in BPGAP1 can interact with Cdc42. Therefore in BPGAP1, BCH domain and GAP domain may compete the interaction with Rho GTPases,

leading to the involvement of BCH domain in the regulation of the GAP activities. BCH domain might be involved in the regulation of GAP activity through other ways such as homophilic and heterophilic interactions with other BCH domain containing protein or intramolecular interactions. It has been previously identified that at least in the BNIP-2 protein, there exits a unique motif in its BCH domain that binds and negatively regulates the activity of Cdc42GAP expressed in the cells (Low *et al*., 2000a). But, it remains to be seen if BPGAP1 homophilic complex has different binding and activity towards specific Rho small GTPases.

The ability of BCH and GAP domains of BPGAP1 in mediating different extents of pseudopodia and their differential binding to members of Rho GTPases are intriguing, especially in light of these two domains being quite diverged in their primary protein sequences. We have seen that the BCH domain of BPGAP1 could interact with Cdc42 very strongly (Figure 3.21) than it would with Rac1 or RhoA. In contrast, although RhoA is the substrate of BPGAP1 in terms of the GTPase activity *in vivo* and *in vitro*, the interaction between RhoA and BPGAP1 is not strong. It appears that *in vivo* RhoA can undergo rapid turnover of binding and dissociation from the GAP domain (kind of “kiss-and-run” scenario) whereas Cdc42 or Rac1 which are not the substrates, are not released from the complex upon their binding.

4.7 Both BCH domain and GAP domain are needed for BPGAP1-induced short and long pseudopodia

Our current studies show that BPGAP1 plays an important role in regulating cell dynamics. Biochemical and cellular functions of its three proteins 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 have uncovered 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.

4.7.1 Regulation of the interaction between BPGAP1 and Rho GTPases

Interactions between BPGAP1 with Rho GTPases are regulated by both its BCH domain and its GAP domain. So far, we are not clear if Rho GTPases interact with BCH domain at the same binding site as with the GAP domain. If the binding sites are different, these separate interactions might facilitate and strengthen the whole protein to interact with Rho GTPases, whereas if the binding sites are the same, their relationship might be competitive rather than cooperative. Meanwhile this interaction is regulated by other factors such as the intramolecular interaction between themselves and the homophilic and heterphilic interactions with other modules. All these multiple factors might contribute to the interaction of BPGAP1 protein as a whole.

Interestingly, there has been a recent report of an *Arabidopsis* RhoGAP, termed RopGAP (Wu *et al*., 2000; Yang, 2002) that utilized Cdc42/Rac-interactive binding motif adjacent to its GAP domain to facilitate both the binding of RopGAP to Rop (Rho equivalents in plants) as well as the formation of transitional state of the Rop during the GTP hydrolysis. While these two classes of proteins seem to share a common strategy of “recruiting” small GTPases, their specific targets and consequences are clearly different.

To fully understand how this mechanism would operate, one needs to identify all necessary interacting motifs by mutational studies for all known interacting partners of BPGAP1, including itself (*via* BCH or GAP domains), other BCH-domain containing proteins as well as the small GTPases. This will allow us systematically and progressively delineate their actual involvements.

4.7.2 BPGAP1 induces short and long pseudopodia through differentially regulating Rho GTPases

These observations of the interaction between BPGAP1 and Rho GTPases raise the question as to whether such differential binding properties in both adjacent domains can account in part, if not entirely, for the observed changes in morphology induced by BPGAP1. Indeed, one could argue that BPGAP1 can interact with different pools of GTPases in the cells at any one time – the equilibrium of which would result in the cellular features seen. These issues were partly resolved by the co-expression studies with either the constitutively active or dominant negative mutants of small GTPases.

Indeed, our results demonstrate 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 (Figrues 3.29-3.31). 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. This is supported by the observation that despite Cdc42 or Rac1 intrinsic activities not being affected (Figure 3.20), the morphological changes induced by BPGAP1 still required the execution of Cdc42/Rac1 pathways, as shown by the strong inhibition from their dominant negative mutants but potentiation by their constitutive active counterparts. Work is currently underway to test this hypothesis further.

However, we cannot exclude the possibility that overexpression of Rac1 dominant negative mutant T17N may lead to nonspecific effect in blocking the BPGAP1-induced pseudopodia formation. It was reported that overexpression of Rac1 T17N could induce some nonspecifice effect (Wennerberg *et al.*, 2002). The inhibition of BPGAP1 induced pseudopodia caused by Rac1(17N) may have blocked some yet undiscovered GTPases. Therefore, the same experiment with the better control of the period of expression time (shorter time) and the expression level (regulatable level) should be performed to further confirm the role of Rac T17N in the inhibition of BPGAP1-induced cell pseudopodia.

These results strongly indicate the involvement of BPGAP1 in differentially regulating distinctive pathways of Rho GTPases that could have important physiological bearings, including 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.

4.7.3 BPGAP1 induces drastic "neurite-like" structure upon Rac1 activation

The revelation that either BCH or the GAP domain could separately induce very similar and drastic "neurite-like" outgrowth upon Rac1 activation represent another novel and intriguing feature of BPGAP function (Figures 3.31 and 3.32). The only difference appears to be on the existence of "nodule-like" structures along the "neurite-like" extensions elicited by BCH domain. While these observations support the involvement of BPGAP1 in Rac1 pathway, it remains to be seen whether the full-length protein could also exert this effect in certain cellular condition(s) or cell types that are yet to be identified. While the actual molecular mechanism awaits further investigation, the disposition of these features could hint towards the likely involvement of Arp2/3 complex in inducing cellular branching and extensions. Arp2/3 complex is a stable assembly of two actin-related proteins, Arp2 and Arp3, with five novel subunits. Biochemical and microscopic studies show that Arp2/3 complex caps pointed ends and initiates growth in the barbed direction as 70 degree. And it has been proved that Arp2/3 functions as a nucleator of actin filaments upon preexisting filaments such as dendritic nucleation (Pollard and Borisy, 2003). It will be interesting to further investigate if the Arp2/3 complex would indeed involve in BPGAP1/Rac-induced "neurite-like" structure.

4.8 BPGAP1-induced cell pseudopodia is not due to cell retraction

Our study shows that expression of BPGAP1 induces long and short pseudopodia of epithelial cells. These long and short pseudopodia are considered to be the cell elongation and protrusion. One may argue that these pseudopodia might

not be cell elongation, but caused by cell retraction. Expression of some RhoGAP proteins can lead to cell area changes. For example, in the early stage of cell spreading, cells expressed wild-type p190 RhoGAP become larger in the area, whereas the cells area become smaller when mutant p190RhoGAP whose catalytic Arginine motif mutated is expressed. In order to confirm if BPGAP1 induced pseudopodia are caused by cell elongation or cell retraction, the cells area are measured in our study. The results demonstrate that there is no difference between the cells area of BPGAP1 transfected cells and the control cells, indicating BPGAP1 induced cell pseudopodia are elongation.

4.9 Roles of domains in the BPGAP1-induced cell migration

4.9.1 BPGAP1 facilitates cell migration through differentially regulating the Rho GTPases activities

Rho GTPases clearly play an key role in regulating actin assembly and disassembly, further inducing cell migration. It has been concluded that Rac, through its ability to promote actin polymerization at the cell periphery, provides the driving force for the protrusive activity required for cell migration, whereas Cdc42 appears to direct and stabilizes Rac activity at the cell front during cell migration. The activation of both Rac and Cdc42 facilitates the cell migration. The role of Rho in cell migration is not very clearly studied so far. It is reported that RhoGTP is excluded from the leading edge of the migrating cells (Worthylake, 2001), suggesting the inactivation of Rho at the cell periphery of cell migration direction where active Rac and Cdc42 localize.

Our current study has added BPGAP1 to the increasing list of proteins that regulate cell morphology and cell motility. Despite various input signals, all stimuli seem to converge to the Rho small GTPases that in turn activate or inactivate the distinctive pathways through a battery of specific regulators (Bishop and Hall, 2000; Narumiya *et al*., 1997). They can either be the effectors that transmit and execute the signal downstream of activated pathways, or a regulator that control the event by affecting the status of the upstream signals. In this regard, we believe that BPGAP1 could serve both functions in a concerted manner- it could inactivate RhoA signaling *via* the GAP domain and yet facilitating the execution of Cdc42/Rac1 pathway probably directly through its BCH domain (since Cdc42 binds to this very strongly) or through its GAP domain where Rac1 and Cdc42 are also shown to interact despite not being the substrates (Figure 3.36). In addition, there are some related reports indicating that inactivation of Rho induces the activation of Cdc42 and Rac1 directly (Cox *et al*., 2001). Therefore differential regulation of the activities of Rho GTPases is required for BPGAP1 induced cell migration. Cdc42 and Rac1 are in the inactive states whereas Rho is in the active state during BPGAP1 induced cell migration, which is consistent with our observation of cell pseudopodia formation.

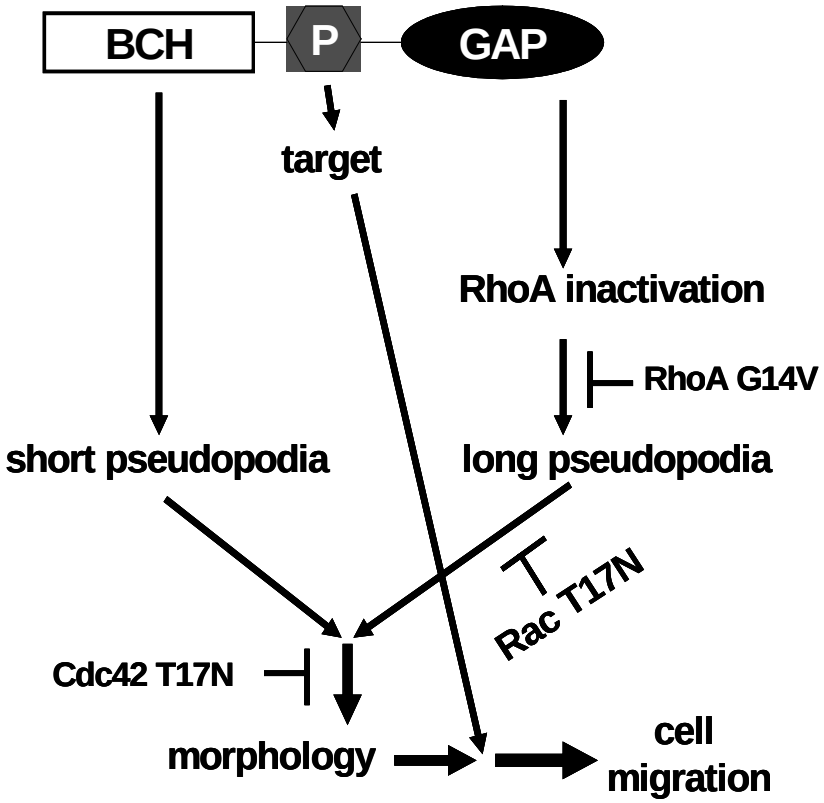

Figure 3.36 Model for the effects of BPGAP1 on cell dynamics control. The three separate domains, BCH, proline-rich and GAP domains of BPGAP1 coordinately regulate distinctive yet concerted pathways in cell dynamics control. Its GAP domain specifically inactivates RhoA pathway and induces long pseudopodia 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 is/are involved in linking cell morphological changes to its migration.

4.9.2 The contribution of proline-rich region to the BPGAP1 induced cell migration

Although proline-rich region of BPGAP1 seems not involved in BPGAP1 induced cell morphological changes, our results show that deletion of this sequence does inhibit the BPGAP1 induced cell migration. There are two possible mechanisms hypothesized. Firstly, we know this proline-rich sequence is localized between the

BCH and GAP domains and may act as "a bridge" of the whole protein structure. Deletion of this region may change structure of the protein, thus influence the functions of BCH and GAP domains. Although this influence might not be involved in the BPGAP1 induced pseudopodia, it may cause other cell physiological changes. The influence of deletion of proline-rich sequence on the function and activity of BCH and GAP domains is to be discovered. Secondly and more possible, proline-rich sequence may facilitate this cell morphological changes by interacting with other proteins. Either this interaction may cause conformational changes or potentiate BPGAP1 to be involved in some signaling pathways is considered.

It is 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 (Macias *et al*., 2002) or WW (Sudol and Hunter, 2000) domains (Figure 3.11). This could point to some common regulatory mechanism among all these proteins. Incidentally, 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 (Bear *et al*., 2000; 2002). 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 (some of this have already been tested, see Figure 3.34 and 3.35), identifying the real interacting partner(s) that mediate this process remains a challenging but exciting prospect for future work.

4.9.3 BPGAP1-induced cell migration requires the interplay of multi-domains

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. 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's wild-type GAP domain alone decreased RhoA activity, promoted the formation of membrane protrusions, and enhanced motility (Arthur and Burridge, 2001). Likewise, DEF1/ASAP1 (the GAP for ADP-ribosylation factor-1, ARF-1) enhanced cell motility *via* a GAP-dependent mechanism (Furman, 2002) but another ArfGAP protein, ARAP1 (which also includes a rhoGAP domain besides the ArfGAP domain) utilizes it RhoGAP domain to cause cell rounding independently of the other domains (Miura *et al.*, 2002). In addition, the rhoGAP domain of p122RhoGAP could only induce an extensive cell rounding and detachment of adherent cells (Sekimata *et al.*, 1999). Our study provides a novel mechanism of the cell regulation through a multi-domain RhoGAP.

4.10 BPGAP1 is ubiquitinated in a Nedd4-dependent manner

4.10.1 Binding motifs of BPGAP1 with Nedd4

Our study shows that BPGAP1 can interact with endogenous Nedd4 both *in vivo* and *in vitro,* leading to the ubiquitination of BPGAP1 in a NEDD4 dependent manner. Overexpression of the Nedd4(CS) mutant impaired ubiquitination of BPGAP1,indicating that the mutant Nedd4 acts in a dominant negative fashion towards the endogenous Nedd4. These results therefore suggest that Nedd4 is likely the E3 ligase involved in the regulation of ubiquitination of BPGAP1.

Nedd4 contains a C2 domain, three or four WW domains and a ubiquitin ligase HECT domain. The WW domains mediate binding to target proteins, usually by associating with their PY motifs (PPxY) (Kikonyogo *et al*., 2001; Staub *et al*., 1996). BPGAP1 does not contain PY motif, suggesting that there might be other binding sites in BPGAP1 with Nedd4. BPGAP1 has two poly proline-rich sequences which potentially encode three types of consensus WW domain binding sites. Further mutation and deletion studies are needed to address the binding motifs of Nedd4. And some other methods such as co-immunofluorescence may also be applied to address the cellular and physiological significance of the interaction between BPGAP1 and Nedd4.

4.10.2 Nedd4 (CS) mutant inhibits the polyubiquitination of BPGAP1

Our current studies show that wild type Nedd4 mediated the polyunbiquitination of BPGAP1, whereas overexpression of Nedd4 (CS) mutant completely abolished ubiquitination of BPGAP1 (Figure 3.39). Interestingly, we found that a band around 55 kDa (Figure 3.39) was pulled down when Nedd4 (CS) was overexpressed with BPGAP1 and ubiquitin, which might be caused by mono-ubiquitination. Monoubiquitination is a modification that is not linked to protein degradation (Moren *et al*., 2003). Instead of sending proteins to their death through the proteasome, monoubiquitylation regulates processes that range from membrane transport to transcriptional regulation (Hicke, 2001; Haglund *et al*., 2002). Monoubiquitylation is involved in at least three distinct cellular functions: histone regulation, endocytosis and the budding of retroviruses from the plasma membrane (Hicke, 2001). Nedd4, as an E3 ubiquitin ligase, accepts ubiquitin from the E2 to

form a ubiquitin-thioester intermediate with the HECT active cysteine. It can also mediate monoubiquitination, but might interact with ubiquitin with different binding motifs (Hicke, 2001; Polo *et al*., 2002). This may explain why it is possible that Nedd4 (CS) mutant could mediate monoubiquitination after inhibiting polyubiquitination.

4.10.3 Not all the BPGAP1 expressed might be ubiquitinated

Interesting, in ubiquitination assay, FLAG-BPGAP1 was coexpressed with Nedd4 (wt) and ubiquitin, BPGAP1 was then immunoprecipitated from transfected cells using anit-FLAG M2 beads and subsequently immunoblotted with anti-HA antibodies to detect conjugation of HA-Ub. As shown in Figure 3.39**,** a high molecular weight smear representing ubiquitinated BPGAP1 (BPGAP1-Ub) is very apparent. However, when this blot was applied to anti-FLAG antibody to detect the FLAG-BPGAP1 that was pulled down, we found there were not detectable high molecular weight smear, whereas only one band about 48 kDa which coincides with the size of unconjugated BPGAP1. Probably, it is because only small amount of BPGAP1 that was expressed was conjugated to ubiqutin and was ubiquitinated subsequently. Most of the BPGAP1 that was expressed might be regulated differently or form complexes with other partners. This small amount of conjugated BPGAP1 was undetectable. This phenomenon was also described by previous reports (Wong *et al*., 2002; Pham and Rotin, 2001).

4.10.4 Implications of the turn-over of BPGAP1 signaling in human disease

The functionality and efficacy of Rho GTPase signaling is essential for a variety of biological processes. Due to the integral nature of these molecules, the dysregulation of their activities can result in diverse aberrant phenotypes. Dysregulation can be based on an altered signaling strength on the level of a specific regulator or that of the respective GTPase itself. Alternatively, effector pathways emanating from a specific Rho GTPase may be under- or overactivated (Boettner and Van Aelst, 2002). Dysregulation of Rho GTPases activities is involved in various disease processes such as cancer progression, mental disabilities and other disorders.

As a regulator of Rho GTPases, both the turn on and turn-over of BPGAP1 cell signaling are very critical in maintaining the normal cycling of Rho GTPases. Protein degradation is one of the tactics employed by the cell for irreversibly inactivating proteins. In eukaryotes, ATP-dependent protein degradation in the cytoplasm and nucleus is carried out by the 26S proteasome. Most proteins are targeted to the 26S proteasome by covalent attachment of a multi-ubiquitin chain. The rapid degradation of protein regulators is especially important when the regulator should act for a short period of time (Hershko and Ciechanover, 1998).

Our research discovered that BPGAP1 could be ubiquitinated in a Nedd4 dependent manner, which provides a possible mechanism for the turn-over of BPGAP1 signaling. The normal activity of BPGAP1 thus can be under control to avoid the dysregulation of Rho GTPases, which may induce a plethora of human diseases.

Chapter 5

Conclusions and Future perspectives

5.1 Conclusions

In this study, we report the cloning of BPGAP families (for BCH domain-containing, Proline-rich and Cdc42GAP-like proteins) and the functional characterization of BPGAP1 which shares high degree of homology with Cdc42GAP and contains multiple protein domains. BPGAP1 forms homophilic and heterophilic complexes with itself, Cdc42GAP and BNIP-2 *via* their homologous BCH domains. We show that BPGAP1 differentially modulates RhoA, Cdc42 and Rac1 signaling pathway by a mechanism that requires cooperation between the BCH and GAP domain. When expressed in non-metastatic human breast epithelial cancer cell lines MCF7 cells, BPGAP1 induces 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 is 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. The interaction between BPGAP1 and Nedd4, a ubqitination ligase, indicates a possible mechanism of the turnover of BPGAP1 induced cell signaling.

5.2 Future perspectives

Our current studies have characterized both the biochemical features and cellular functions of BPGAP1. Meanwhile, preliminary results and reliable clues have been raised for the future investigation related to the BPGAP family proteins.

First of all, our current study shows that BPGAP1 targets a variety of

interacting partners through it multiple protein domains. Most interestingly, we find Rho GTPases can be targeted by both BCH domain and GAP domain, and these two domains can even form intramolecular interaction themselves. Therefore, it is a challenge to uncover the regulation of all these protein-protein interactions and their significance in cell signaling.

To fully understand how this mechanism would operate, one needs to identify all necessary interacting motifs by mutational studies for all known interacting partners of BPGAP1, including itself (*via* BCH or GAP domains), other BCH-domain containing proteins as well as the small GTPases. This will allow us systematically and progressively delineate their actual involvements.

To date, we have characterized BPGAP1 of the BPGAP family. However, based on our molecular cloning results and bioinformatics analyses of various EST and putative/uncharacterized sequences, there exist at least four more closely-related members namely BPGAP2 to BPGAP5. Interestingly, when compared to BPGAP1, both BPGAP2 and BPGAP4 were identified to have an insertion of 31 amino acids in their BCH domain whereas BPGAP4 has an even longer extension at the N-terminal sequence. Our preliminary results have shown that expression of BPGAP2 can not induce cell pseudopodia/ protrusions as that of BPGAP1. Therefore, it is potentially interesting to see what effect the insertion within the BCH domain will have on its interactions with other BCH domains, other small GTPases as well as in regulating its full-length activity.

Small interfering RNAs (RNAi) is a broadly applicable method for gene silencing *in vitro*. It has rapidly become a well recognized tool for characterizing the functions of a variety of genes. Moreover, RNA interference using short dsRNA oligonucleotides will permit to decipher the functions of genes which are only

partially sequenced (http://www.eurogentec.be). RNAi will therefore become inevitable in studies such as: inhibition of gene expression at the post-transcriptional level in eucaryotic cells; development of the RNAi technology for use in post-implantation embryos; application as a therapeutic principle to yield RNA-based drugs to treat human diseases (http://www.eurogentec.be). Studies using RNAi experiments, may also help elucidate the *in vivo* function of RhoGAP proteins. This method has been used to examine RhoGEF functions (Peck *et al*., 2002). For our future studies, RNAi may be applied to investigate the functions of BPGAP family.

Genetically altered mice, such as knockout mice and transgenic mice, are used increasingly as tools to define or clarify the *in vivo* function of molecules that have been studied *in vitro*. The phenotypes of a mutant mouse can be studied at many levels from biochemistry to cell biology to systems physiology to behavior. One of the advantages of a mutant mouse that survives to adulthood is that the effect of a single gene alteration on a complex behavior can be studied (Picciotto and Wickman, 1998). For example, the role of many signal transduction pathways in learning and memory has been investigated using mutant mice. Gene knockouts in mice, such as those for BCR (Voncken *et al*., 1995), p190-A (Billuart *et al*., 2001), and p190-B (Sordella *et al*., 2002) may play an important role in studying the normal *in vivo* function of RhoGAP domain-containing proteins (Peck *et al*., 2001). This also might be involved in our future studies of BPGAP families.

BPGAP1, as a regulator of Rho GTPases, may also play important role in development biology. Currently, we are using the mice as the model to study the expression profiles at different development stages.

Various forms of GAPs 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 the wider applications of bioinformatics for data-mining, more sophisticated tools available for real-time imaging of protein-protein interaction and cell dynamics *in vivo* (Van Roessel and Brand, 2002), more established proteomics tools for rapid identification of novel interacting partners (Blagoev *et al*., 2003), as well as good animal model systems (Figure 4.1), one could foresee an increasing number of multi-domain proteins to be uncovered for molecular dissection and functional characterization in cell dynamics control. Then, we will have a better understanding on the significance of multi-domain GAPs in controlling the specificity, redundancy and regulation, and their roles in normal and pathophysiological situations.

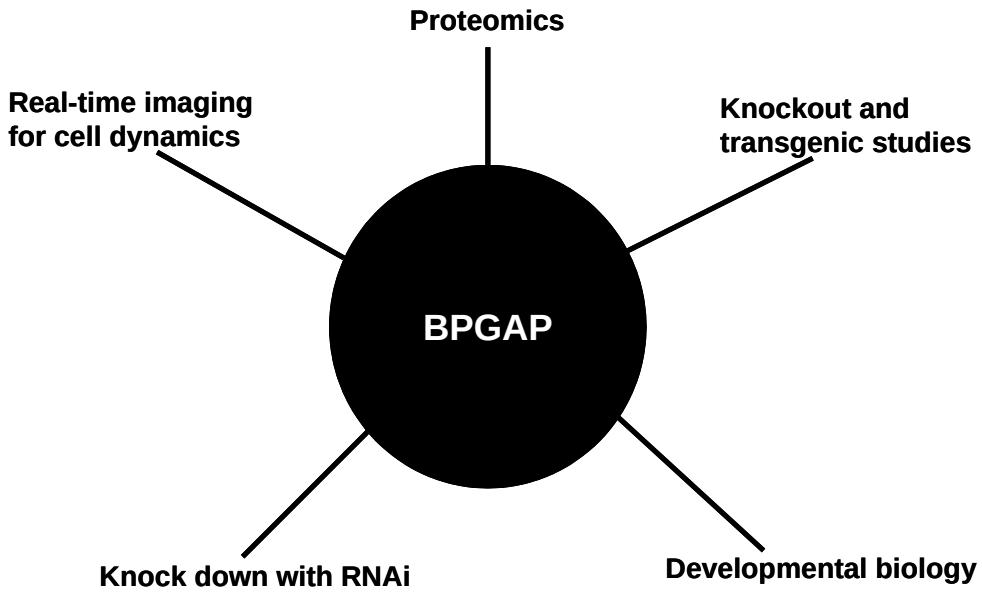

Figure 5.1 Future perspectives for the studies of BPGAP family.

Chapter 6

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Appendix

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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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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 circuitry 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 GTP-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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[1] 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.

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 diversed 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: 260–439) was used as query sequence in the "position-specific iteractive 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 Isoforms 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 *Taq* polymerase enzyme (Roche Applied Science) 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, several clones were chosen and sequenced entirely in both directions using the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystem). 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 R232A 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 MCN45 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% CO_2 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 indicated plasmids using FuGENE 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) Trition 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 directly under 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 $NaCl_2$, 1 mM EDTA, multiple protein inhibitors (Roche Applied Science), 0.1% (w/v) β-mercaptoethanol, and 0.1% (w/v) Triton-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 Vitro 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 nmol 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-methylpurine ribonucleoside, 10 μl (1 unit) of purine nucleotide phosphorylase, and 0.78 ml of HEPES buffer (pH 7.5). The cuvette was immediately placed in the spectrophotometer to monitor absorbance at 360 nm (A_{360}). 10 μl of 1 M $MgCl_2$ solution was added to 0.25 nmol 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 A_{360}, the second mixture of small GTPase solution was added to initiate the reaction. The reading at A_{360} was recorded every 10 s.

In Vivo GTPase Activity and Binding Assay—GTP-bound Cdc42, Rac1, or RhoA was determined by specific binding to the p21-binding domain of PAK1 (GST-PBD) (27) or rhotekin (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). Whole cell lysates were also analyzed for the presence of expressed Cdc42, Rac1, RhoA, and BPGAP1 for normalization. For detecting binding of endogeneous 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—MCF7 cells were transfected with GFP control or GFP-tagged BPGAP1 full-length, NP, and PC domains. After 20 h, the longest diameter (LD) and shortest diameter (SD) that bisected 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 previous described (30). The lower surface of the filters was coated with 0.5-μg fibronectin (Sigma) as a chemoattractant. MCF7 cells transiently transfected with GFP vector, GFP-BPGAP1 full-length, different fragments, or mutants were seeded at a density of 3×10^5 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% CO_2, 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 BNIP-2 and Cdc42GAP homology (BCH)/Sec14p-like domain that we first described in the BNIP-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. 1*A*), which codes for a protein that differs from BAA91614 by lacking 31 amino acids (Fig 1*B*, *upper line*). The protein also differs at the N terminus, from two putative proteins encoded from the same human ARHGAP8 locus (GenBank™ 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 (GenBank™ 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_082731 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. 2*A*). 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. 2*B*) while its GAP domain also shares the highest degree of homology with that of Cdc42GAP (Fig. 2*C*). More importantly, BPGAP1 contains an invariant arginine at residue 232 (Fig. 2*C*, 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. 2*D*). 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.

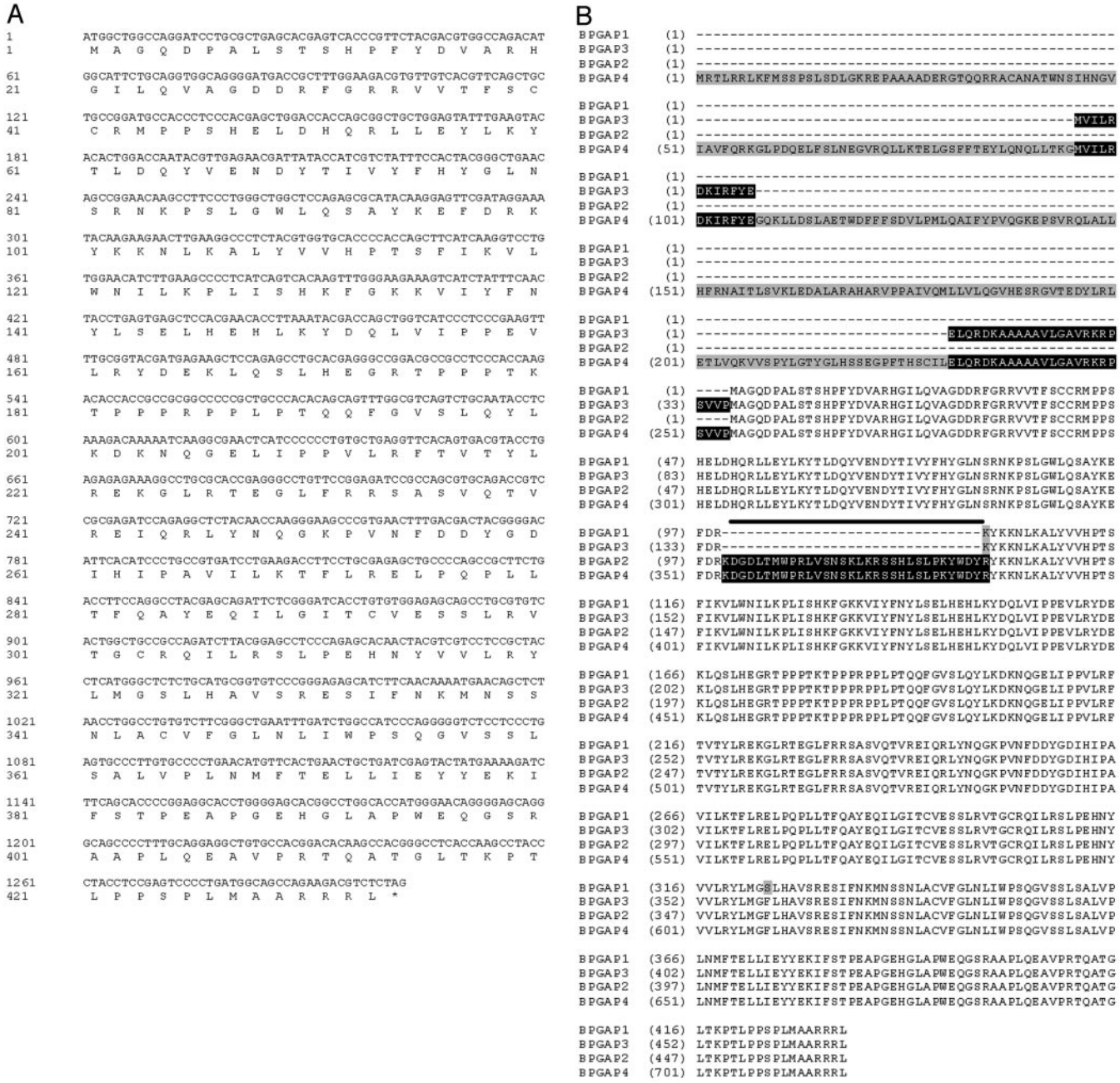

FIG. 1. **Cloning of BPGAP1.** *A*, depicted is the unique coding region and the translated protein sequence for BPGAP1 cDNA (accession number: AF544240) isolated by RT-PCR from MCF7 cells. *B*, comparison of BPGAP1 with three other putative isoforms derived from sequences deposited in GenBank™. Regions predicted to be encoded by alternative RNA splicing are highlighted in *black* and *gray*. The *upper line* represents unique differences among the BCH domain of BPGAP isoforms. BPGAP2, BPGAP3, and BPGAP4 are derived from BAA91614, AF195968, and Q9NSG0, respectively. The mutation F324S present in BPGAP1 may be due to polymorphism.

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. 3*A*). 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. 3*B* 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. 3*C*

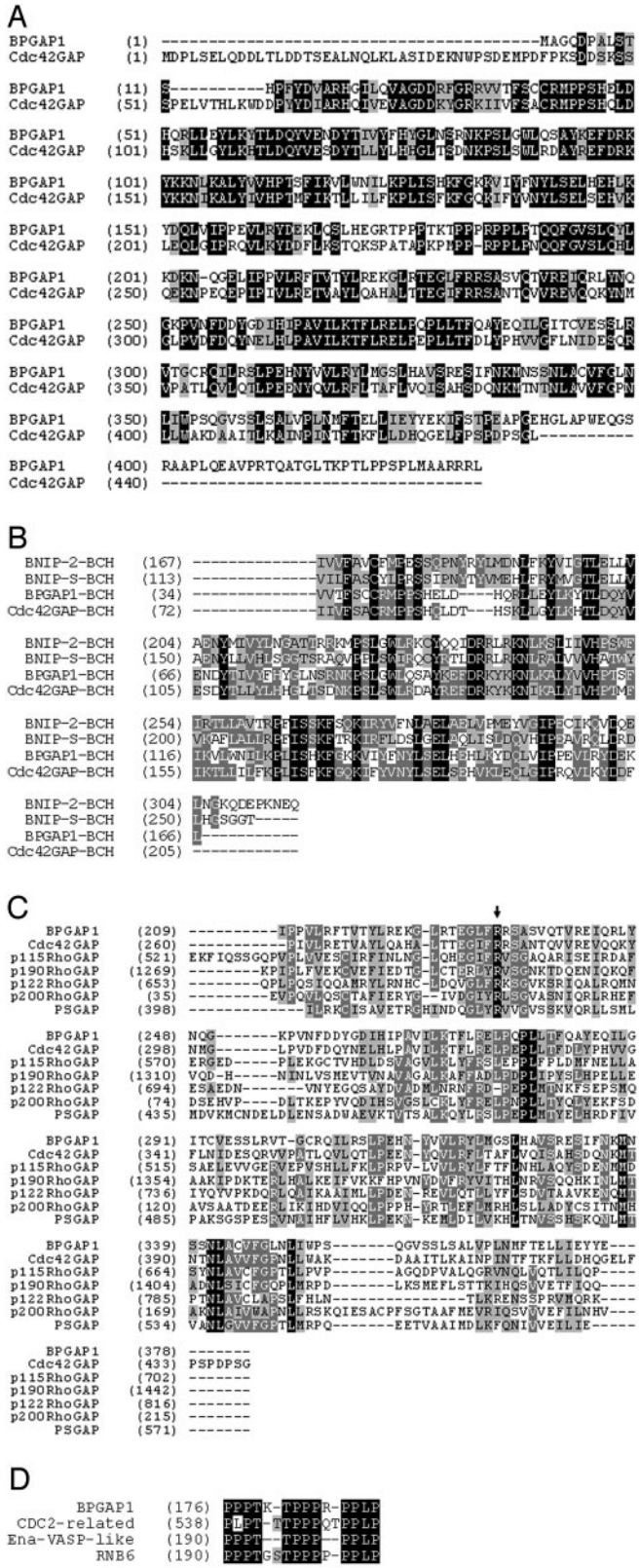

FIG. 2. **Analyses of BPGAP1 domains.** *A*, alignment of BPGAP1 with Cdc42GAP protein sequences reveals regions of homology and divergence. *B*, alignment of BCH domains among BPGAP1, Cdc42GAP, BNIP-2, and BNIP-Sα. *C*, alignment of GAP domains among BPGAP1, Cdc42GAP, PSGAP, p115RhoGAP, p122RhoGAP, p190RhoGAP and p200RhoGAP. *Arrow* indicates the invariant arginine-finger necessary for its GAP function. *D*, alignment of the proline-rich regions of BPGAP1, RNB6, Ena-VASP-like protein, and Cdc2-related kinase 7. All sequence alignments were performed using Vector NTI Suite. Residues that are totally conserved in all members are shaded *black*, those that are conserved in most of the members are in *dark gray* while the significant but least conserved ones are in *light gray* shading. GenBank™ accession numbers used for all alignments above are human BPGAP1 (AF544240), human Cdc42GAP (Q07960), human BNIP-2 (U15173), human BNIP-Sα (NM_138278), mouse PSGAP (NP_084389), human p190RhoGAP (NP_001164), mouse p115 RhoGAP (NP_619536), mouse p122RhoGAP/DLC1 (NP_056617), human p200RhoGAP (NP_055530), rat RNB6 (NM_024147), mouse Ena-VASP-like isoform (AF279662), and human CDC2-related protein kinase 7 (NM_016507).

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 could now form a heterophilic partner with this fragment. Furthermore, the interaction with Cdc42GAP or its BCH domain was apparently enhanced. These results indicate that BPGAP1 can form a heterophilic complex with Cdc42GAP more readily than it can with BNIP-2. Under these experimental conditions, the fragment C that carried the GAP domain (but without the 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. 3*D* 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. 3*E*). 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. 2*C*), 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. 4*A* 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, but devoid of the GAP domain. Furthermore, such activation was abrogated after introducing a mutation R232A at the invariant arginine residue (see Fig. 2*C*).

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 vivo*. 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. 4*B* 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

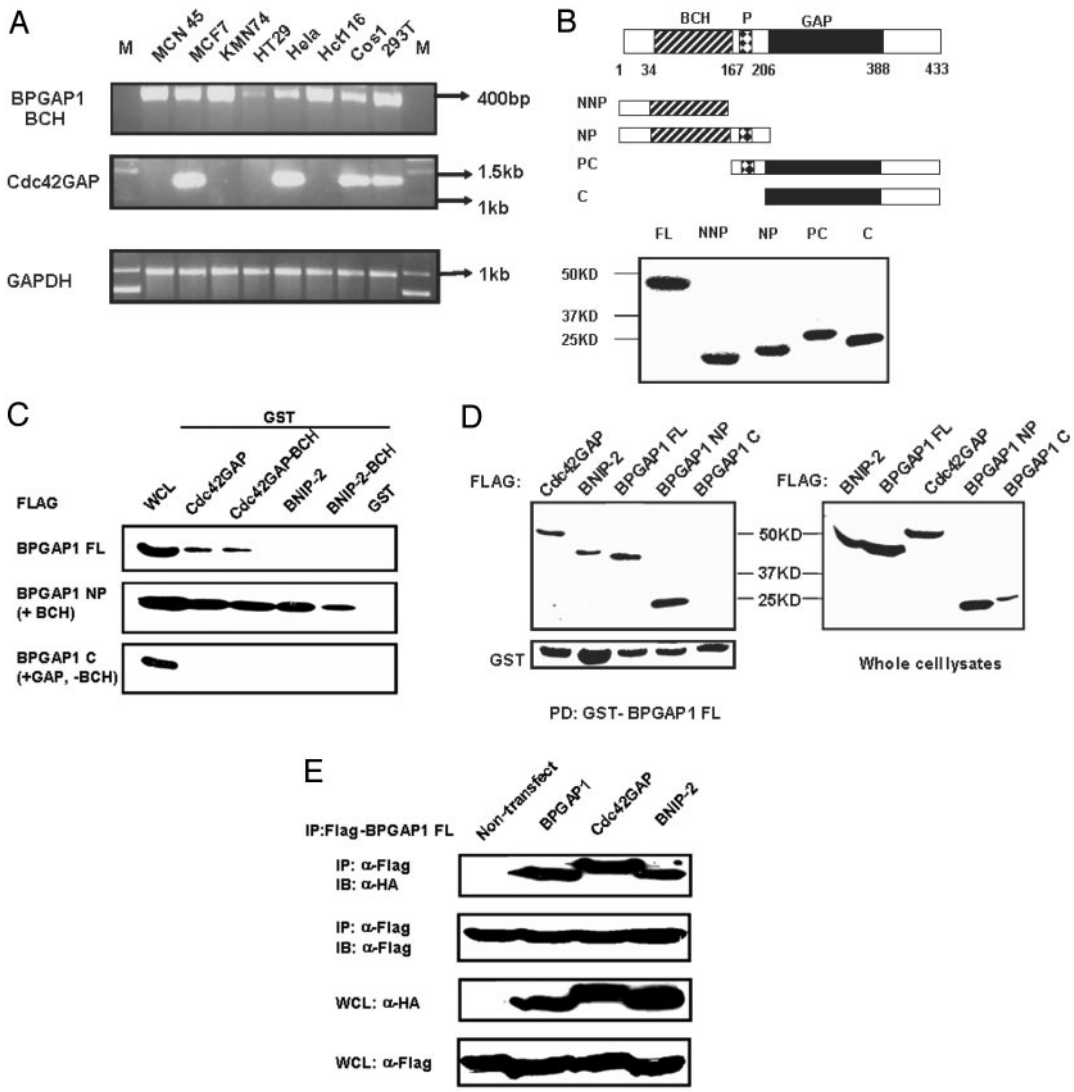

FIG. 3. **BPGAP1 expression and its multiple interacting partners.** *A*, cells were grown in appropriate media containing 10% serum and total RNA isolated for semi-quantitative RT-PCR to detect the expression of BPGAP1 as described under "Materials and Methods." Expression of Cdc42GAP and GAPDH were analyzed for comparison and normalization of samples respectively. *M*, markers. *B*, various expression constructs were tagged with FLAG epitope as indicated: *NNP* (N terminus, non-proline region; amino acids 1–167), *NP* (N terminus, with proline; amino acids 1–206), *PC* (proline-containing carboxyl end; amino acids 168- 433), and *C* (carboxyl end without proline; amino acids 207–433). Cells were transfected with plasmids expressing these domains and analyzed for their expression by anti-FLAG Western blot analyses. Same regions were also tagged with either GST or GFP for use in subsequent experiments. *C* and *D*, GST recombinants of various proteins were prepared as agarose beads and used for pull-down assays using cell lysates expressing FLAG-tagged proteins as indicated in separate experiments. Beads were then washed and processed for Western blot analyses using FLAG antibodies as described under "Materials and Methods." *E*, *in vivo* binding of BPGAP1 with itself and other BCH domain-containing proteins. Cells were cotransfected with plasmids expressing FLAG-tagged full-length BPGAP1 and HA-tagged full-length BPGAP1, Cdc42GAP, or BNIP-2. Lysates were immunoprecipitated (*IP*) with anti-FLAG M2 beads, and the associated proteins were separated on SDS-PAGE and probed with HA antibody (*IB*) as described under "Materials and Methods." Expression of HA-tagged BPGAP1, Cdc42GAP, and BNIP-2, and FLAG-tagged BPGAP1 were verified by anti-HA (*third panel*) or anti-FLAG (*fourth panel*) Western analyses of whole cell lysates (*WCL*). Equal loading of anti-FLAG M2 beads is shown by applying the IP blot with anti-FLAG antibody (*second panel*).

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 BPGAP1. Fig. 4*C* 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. 4*D*). Consistent with this was the observation that endogenous RhoA could be co-immunoprecipitated with overexpressed BPGAP1 (Fig. 4*E*), and this level of interaction was further increased when RhoA itself was also overexpressed (Fig. 4*F*). 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

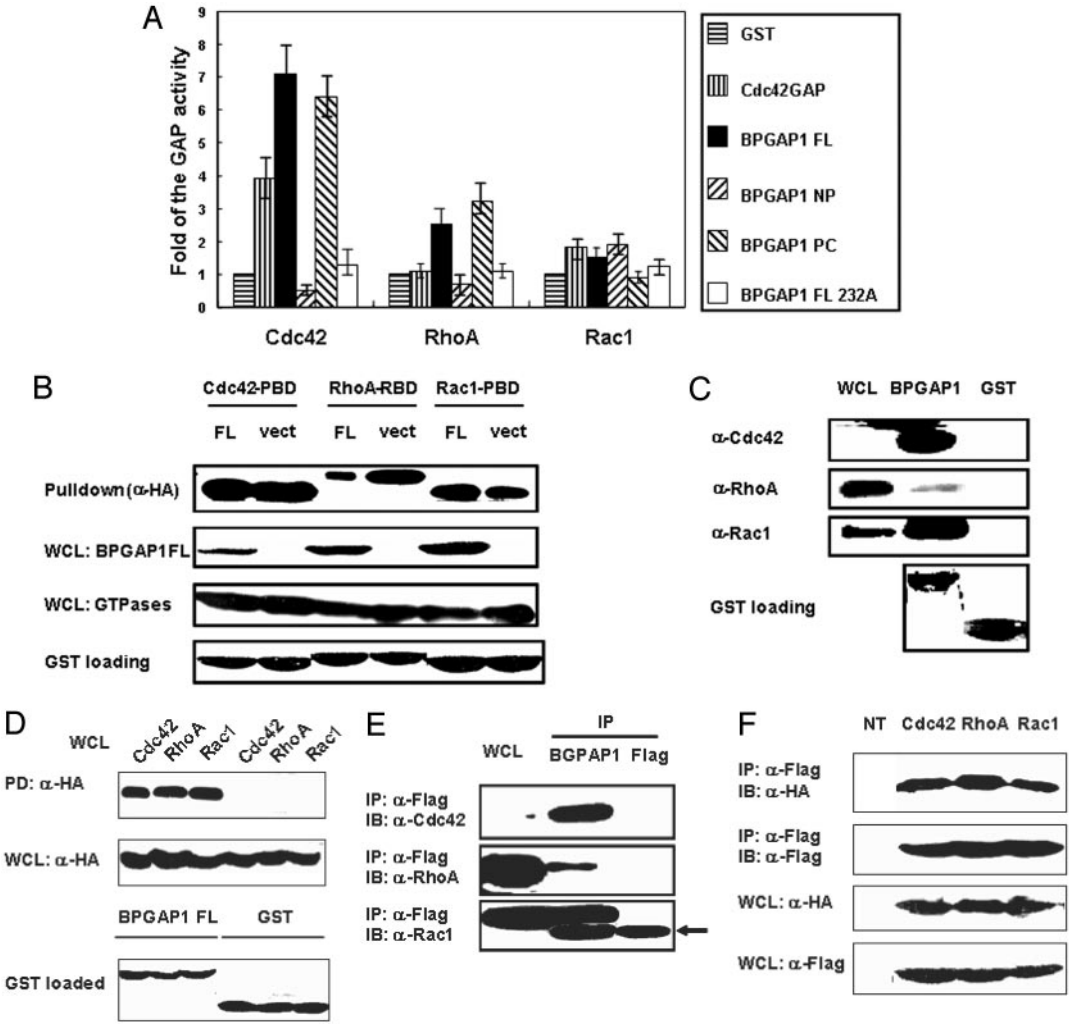

FIG. 4. **Effects of BPGAP1 on Rho GTPase activity and their binding *in vitro* and *in vivo*.** *A*, *in vitro* GAP assays. Purified Cdc42, RhoA, or Rac1 were loaded with GTP and their GTPase activity determined in the absence or presence of Cdc42GAP, BPGAP1 full-length or mutants using an enzyme-coupled assay as described under "Materials and Methods." The activity was expressed as fold over the control using GST alone. Results are means ± S.D. of three replicate determinations. *B*, *in vivo* GTPase binding assays. Cells were transfected with HA-tagged wild-type Cdc42, RhoA, or Rac1 in the presence of either the vector control or FLAG-tagged BPGAP1. Cell were lysed and incubated with GST-PBD or GST-RBD immobilized on beads to assess the ability of BPGAP1 in down-regulating GTPase pathway as described under "Materials and Methods." Bound GTPases were resolved on SDS-PAGE and detected by immunoblotting with HA-antibody (*top panel*). Expression of BPGAP1 and GTPases were verified by anti-FLAG (*second panel*) or anti-HA (*third panel*) Western analyses of the whole cell lysates, respectively. Equal loading of GST fusion proteins is shown in the *bottom panel*. *C*, *in vitro* binding of BPGAP1 with endogenous Rho GTPases. GST-recombinant of BPGAP1 or GST control were used for pull-down assays using normal cell lysates as described under "Materials and Methods." Beads from the pull-down experiments were washed and processed for Western analyses using anti-Cdc42, anti-RhoA, or anti-Rac1 antibodies. Blots were stripped and stained with Amido Black to reveal loading of GST-recombinants. Under this level of film exposure, the endogenous level of Cdc42 was too low to be detectable. *D*, *in vitro* binding of BPGAP1 with overexpressed Rho GTPases. Cells were transfected with HA-tagged Cdc42, RhoA, or Rac1, and lysed for pull-down (*PD*) assays with either the GST control or GST-recombinant of BPGAP1. Bound proteins were detected with anti-HA antibodies while GST staining reveals equal loading of samples. *E*, *in vivo* binding of BPGAP1 with endogenous Rho GTPases. Cells were transfected with expression vectors for full-length FLAG-tagged BPGAP1 and FLAG vector control. Lysates were subjected to immunoprecipitation with anti-FLAG M2 beads and the associated proteins were separated on SDS-PAGE, and probed with anti-Cdc42, anti-RhoA, or anti-Rac1 antibodies to reveal the binding of targets. *Arrow* indicates the light chain of the antibody. *F*, *in vivo* binding of BPGAP1 with overexpressed Rho GTPases. Cells were transfected with expression vectors for full-length FLAG-tagged BPGAP1 or FLAG vector control, together with either HA-tagged Cdc42, RhoA, or Rac1. Lysates were subjected to immunoprecipitation with anti-FLAG M2 beads, and the associated proteins were separated on SDS-PAGE and probed with anti-HA antibodies to reveal the binding of targets. Expression of HA-tagged GTPases and FLAG-tagged BPGAP1 were verified by anti-HA (*third panel*) or anti-FLAG (*fourth panel*) Western analyses of the whole cell lysates, respectively. Equal loading of anti-FLAG M2 beads is shown by applying the immunoprecipitation blot with anti-FLAG antibody (*second panel*). *NT*, non-transfection control.

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. 5*A* 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-

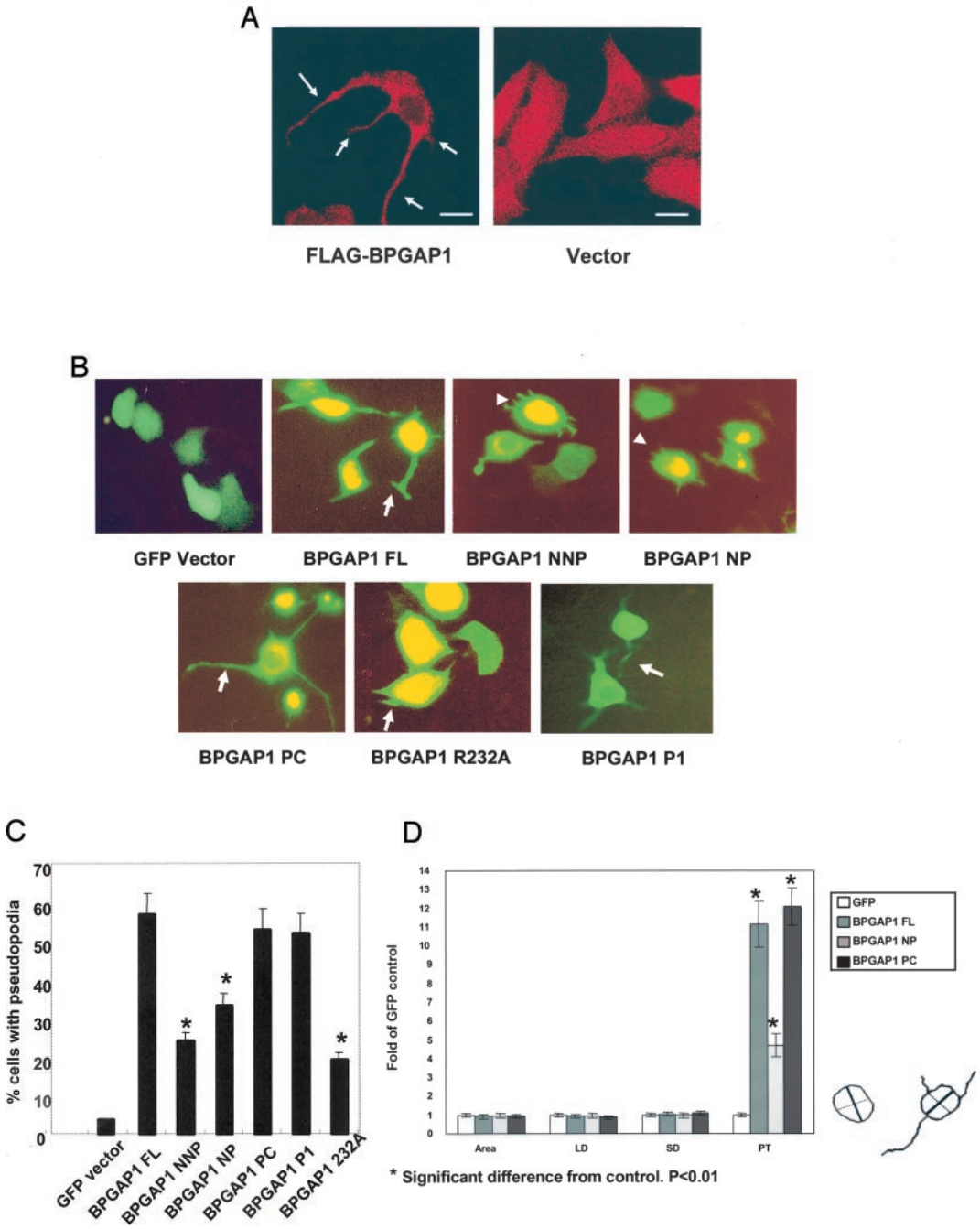

FIG. 5. **BPGAP1 induces pseudopodia via BCH and GAP domains.** *A*, MCF7 cells were transfected with FLAG-tagged expression plasmids for BPGAP1 full-length or control vector. Cells were then fixed, permeabilized, and incubated with anti-FLAG monoclonal, followed by Texas Red dye-conjugated rabbit anti-mouse 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.

rescent protein (GFP) fusion of BPGAP1 and its various deletion mutants (refer to Fig. 3*B*). 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

projections of more than 30 micron from the opposing ends of the cell bodies, accompanied by occasion branching off the main pseudopods (Fig. 5*B*). 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. 2*D*) 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. 5*D*). 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. 6*A*). 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 phalloidin 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. 6*B*). 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. 6*C*). 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. 6*D* 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.

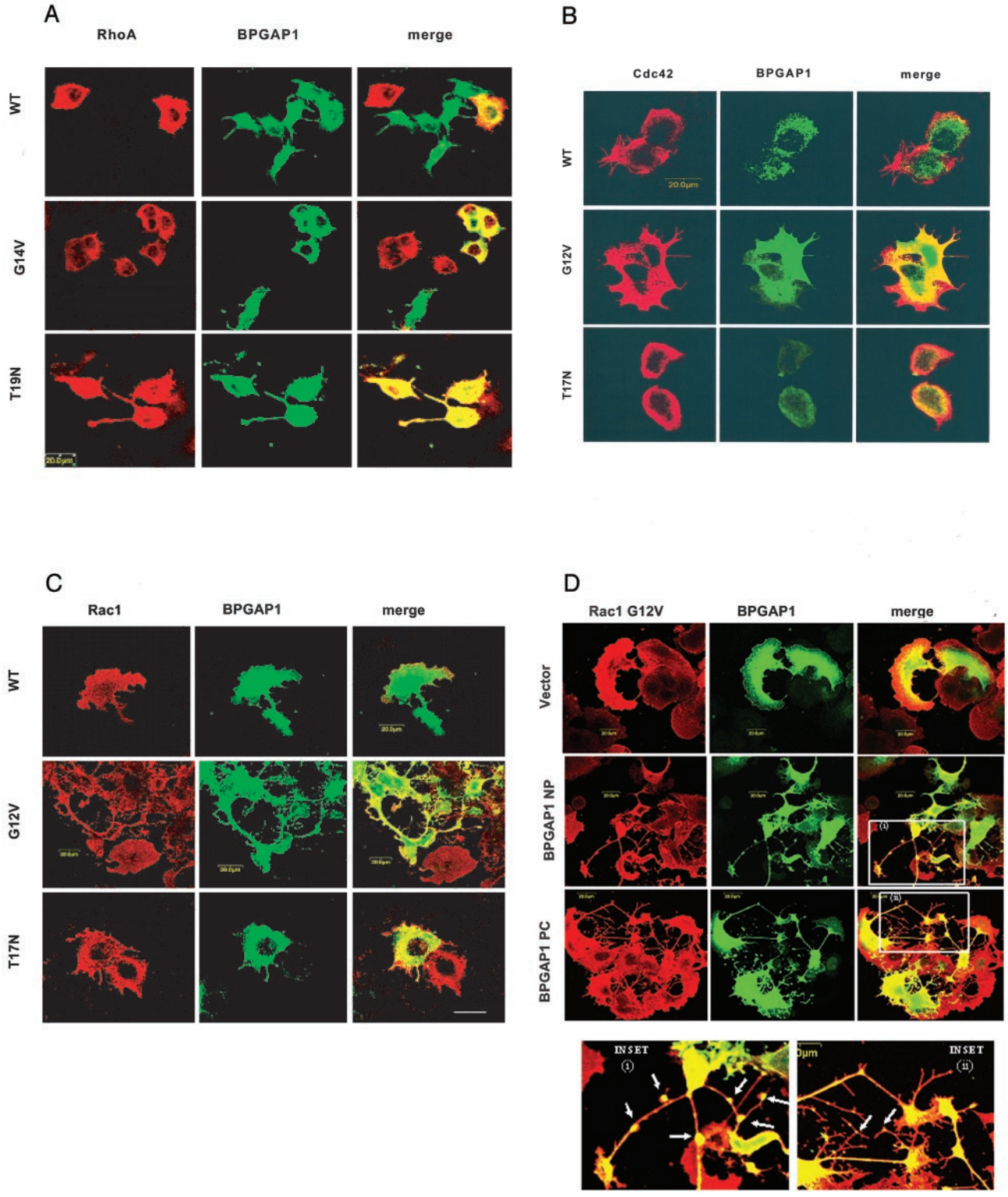

FIG. 6. **BPGAP1-induced pseudopodia involve differential regulation of Rho GTPases.** MCF7 cells were cotransfected for 16 h with GFP expression plasmid for BPGAP1 full-length and HA-tagged expression plasmids for wild-type, constitutively active, or dominant negative mutants of either RhoA (*A*), Cdc42 (*B*), or Rac1 (*C*). Cells were then fixed, permeabilized, and incubated with anti-HA polyclonal antibodies, followed by Texas Red dye-conjugated goat anti-rabbit IgG (*red*) for fluorescence detection as described under "Materials and Methods." Merged signals are presented as overlaid staining (*yellow*). GFP controls did not result in any perturbation to the cell dynamics under all these studies (data not shown). *D*, MCF7 cells were cotransfected for 16 h with GFP expression plasmid for BPGAP1 FL, NP, or PC domains and HA-tagged expression plasmids for constitutively active mutants of Rac1 (G12V) and then monitored for cell dynamics changes as described above. Result for the full-length (*FL*) is essentially the same as in *C*. *Inset (i)* and *inset (ii)* are magnified images to highlight distinctive miscroscopic features described in text. *Arrows* indicate nodule-like structure.

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

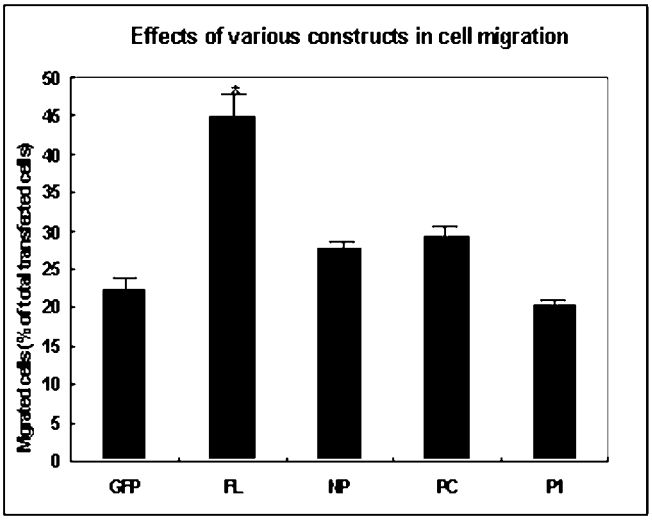

FIG. 7. **Effects of BPGAP1 on cell migration.** MCF7 cells were transfected with either GFP vector control or GFP fusion of BPGAP1 full-length (*FL*), *NP*, *PC*, or proline-deletion mutant (*P1*), and seeded on special chamber for 24 h to monitor their effects on cell migration, as described under "Materials and Methods." Cells that had migrated through the pores in the filter were scored by microscopy visualization and expressed as percentage over the total transfected cells. Results are means ± S.D. of three independent experiments performed in duplicates. *Asterisk* indicates $p < 0.01$ *versus* GFP control.

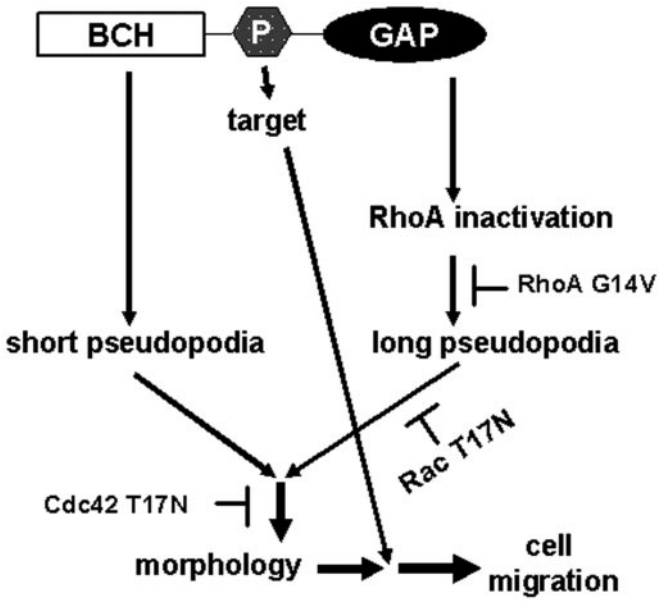

FIG. 8. **Model for the effects of BPGAP1 on cell dynamics control.** The three separate domains, BCH, proline-rich, and GAP domains of BPGAP1 coordinately regulate distinctive yet concerted pathways in cell dynamics control. Its GAP domain specifically inactivates RhoA pathway and induces long pseudopodia 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 is/are involved in linking cell morphological changes to its migration.

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.[2] To date, we have discovered and characterized at least one member of the BPGAP family. Its wide distribution in tissue suggests that it could play a very common cellular function such as the control of cell morphology and cell motility as shown in the current study. However, based on our bioinformatics analyses of various EST and putative/uncharacterized sequences, there exist at least three more closely related members, namely BPGAP2 to BPGAP4, all expected to be derived through alternative RNA splicing. It remains to be seen how such sequence variations might influence the properties of these isoforms.

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, DEF1/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

[2] 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 (32) or WW (33) domains (Fig. 2*D*). 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.

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