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乳癌激酶促進癌細胞生長及移動之機制研究

The Mechanisms of Breast Tumor Kinase in Promoting Tumor Cell Growth and Migration

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中文摘要

乳癌激酶是一種非受體型酪胺酸激酶與 Src 有高度相似性，其蛋白質結構由典型的 SH3 區塊、SH2 區塊以及酪胺酸激酶區所組成。乳癌激酶已於乳癌、黑色素細胞瘤以及大腸癌等癌症中被報導具有高度表現性，也被認為在細胞癌化機轉(oncogenesis)中扮演重要角色。但是目前對於乳癌激酶的活性調控及其所參與的信息傳導路徑，乃至於生物功能的了解均十分有限。因此，探查乳癌激酶所作用的受質與其所參與的信息傳導途徑，能進一步揭開乳癌激酶的生物功能及致癌機轉。在此，我們找到了一新的乳癌激酶受質－p190RhoGAP-A (p190)。乳癌激酶透過磷酸化 p190 促使 p190 與 p120RasGAP (p120)結合，此蛋白質複合體具有能抑制 Rho 及活化 Ras 的能力。乳癌激酶也藉由磷酸化 p190 抑制 Rho 及活化 Ras，並且刺激細胞移動、促進細胞的侵入行為以及導致細胞增生。此外我們也發現若利用干擾蛋白破壞 p190 與 p120 之間的結合，將弱化乳癌激酶調控 Rho 及 Ras 的能力，也減弱其對細胞行為及生長的影響，並進一步降低乳癌激酶的致癌性(tumorigenicity)。因此 p190 在乳癌激酶所作用的信息傳導路徑及於生物功能上扮演重要功能。

Abstract

Breast tumor kinase (Brk), a Src-like nonreceptor tyrosine kinase, is overexpressed in breast cancer and several other cancer types. Our previous study indicates that Brk promotes cell migration and tumor invasion by phosphorylating the focal adhesion protein paxillin. Here, we report the identification of p190RhoGAP-A (p190) as a Brk substrate. Brk phosphorylates p190 at the Y1105 residue both *in vitro* and *in vivo*, thereby promoting the association of p190 with p120RasGAP (p120). As a consequence, Brk stimulates p190 and attenuates p120 functions, leading to RhoA inactivation and Ras activation, respectively. In carcinoma cells expressing high levels of Brk, endogenous Brk functions as a key contributor to EGF-induced p190 tyrosine phosphorylation. We present evidence showing that p190 phosphorylation plays essential roles in both migratory and proliferative effects of Brk. Furthermore, disruption of p190 phosphorylation-induced p190/p120 complex in breast cancer cells abolishes not only the abilities of Brk to regulate RhoA and Ras, but also the stimulatory effects of Brk on proliferation, migration, invasion, transformation and tumorigenicity. Together, our findings reveal a previously unknown function of Brk in regulating both RhoA and Ras by phosphorylating p190, and provide evidence for the crucial roles of this Brk-elicited signaling pathway in promoting breast malignancy.

Literature Review

1. Mechanisms that control tumor progression

Tumor formation is a long-term event which usually spans several years. Accordingly, tumorigenesis is a multistep process involving the accumulations of multiple genetic alterations, which ultimately change natural cell physiology of normal cells thereby transforming them to malignancy. It has been proposed that tumorigenesis needs to acquire six essential alterations, including self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and migration (Hanahan and Weinberg 2000).

The first capability that tumor cell acquired is self-sufficiency in growth signals. Different from normal cells, tumor cells generate many growth signals by themselves to relief the dependence on normal environment supporting. Growth factors can be produced by tumor cells to create autocrine stimulations, such as PDGF from glioblastomas and TGFα from sarcomas. In other cases, overexpressed and/or modified growth factor receptors on the cell membrane can also render tumor cells becoming hypersensitive to or even independent of growth factors. For example, EGF receptor is upregulated in stomach and breast carcinomas, and certain modifications of cytoplasmic domain maintain EGFR in a constitutive active state (Aaronson 1991).

Insensitivity to antigrowth signal would lead to the failure of maintaining cell

quiescence and tissue homeostasis. For instance, certain tumor cells can escape the cell-cycle arrest signal transduced by TGF-β (Hannon and Beach 1994), while other tumors ignore antigrowth signals by turning off the expression of cell adhesion molecules such as integrin. Besides insensitivity to antigrowth signal, resistance to apoptosis is essential for preventing tumor cells from program cell death and expanding their populations. Telomere maintenance is another notable difference between normal and tumor cells. This alternation allows tumor cells to replicate without shortening their telomere and is essential for cellular immortalization. At the initial stage, tumor development relies on diffusion transport for oxygen, nutrients and wastes. However, to expand to a larger size and to form neoplasia, formation of new capillaries is essential. Once angiogenesis is activated, tumors appear to undergo malignant transformation (Hanahan and Folkman 1996).

Up to 90% of the human cancer deaths result from tumor cell metastasis (Sporn 1996). Invasion and migration abilities enable cells to move out of primary tumor mass and travel to other parts of body where they may form new colonies. In this way, nutrients and oxygen supporting, wastes removing, and spaces are not limitations for tumor growth. Different from normal cells tethered to the surroundings, tumor cells appear to lose cell-cell and/or cell-matrix interactions. This can be achieved by altering the expression or function of E-cadherin and/or integrin (Aplin, Howe et al. 1998). In addition, expression of active extracellular proteases on the tumor cell surface can also facilitate their invasion into nearby stroma, through basal epithelial cell layer, and across blood vessel wall (Coussens and Werb 1996). Once malignancy cells invade into the circulatory or lymphatic system, they spread to

everywhere and increase the mortality rate.

2. The roles of protein tyrosine kinases in cancer

Protein tyrosine kinases (PTKs) are enzymes that catalyze the phosphorylation of tyrosine residues in proteins and form a large multigene family. PTKs are capable of catalyzing the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosine residues on target proteins. This tyrosine phosphorylation changes protein conformation leading to either modulating the enzymatic activity or creating binding sites for the recruitment of downstream signaling proteins. PTK-mediated tyrosine phosphorylation cascades play significant roles in regulating and coordinating a wide variety of biological processes such as cell growth, differentiation, adhesion, motility and death, and are involved in many diseases such as diabetes and cancer in human.

2.1 PTK classification

Through BLAST searching for genes that possess a highly conserved kinase domain, it is found that human genome contains 90 tyrosine kinase genes and 5 pseudogenes. The 90 tyrosine kinases are divided into two categories, receptor and non-receptor tyrosine kinases. Based on whether their sequences contain a predicted transmembrane domain, 58 of the 90 tyrosine kinase genes are classified as receptor protein tyrosine kinases (RTKs), and the remaining 32 kinase genes fall into non-receptor protein

tyrosine kinases (NRTKs) (Robinson, Wu et al. 2000).

All RTKs possess an extracellular ligand-binding domain and a large intracellular domain. The extracellular and intercellular domains are linked by a hydrophobic transmembrane domain thereby anchoring RTKs to the plasma membrane. The structurally diverse ectodomains and their glycosylation pattern of extracellular domain determine the ligand-binding specificity. The intracellular domain contains kinase domain and additional regulatory motifs and is responsible for the catalytic activity of RTKs. Based on the domain structure and motif arrangement, RTKs are grouped into 20 subfamilies (Yarden and Ullrich 1988). EGFR, the first characterized RTK, is considered as the prototype of RTKs (Carpenter, King et al. 1978). The signaling circuits mediated by EGFR regulate a variety of cellular functions and play important roles in the tumor pathogenesis and progression (Normanno, Bianco et al. 2003).

32 cytoplasmic NRTKs lack for receptor-like features. Depending on their structural similarities, these NRTKs are classified into 10 families: Src, Abl, Jak, Ack, Csk, Fak, Fes, Frk, Tec and Syk (Blume-Jensen and Hunter 2001). The NRTKs have a kinase domain and several additional domains, such as SH2, SH3, and PH domain. These additional domains are required for regulating kinase activity and mediating the interactions with proteins, lipids, and/or DNA. The largest subgroup among NRTKs, with nine members, is the Src family. The members of this family are known to regulate a wide variety of cellular functions (Tatosyan and Mizenina, 2000; Thomas and Brugge, 1997).

2.2 The mechanisms of PTK activity regulation

The kinase activities of RTKs are activated by many growth factors, differentiation factors, and hormones. In the rest state, RTKs are unphosphorylated and monomeric, and their kinase domains are inactive. In some cases, the cytoplasmic juxtamembrane domain interacts with kinase domain leading to further inhibition of the kinase activity (Griffith, Black et al. 2004). In response to extracellular stimulation, RTKs increase their catalytic activity and create protein-binding sites for docking the downstream signaling transducers leading to the initiation of intracellular signaling events. In general, the initial step for RTKs activation is ligand-induced extracellular domain conformational change, receptor dimerization (or oligomerization) and disruption of the juxtamembrane interaction. Dimerized (or heterodimerized) receptor facilitates the autophosphorylation and/or transphosphorylation of tyrosine residues either in the tyrosine kinase domain to enhance enzymatic activity, or in the regulatory regions to create docking sites for SH2 and/or phosphotyrosine-binding (PTB) domain-containing proteins (Kuriyan and Cowburn 1997). This results in the recruitment of signaling protein complexes to activated receptor and subsequent activation of intracellular signaling cascades, which lead to regulation of various biological responses. During these processes, the receptor migrates and clusters on the plasma membrane, and internalizes via the formation of clathrin-coated endocytic vesicle. In accompanied with receptor internalization, the ligand-receptor complex is dissociated, resulting in the termination of RTK signaling. The internalized receptor may be degraded through lysosomal hydrolysis or recycled to the plasma membrane via endosome recycling pathway (Paul and Mukhopadhyay

2004).

In contrast to RTKs, NRTKs are localized in the cytoplasm, nucleus or anchored to the inner leaflet of the plasma membrane. In the inactive state, their enzymatic activities are inhibited through the interaction with inhibitory proteins, lipids, and/or intramolecular interactions (Van Etten 2003). The activation of NRTKs is more complex than RTKs and can be achieved by dissociation from inhibitory factors, autophosphoylation or transphosphorylation. Some NRTKs link to membrane receptors and are activated when extracellular signals (ligand) bind to their associated receptors (Taniguchi 1995). These activated NRTKs in turn transduce signals to regulate key cellular functions such as proliferation, differentiation, anti-apoptotic signaling and neurite outgrowth. c-Src is the first and best characterized NRTK (Bjorge, Jakymiw et al. 2000), and the mechanisms that regulate c-Src activity illustrate some basic principles of NRTK activation. c-Src contains two important regulatory tyrosine residues: Y416 and Y527. Phosphorylation of Y527 by Csk induces an intramolecular interaction with its SH2 domain which results in a negative regulation of the kinase activity. In contrast, autophosphorylation of Y416 and/or dephosphorylation of phospho-Y527 by PTPs result in kinase activation. Deregulated Src activation/signaling is known to be able to transform cells and is highly associated with cancer development (Hubbard and Till 2000; Blume-Jensen and Hunter 2001).

2.3 PTK activation in cancers

In normal cells, PTK-mediated signaling pathways regulate diverse

biological processes in response to external and/or internal stimuli and therefore their activities have to be tightly regulated. Deregulated or constitutive activation of PTKs has been found in numerous types of cancers and such activation can be achieved by a variety of mechanisms, such as chromosomal translocation, gain of function mutations and/or deletions, and overexpression of the kinase, its receptor, or ligand (Nichols 2003).

Chromosomal translocations involving PTK genes may generate constitutively active TK fusion proteins, as the partner gene may provide a sequence to activate the kinase activity of PTK or remove the repression sequences. The *bcr-abl* translocation which results from the chromosome fusion of the *Bcr* gene on chromosome 22 with the protooncogene *c-Abl* on chromosome 9 is essential for the development of over 95 % of chronic myelogenous leukemia (CML) and 20% of acute lymphoblastic leukemias (ALL). Such chromosomal translocation produces a constitutively active chimera Bcl-Abl kianse, which leads to the transformation of hematopoietic progenitor cells (Chan, Karhi et al. 1987).

Gene amplification and/or protein overexpression of PTKs or its receptors and ligands are commonly found in many types of cancers. Patients with overexpressed PTKs often associate with poor response to therapy and short survival. In some cases, co-overexpression of two components in a signaling pathway is observed. For instance, EGFR and its ligands (EGF and TGFα) are frequently coexpressed in human breast carcinomas. This finding suggest an autocrine stimulation loop in the breast cancer development (Kolibaba and Druker 1997).

Mutations in PTKs have been associated with constitutive PTK activation and development of tumor malignancy. Mutations that lead to oncogenic activation of PTKs can occur in extracellular domain, kinase domain, or inhibitory region (Nichols 2003). For instance, EGFR mutant which lacks amino acid 6-273 within the extracellular domain is constitutively active even in the absence of ligand binding and is associated with the development of globlastomas, ovarian tumors, and non-small lung cancer (Nishikawa, Ji et al. 1994). The internal tandem duplication in juxtamembrane domain of FTL3 receptor renders this kinase active. This gain of function mutant is found in nearly 20% of acute myeloid leukemia (Kiyoi, Towatari et al. 1998). Tyr-530 truncated form of c-Src has been detected in human colon cancers. This Src constitutively active form can be detected in highly metastatic colon cancers (Irby, Mao et al. 1999). These findings suggest that oncogenic PTKs are created by a variety of mechanisms, and their constitutive activity is tightly associated with tumorigenesis and tumor malignancy.

3. Overview of Brk: expression, regulation and functions

3.1 Brk sequence features and domain structure

Breast tumor kinase (Brk), also known as protein tyrosine kinase 6 (PTK6), was first identified from human melanocytes (Lee, Strunk et al. 1993) and human metastatic breast tumors via PCR-based differential screening method (Mitchell, Barker et al. 1994). Subsequently, the mouse homologue of Brk, also

called Src-related intestinal kinase (Sik), was identified from a screen of tyrosine kinases expressed in the mouse small intestine and this protein shares 80 % amino acid identity to human Brk (Siyanova, Serfas et al. 1994; Vasioukhin, Serfas et al. 1995). At the genomic level, the human Brk lies within the chromosome 20q 13.3-13.4 and consists of 8 exons (Mitchell, Barker et al. 1997; Serfas and Tyner 2003). At the protein level, Brk encodes a 451 amino-acid protein and possesses one SH3 (aa.11- aa.71), one SH2 (aa.75-aa.161), and one kinase domain (aa.191- aa.441). Human Brk shares 46% amino acid sequence identities with c-Src and its domain arrangement is also similar to that of Src family members (Mitchell, Barker et al. 1994). Despite these similarities, Brk differs from Src family kinases in some aspects. First, the genomic structure and exon boundaries of Brk are distinct from those of Src family members, suggesting that Brk has diverged from Src family. Second, Brk kinase domain contains the RDLLARN motif rather than the RDLRAAN motif which is considered as a signature sequence for Src family. Third, Brk lacks an N-terminal extension sequence and the consensus myristoylation sequence, which are found in all Src family members (Mitchell, Barker et al. 1997). Because of these differences, Brk is considered to be a member of a separate family (Frk family) (Serfas and Tyner 2003).

Similar to Src family members, the conformation of Brk can be stabilized by a number of intramolecular interactions. First, the interaction between the C-terminal phosphotyrosine residue (pY447, equivalent to pY527 in Src) and the SH2 domain is revealed by NMR and surface plasmon resonance analyses (Hong, Shin et al. 2004). The inhibitory effect of this interaction on Brk catalytic activity is evident by mutagenesis study (Qiu and Miller 2002). However, the

tyrosine kinase and phosphatase responsible for phosphorylating and dephosphorylating the Y447 residue, respectively, have not been identified. Another autoinhibitory interaction occurs in between proline residues (P175, P177, P179) in the Linker region (aa.161- aa.191) and the SH3 domain (Qiu and Miller 2002; Qiu and Miller 2004; Kim, Jung et al. 2007), and substrate binding to the SH3 domain is expected to relief such inhibition (Qiu and Miller 2004). Finally, an intramolecular interaction between the Liner region and Kinase domain is identified recently. Intriguingly, mutagenesis analysis indicates such interaction is essential for Brk catalytic activity (Kim and Lee 2005), whereas equivalent interaction in Src family members plays inhibitory role by disturbing the N-lobe conformation of kinase domain (Sicheri, Moarefi et al. 1997; Xu, Harrison et al. 1997). Understanding of the basis of this discrepancy would require the solution of Brk crystal structure. In addition to intramolecular interaction, Brk activity can also be regulated by autophosphorylation at Y342 in the kinase activation loop (Qiu and Miller 2002), which is equivalent to the Y416 residue in Src.

3.2 Brk expression in normal and cancer tissues

In normal tissues, Brk expression is detected in the differentiating epithelial cells in gastrointestinal tract and prostate as well as in skin keratinocytes and oral epithelial cells (Llor, Serfas et al. 1999; Derry, Prins et al. 2003; Haegebarth, Heap et al. 2004; Petro, Tan et al. 2004). Consistent with these expression patterns, Brk is implicated in the regulation of keratinocytes differentiation in both human (Wang, Jee et al. 2005) and mouse (Vasioukhin and Tyner 1997) and is crucial for mouse enterocytes differentiation thereby

maintaining homeostasis of the intestinal tissues (Haegebarth, Bie et al. 2006).

Although Brk expression is undetectable in normal breast tissue, about 2/3 breast tumors express a significantly high level of Brk (Barker, Jackson et al. 1997). Furthermore, overexpression of Brk is detected in 86% of 250 samples of invasive breast cancer (Ostrander, Daniel et al. 2007). High level of Brk expression is also found in metastatic melanoma (Easty, Mitchell et al. 1997), prostate cancer (Derry, Prins et al. 2003), oral squamous cell carcinomas (Born, Quintanilla-Fend et al. 2005), T and/or B cell lymphomas (Kasprzycka, Majewski et al. 2006), head and neck cancers (Lin, Berry et al. 2004), ovarian cancers (Slamon, Godolphin et al. 1989; Schmandt, Bennett et al. 2006), and colon cancers (Llor, Serfas et al. 1999). In line with this increased expression of Brk in tumors, *Brk* gene is located in the chromosomal region 20q13, which is frequently amplified in primary breast tumors, gastric and gastro-esophagel tumors, and colon tumors (Llor, Serfas et al. 1999). In prostate cancers, Brk undergoes a cytosol to nucleus translocation during tumor progression, even though its expression level is unaltered (Derry, Prins et al. 2003). These findings suggest that deregulated Brk expression or subcellular localization may contribute to tumorigenesis.

3.3 Brk-mediated signaling pathways and its biological functions

Several Brk substrates have been identified to date. Sam68 (Src-associated protein in mitosis) is the first identified Brk substrate, followed by two Sam68-like proteins, SLM-1 and SLM-2. Brk-induced phosphorylation

of these proteins leads to the inhibition of their RNA-binding ability and mRNA nuclear exporting function (Derry, Richard et al. 2000; Haegebarth, Heap et al. 2004). In addition, such phosphorylation induces a relocalization of Sam68 to SNBs (Sam68 nuclear bodies) and inhibition of Brk-increased cell cycle progression, suggesting that Brk neutralizes the anti-proliferative function of Sam68 (Lukong, Larocque et al. 2005). BKS, an adaptor-like protein regulating STAT3 activation ((Minoguchi, Minoguchi et al. 2003), was found as a Brk substrate (Mitchell, Sara et al. 2000), but the biological consequence of this phosphorylation event remains uncharacterized. Notably, Brk can directly phosphorylate STAT3 and STAT5b, thereby increasing their transcriptional abilities and proliferative functions (Liu, Gao et al. 2006; Weaver and Silva 2007). With respect to another functional arm of Brk, that is, cell migration, Brk was found to phosphorylate the focal adhesion protein paxillin, which leads to the activation of CrkII/Elmo/Rac pathway to promote migration (Chen, Shen et al. 2004). Finally, Brk is capable of phosphorylating kinesin associated protein 3A, and this phosphorylation is implicated in the migration function of Brk (Lukong and Richard 2008).

Several lines of evidence support the involvement of Brk in the signaling pathways and biological functions of EGF family receptors. First, Brk overexpression in epithelial cells enhances EGF-induced anchorage-independent proliferation ability and transforming phenotype (Kamalati, Jolin et al. 1996). Second, Brk overexpression potentiates EGF-induced PI3K-AKT activation via an increase in EGFR-associated erbB3 phosphorylation (Kamalati, Jolin et al. 2000). Brk is also involved in EGF-induced Rac activation via paxillin phosphorylation (Chen, Shen et al.

2004). More recently, Brk is found to mediate EGF- and heregulin-induced activation of Rac, p38 MAPK, and ERK5 (Ostrander, Daniel et al. 2007). In line with the crosstalk of Brk with EGF signaling pathways, Brk is associated with and activated by EGFR and erbB receptors following receptor activation (Kamalati, Jolin et al. 2000; Chen, Shen et al. 2004; Ostrander, Daniel et al. 2007). Furthermore, it has been reported that Brk overexpression correlates significantly with the expression of erbB2 (Born, Quintanilla-Fend et al. 2005), erbB3 and erbB4 (Aubele, Auer et al. 2007) in human breast tumors. Such coexpression of Brk and EGF family receptors may lead to a synergistic activation of downstream pathways of EGF family receptors.

In addition to mediating EGF signaling, Brk is implied to be involved in insulin-like growth factor 1 signaling through binding to insulin receptor substrate 4 thereby promoting IGF-1-induced cell proliferation and migration (Qiu, Zappacosta et al. 2005). Furthermore, a recent study reveals that Brk is involved in osteopontin (OPN)-induced NF-κB/ATF4 activation which ultimately augments the expression of VEGF and tumor angiogenesis and that Brk catalytic activity is elevated in response to OPN stimulation (Chakraborty, Jain et al. 2008). Together, these findings indicate that Brk is involved in the signaling pathways of several growth factors and their associated RTKs, thereby promoting a number of critical processes in tumorigenesis, such as proliferation, migration and angiogenesis.

4. Ras and Rho small GTPases

4.1 Overview of small GTPases

4.1.1 Discovery, classification, and structure of small GTPases

Small GTPases are monomeric G proteins with ~20-40 kDa in size and cycle between a GDP-bound (inactive) form and a GTP-bound (active) form. Ras was the first discovered small GTPase through the identification of v-Ras oncogene of sarcoma virus around 1980 (Chien, Lai et al. 1979). Subsequently, Rho was discovered as a homolog of the Ras gene in *Aplysia* in 1985 (Madaule and Axel 1985). Arf was identified as a cofactor for the cholera toxin-catalyzed of G_s in 1984 (Kahn and Gilman 1984). Now, at least 154 small GTPases have been systematically isolated and characterized, and they comprise a superfamily called Ras superfamily. Based on their sequences and protein structures, this superfamily is divided into 5 families including Ras, Rho, Rab, Arf and Ran families (Wennerberg, Rossman et al. 2005). They display different intracellular localizations and downstream effectors leading to the diverse cell functions such as gene expression and proliferation (Ras), cytoskeleton reorganization (Rho/Rac/Cdc42), vesicle trafficking (Ran and Arf), and cell cycle (Ran).

All small GTPases have consensus amino acids position in G domain that is required for the binding and hydrolysis of guanine nucleotides. The G domain contains N/TKXD motif (where X is any amino acid) for the interaction

with nucleotide base, conserved P loop for the binding of β-or γ-phosphates, DXXG motif for the guanine specificity, GXXXXGKS/T motif, as well as switch I and switch II region (Saraste, Sibbald et al. 1990). Through crystallographic and NMR analysis, many of small GTPases, such as Ras, Rap2A, RhoA, Rac1, Rab, Arf, and Ran, have a common topology of their G domain and are suggested a universal GTP/GDP switch mechanism (Vetter and Wittinghofer 2001). The switch region within G domain were first observed and named in H-Ras (Milburn, Tong et al. 1990). This region contains two highly exposed and flexible arms, which are called as switch I and switch II. They surround the γ-phosphate of GTP through the hydrogen bonds from two γ-phosphate oxygens to Thr35 of switch I and Gly60 of switch II. Both switch I and switch II together with P loop and magnesium ion stabilize the interaction between GTP and small GTPase.

In addition to the consensus G domain, the posttranslational modifications of their C termini are also play an important function for their subcellular localization and signaling. Ras, Rho/Rac/Cdc42, and Rab are modified by lipid at their C termini. A C-terminal CAAX (X=any amino acid) motif within Ras and Rho family is recognized by farnesyltransferase and geranylgeranyltransferase I, which catalyze the covalent addition of a farnesyl or geranylgeranyl group, respectively, to the cysteine residue. The C-terminal of the Rab family contains cysteine-containing motif (CC, CXC, CCX, CCXX, or CCXXX) is modified by geranylgeranyltransferase II, which also adds a geranylgeranyl group to the cysteine residue. In addition, Arf proteins are modified with myristic acid at the N terminus. These translational modifications are required for their binding to the membrane and/or regulators and also necessary for the activation of their

downstream effectors (Takai, Sasaki et al. 2001; Wennerberg, Rossman et al. 2005).

4.1.2 The regulation of GTPases activity of small G proteins

The GDP/GTP cycle of small GTPases is highly regulated by GEFs (Guanine nucleotide exchange factors) and GAPs (GTPase-activating proteins). GEFs activate small GTPases through accelerating the dissociation and replacement of the bound GDP with GTP. On the other hand, GAPs inactivate small GTPases by proving an essential catalytic group for GTP hydrolysis. In addition, GDIs (Guanine nucleotide dissociation inhibitors) are also involved in regulating GTPases activities through keeping the GTPases in GDP-bound state (Takai, Sasaki et al. 2001).

GEFs catalyze the dissociation of nucleotide from the G proteins by modifying their nucleotide binding site. As cellular concentration of GTP is ~10 fold higher than that of GDP, the release of nucleotide from G protein leads to an increase of GTP bound over GDP bound form. GEFs are classified into families on the basis of both sequence similarity and the small GTP-binding domain (Cherfils and Chardin 1999). For instance, the GEFs that regulate Rho family contain a DH domain (RhoGEF domain) in adjacent to a PH domain. The RasGEFs contain a CDC25 homolog domain (RasGEF domain) together with a REM (Ras exchange motif) domain. ArfGEFs possess Sec7 domain, whereas the RabGEFs contain Vps9-, Sec2-, or Mss4-like domain. Intriguingly, certain proteins contain two GEF domains for different small GTPase, such as Sos with both RasGEF and RhoGEF domains (Bos, Rehmann et al. 2007).

Although the catalytic domains of the various families of GEFs are structurally unrelated, they all use similar principle to deform the nucleotide binding site. In general, GEFs binding induces the conformation change of the switch I and II regions and the P loop of GDP-bound small GTPase. This perturbs the interaction surface in the phosphate-binding region while leaving the base-binding region mostly unperturbed. In the cases of RasGEFs and RhoGEFs, GEF binding pushes the switch I out of its normal position and pulls the switch II toward the nucleotide-binding site. Concomitantly, Mg^{2+} ion is pushed out of its position and the structure of P loop is disturbed and reoriented (Vetter and Wittinghofer 2001). These changes collectively cause the disruption of G domain leading to a reduced binding affinity and release of the GDP.

The reaction mechanism of GAPs is unraveled by several structural and biophysical studies. In the structure of Ras bound to GppNHp, a nonhydrolyzable GTP analog, a H_2O molecule is positioned optimally for an in-line nucleophilic attack. GAPs catalyze the effective phosphoryl transfer via several elements: the proper orientation of the attacking H_2O and its polarization, occlusion of H_2O from active site, and the stabilization of the transition state. In the case of RasGAP (Scheffzek, Ahmadian et al. 1997), its binding to Ras stabilizes the position of Gln61 at switch II region of Ras, which in turn coordinates the H_2O attacking on the γ-phosphates of GTP. In addition, an arginine residue on GAP is positioned into the phosphate-binding site and stabilizes the transition state by neutralizing negative charge at the γ-phosphates. In this way, RasGAP stimulates the weak intrinsic GTPase activity and catalyzes GTP hydrolysis. A similar mechanism is applied to

RhoGAP-assisted Rho-GTP hydrolysis (Rittinger, Walker et al. 1997). Although other families of GAPs use slightly different ways to enhance the GTPase activities, their main contribution is the stabilization of the intrinsically mobile catalytic machinery of the G protein and , in most cases, the insertion of a catalytic residue in *trans*.

4.2 Ras GTPases

4.2.1 Ras family

The Ras family consists of the well-known classical p21 Ras protein, H (*Harvey*)-Ras, N (*Neuroblastoma*)-Ras and the two splice-variants of K (*Kirsten*)-Ras, K-*RasA* and K-*RasB* (Pells, Divjak et al. 1997). Besides, R-Ras, TC21(R-Ras2), M-Ras (R-Ras3), Rap1A/1B, Rap2A/2B, RalA/B, Rin, and Rit are members of this family. H- and K-Ras were first identified as viral (v-Ras) oncoproteins of Harvey and Kirsten murine sarcoma viruses. N-Ras was isolated from a neuroblastoma cell line. The H-*Ras* (3 Kb), K-*Ras* (35 Kb), and N-*Ras* (7 Kb) span in the human genomic DNA and locate in chromosome 11p15.5, 12p12.1, 1p13, respectively (Lowy and Willumsen 1993). These members of the Ras family genes share at least 50% sequence identity (Macaluso, Russo et al. 2002; Giehl 2005).

H-, K-RasA/B, and N-Ras contain an N terminal catalytic domain (a.a. 1-165) and a C terminal hypervariable region (a.a. 166-188/189). The catalytic domains are highly conserved (> 85 % identity) and the hypervariable region exhibits 4 % amino acid identity (Giehl 2005). The hypervariable region is

divided into the linker region and the anchor region. The linker region encodes Ras trafficking signals and the anchor region comprises CAAX motif for posttranslational modifications, such as farnesylation (Hancock 2003). Correct posttranslational modifications of the hypervariable region within Ras proteins are essential for their membrane targeting and their functionally activation. H- and N-Ras are targeted to plasma membrane via exocytotic pathway through the Golgi apparatus, but the mechanism for K-Ras membrane targeting is unknown (Apolloni, Prior et al. 2000; Silvius 2002). Activated H-Ras is localized on the plasma membrane and displays a different membrane localization pattern with that of K-Ras. The difference in the membrane localization of activated H- and K-Ras accounts for their interacting with distinct effectors and/or cofactors thereby triggering different downstream signaling pathways (Prior, Muncke et al. 2003).

4.2.2 Effectors and functions of Ras

Ras-GTP interacts with more than 20 effectors, including Raf serine/threonine kinase and phosphoinositide 3-kinase (PI3K), and regulates various cell processes and behavior. The Raf-Mek-Erk cascade is the best characterized Ras effector pathway. In mammals, there are three highly conserved Raf serine/threonine kinases, A-Raf, B-Raf, and Raf1. All three Raf isoforms are able to phosphorylate and activate Mek, but B-Raf is the strongest and A-Raf is the weakest activator. Ras-GTP does not directly activate A-Raf and Raf-1, but translocates them to the plasma membrane where they are activated by membrane-bound tyrosine kinases, such as Src tyrosine kinase family. B-Raf is not directly activated by Ras but requires

Ras-GTP for the correct membrane targeting (Marais, Light et al. 1997). Activated Raf phosphorylates the dual-specific kinase Mek1/2 which in turn phosphorylates Erk1/2. Activated Erk phosphorylates several cytoplasmic substrates, such as Sos and Rsk. Phosphorylated Sos dissociates from Grb2 thus resulting in the negative feedback control of Ras signaling. Activated Rsks (90 kDa Ribosomal protein S6 Kinases) translocates into nucleus and regulates transcription by phosphorylating transcription factors, such as c-fos, and promotes cell survival by phosphorylating pro-apoptotic proteins, such as Bad. In accompanied with phosphorylation, Erk translocates into the nucleus where it elicits functions in both gene expression and DNA replication. The best-characterized nuclear substrates of Erks are TCFs (Ternary Complex Factors) including Elk1. Elk1 forms complex with SRF (Serum Response Factor) which regulates the expression of numerous genes to control proliferation, differentiation, survival and apoptosis (Kolch, Kotwaliwale et al. 2002).

The interaction of Ras-GTP and p110 catalytic subunit of type I PI3Ks results in the translocation of PI3K to the plasma membrane. PI3K phosphorylates phophatidylinostol-4,5-bisphosphate (PIP2) to produce phophatidylinostol-3,4,5-bisphosphate (PIP3) at the inner leaflet of the plasma membrane. PIP3, a secondary messenger, is involved in the recruitment and activation of a wide variety of downstream targets, including PDK1 and Akt (Bader, Kang et al. 2005). Akt pathway is a key regulator of cell survival by antagonizing apoptosis via phosphorylating and inactivating several components of the apoptosis, such as Bad and Forkhead transcription factors (Vivanco and Sawyers 2002).

4.2.3 Ras and cancers

Ras proteins are signal switches that regulate cell fates by coupling receptor activation to downstream signal cascades that control a variety of cellular processes such as proliferation, differentiation, and survival. Ras mutants are found in 30% of all human cancers. These mutations are mainly occurred in K-*Ras* gene; the N-*Ras* mutations are moderately prevailed, and the mutations in H-*Ras* are quite rare (Bos 1989). K-*Ras* mutants are common in lung (Rodenhuis, Slebos et al. 1988), colorectal cancer (Bos, Fearon et al. 1987), and in almost all pancreatic adenocarcinomas (Almoguera, Shibata et al. 1988). N-*Ras* mutants are found in melanoma, liver cancer, and acute myelogenous leukemia (Bos 1988; Adjei 2001), H-*Ras* mutants are found in some types of bladder and kidney cancer. Evidence for the tumorigenic roles of these Ras mutants comes from a number of mouse tumor models that express such somatically obtained oncogenic Ras mutants. For instance, mice carrying K-Ras mutations are highly predisposed to a range of tumor types, with the highest incidence in early-onset lung cancer (Johnson, Mercer et al. 2001). Transgenic mice strains that carry a v-H-*ras* oncogene under the control of a viral promoter demonstrate that overexpression of oncogenic H-Ras perturbs cell growth, resulting in malignant tumor growth in several tissues and organs (Sinn, Muller et al. 1987). Furthermore, using transgenic mice in which H-Ras expression is controlled by tetracycline, it has been demonstrated that the activated H-Ras is not only necessary for tumor initiation, but also for maintaining an oncogenic phenotype (Chin, Tam et al. 1999). Of note, these oncogenic Ras proteins possessing mutations in codon 12, 13, or 61 are impaired in its intrinsic GTPase activity and are insensitive to

GAPs. Therefore, they are constitutively present in the GTP-bound state and are independent of the extracellular stimulations (Scheffzek, Ahmadian et al. 1997). This deregulated Ras activity and signaling promote cell transformation and human cancer malignancy.

4.2.3.1 Ras in cell transformation

Ras-transformed fibroblasts display typical anchorage-independent growth and morphological changes which are mediated by Ras-induced gene expression, proliferation, cytoskeleton rearrangement and cell adhesion. Oncogenic Ras activates the Ets family transcription factors via the function of Erk to regulate the expression of Fos (Yordy and Muise-Helmericks 2000). Fos forms complex with Erk-phosphorylated c-Jun to upregulate cell-cycle regulatory proteins, such as cyclin D (G1 phase regulators) thereby inducing cell cycle entry and cell proliferation (Pruitt and Der 2001). Besides, Ras/PI3K/AKT signaling has a strong anti-apoptotic function. Both Ras triggered cell proliferation and survival signals are important in cell transformation. In addition, activated Ras directly binds to and activates the RacGEF, Tiam1 (tumor invasion and metastasis inducing protein 1), leading to the activation of Rac and actin cytoskeleton reorganization. This pathway is important for Ras-induced cell transformation and is necessary for the development of Ras-dependent skin tumor (Malliri, van der Kammen et al. 2002). Through the coordination of Ras-elicited signaling pathways mentioned above, the expression of activated Ras mutant promotes malignant cell transformation.

4.2.3.2 Ras in tumor cell invasion and metastasis

The key steps in the invasion and metastasis include alterations in cell-cell or cell-ECM interaction and increase in cell motility. Matrix metalloproteinases (MMPs) are critical for degrading the ECM to allow tumor cell migration. Regulation of MMPs expression has been shown to be downstream of Ras signaling cascades. Oncogenic Ras activates transcription factors, AP-1 and Ets-1, via Erk pathway leading to MMPs expression (Westermarck, Li et al. 2001). Ras can also act through NFκB to simultaneously increase MMP9 expression and decrease TIMP-1 (as a MMP-9 inhibitor) expression (Yang, Zhao et al. 2001). These observations demonstrate that Ras is able to upregulate ECM proteases and downregulate the protease inhibitors through multiple mechanisms, thereby promoting basement membrane degradation. In addition to affecting ECM compositions, Ras signaling also regulates actin cytoskeleton organization mainly through Rho family GTPases to impact on cell migration and invasion. The Ras-induced Rac activity is important for the membrane ruffle formation that is necessary for increased cell migration and invasion. Ras can upregulate Rac1 by directly activating its GEF Tiam1, Alternatively, Ras can act through PIK3 to activate another GEF Vav1 (Han, Luby-Phelps et al. 1998) or to induce the formation Eps8/E3B/Sos1 complex, thereby promoting Rac activation (Innocenti, Frittoli et al. 2003). Finally, oncogenic Ras has been reported to protected cell from suspension-induced programmed cell death (Khwaja, Rodriguez-Viciana et al. 1997; McFall, Ulku et al. 2001). The inhibition of such matrix deprivation-induced apoptosis, anoikis, prevents the detached tumor cells from apoptosis and allows them to migrate to distal tissues.

4.2.3.3 Ras in angiogenesis

Angiogenesis is a process that involves in the growth of new blood vessels from pre-existing vessels. This is essential for metastatic cells moving out of primary tumor mass and traveling to other parts of body. Oncogenic Ras not only promotes the initiation of tumor growth by stimulating tumor cell proliferation, but also ensures tumor progression by stimulating tumor-associated angiogenesis (Rak, Yu et al. 2000). Of note, even in tumor cells that do not harbor oncogenic Ras mutations, endogenous Ras can be transiently activated to induce angiogenesis in response to hypoxia (Jung, Haendeler et al. 2002) or acidosis (Xu, Fukumura et al. 2002), the condition commonly occurred in intratumoral microenvironment. VEGF, the most potent angiogenesis-stimulating factor, is a well-known Ras target. Consistently, a high correlation between oncogenic K-*Ras* mutations and elevated VEGF level has been observed in a number of cancer types (Konishi, Huang et al. 2000; Ikeda, Nakajima et al. 2001). Activated Ras stimulates VEGF production via multiple mechanisms involving both the transcriptional and translational upregulation of VEGF. First, the Ras/Raf/Erk pathway induces the phosphorylation and activation of transcription factors HIF-1, SP1/3 , and AP-2, which act together on the promoter of VEGF to stimulate its transcription (Milanini, Vinals et al. 1998; Richard, Berra et al. 1999; Berra, Pages et al. 2000). Ras can also lead to the stabilization of HIF-1 via the PI3K/Akt/GSK3 pathway (Sodhi, Montaner et al. 2001), further enhancing VEGF synthesis. Furthermore, the Ras/Raf/Erk pathway and Ras/PI3K/Akt pathway can both stimulate eIF-4E-dependent translation initiation, which in turn elevates the VEGF protein level (Gingras, Gygi et al. 1999; Herbert, Tee et al. 2002). Finally,

activated Ras upregulates COX-1/2 leading to the production of postaglandins (Dubois, Abramson et al. 1998; Sheng, Williams et al. 1998). The postaglandins act on either tumor cells via autocrine signaling or the nearby stromal cells via paracrine signaling to stimulate the production of VEGF. In addition to upregulating VEGF, activated Ras also represses the anti-angiogenic factor thrombospondin-1 (Zabrenetzky, Harris et al. 1994) and stimulates matrix remodeling factors urokinase plasminogen activator (Brunner, Pohl et al. 1989; Testa, Medcalf et al. 1989) and MMP-2/9 (Sato, Kida et al. 1992; Gum, Lengyel et al. 1996), thereby liberating growth factors from ECM to promote endothelial cell activation and migration. Thus, through a complex signaling network, Ras stimulates the endothelial cells in nearby vascular beds to enhance angiogenesis.

4.3 Rho GTPases

4.3.1 Rho family

Rho family proteins are ~30% homologous to Ras and ~50% homologues to each other (Valencia, Chardin et al. 1991). Rho members differ from other Ras-like GTPases by the presence of a Rho-specific insert region. This insert region (12 amino acids) locates to the GTP binding domain and has been suggested to be involved in the interactions of effectors and regulators (Freeman, Abo et al. 1996). To date, over 20 Rho GTPases have been isolated and divide into 6 groups: the Rho subfamily (RhoA, RhoB, RhoC), the Rac subfamily (Rac1, Rac2, Rac3, RhoG), the Cdc-42 like subfamily (Cdc42, TC10, TCL, Wrch1, Chp), the Rnd subfamily (Rnd1, Rnd2, Rnd3), the RhoBTB

subfamily (RhoBTB1, RhoBTB2, RhoBTB3) and the Miro subfamily (Miro1, Miro2). Of note, the last two subfamilies are sometime excluded to the Rho family because they lack the Rho-specific insert region and are of distinct phylogenetic origin (Boureux, Vignal et al. 2007). There are three additional members, i.e., RhoD, Rif and TTF, that do not belong to any of these subgroups (Ellenbroek and Collard 2007).

Similar to other small GTPases, most Rho family members cycle between GTP-bound active forms and GDP-bound inactive forms, and their activities are tightly regulated by RhoGEFs and RhoGAPs. Rho proteins are frequently posttranslational modified at the C-terminal by prenylation and/or palmitoylation. Such modifications enhance their interaction with membrane where they bind to downstream effectors. RhoGDIs (Rho guanine-nucleotide-dissociation inhibitors) regulate Rho GTPases activity by binding to their C-terminal prenyl group, preventing their membrane association, and sequestering them in the cytoplasm, thus inhibiting their downstream signaling (Dovas and Couchman 2005).

Some Rho proteins are atypical because they are not regulated as the classical Rho GTPases. Rnd subfamily, RhoBTB subfamily, and TTF have key amino acid substitutions that make them lacking GTP hydrolysis activity and therefore they are permanently bound to GTP (Chardin 2006; Aspenstrom, Ruusala et al. 2007). These atypical Rho GTPases are likely to be regulated by expression level, phosphorylation, and/or protein-protein interactions (Vega and Ridley 2008). In addition, Miro subfamily (Miro for mitochondrial Rho) has an additional C-terminal transmembrane domain. This domain is unrelated to

the Rho GTPases but is required for their mitochondria targeting (Fransson, Ruusala et al. 2006).

4.3.2 Effectors and functions of Rho GTPases

Activated Rho GTPases recruit a number of effectors thereby triggering downstream signaling cascade to direct cellular responses. Over 70 effectors of Rho GTPases have been identified including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases, and scaffold proteins. Through the interactions with these diverse effectors, Rho GTPases exhibit their distinct cellular functions, including actin and microtubule cytoskeleton organization, cell adhesion and motility, vesicular trafficking and gene transcription (Schmitz, Govek et al. 2000; Hall 2005). Much of our knowledge on the functions of Rho family protein has been derived primarily from the studies of Rac1, RhoA and Cdc42. They have been suggested to play roles in regulating actin and microtubule cytoskeleton reorganization, as well as gene transcription.

Rac1, RhoA and Cdc42 stimulate actin cytoskeleton reorganization leading to the formation of sheet-like lamellipodia and membrane ruffles, contractility and stress fiber formation and finger-like filopodia formation, respectively. Rac1 and Cdc42 acting through WASP family protein and WAVE family protein, respectively, activate Arp2/3 complex (actin-related protein 2/3) which induces the branch of actin filaments from the pre-existing filaments leading to membrane protrusion such as lamellipodia and filopodia. RhoA stimulates actin polymerization through direct binding to diaphanous-related forming

(mDia). mDia interacts with and delivers profilin/actin complex to the barbed end of actin filaments resulting in filament elongation. RhoA also exhibits a significant role in bundling actin filament. RhoA stimulates myosin light chain (MLC) phosphorylation via activating Rho-kinases (ROCKs) or inhibiting MLC phosphatase. Phosphorylated MLCs bundle actin filament leading to RhoA-induced actin stress fibers formation (Jaffe and Hall 2005).

In addition to regulating actin cytoskeleton, the Rho GTPases also exert functions in reorganizing microtubule cytoskeleton. RhoA signaling promotes the formation of stable and aligned microtubules via binding to mDia (Palazzo, Cook et al. 2001). Rac1 and Cdc42 can stabilize microtubules through activating their common effector PAK, which in turn phosphorylates and inactivates microtubule destabilizing factor stathmin (Daub, Gevaert et al. 2001). Rac1 and Cdc42 can also act through IQGAP to simulate the microtubule plus end binding protein CLIP-107, which subsequently captures microtubule at the cell cortex (Fukata, Watanabe et al. 2002). Microtubule capture is necessary for microtubules stabilization cell polarity (Fukata, Watanabe et al. 2002). Cdc42 also facilitates the microtubule stabilization and rearrangement through regulating another microtubule plus end capture protein, EB1, which forms complex with APC. During polarization and directional cell migration, Cdc42 acts together with Par6/aPKC to phosphorylate and to inactivate GSK3, thereby promoting the association of EB1/APC complex with microtubule plus ends. This association is essential for microtubule stabilization and consequently the reorientation of the Golgi apparatus and MTOC (microtubule organizing centre),which collectively induce cell polarization (Etienne-Manneville and Hall 2003; Etienne-Manneville

2004).

Rho GTPases have also been reported to regulate gene expression by activating several transcription factors, such as Ets, AP1 and NFκB, via modulating a number of signal transduction pathways. For instance, Rac1 and Cdc42 can activate JNK and MAPK cascades leading to phosphorylation and activation of nuclear transcription factor AP-1 components JUN and ATF (Vojtek and Cooper 1995; Hinz, Krappmann et al. 1999). Many Rac1 and Cdc42 effectors are implicated in the activation of JNK and p38MAPK, such as PAKs, MLKs (mixed-lineage kinases), MAPKKs and POSH (plenty of SH3 domains). RhoA has been demonstrated to regulate gene transcription via activating SRF (serum response factor) and MEF2C (myocyte-enhancer factor 2C), which lead to increased transcription of AP-1 components JUN and FOS, respectively (Hill, Wynne et al. 1995; Marinissen, Chiariello et al. 2001). Rac1 also activates NFκB by promoting phosphorylation and degradation of IκB (Cammarano and Minden 2001). Through activating these nuclear transcription factors, Rho GTPaes upregulate cyclin D1 for cell proliferation (Albanese, Johnson et al. 1995; Hinz, Krappmann et al. 1999; Shaulian and Karin 2001), anti-apoptosis proteins for cell survival (Sonenshein 1997), as well as MMPs (metalloproteinases) and TIMPs (inhibitors of metalloproteinases) for invasive potential of tumor cells (Benbow and Brinckerhoff 1997; Kheradmand, Werner et al. 1998).

4.3.3 Rho GTPases and cancers

4.3.3.1 Altered Rho GTPases signaling in cancers

Alterations of Rho proteins in cancers

Only one human Rho GTPase has been found to be genetically altered in human cancers. Rearrangement of the *TTF* (also known as *RhoH*) gene is found in non-Hodgkin's lymphomas and multiple myeloma (Preudhomme, Roumier et al. 2000), as well as mutations in the 5′ UTR in diffuse large-cell lymphomas (Pasqualucci, Neumeister et al. 2001). However, the contribution of *TTF* gene mutations in the pathology has not been elucidated yet. Several Rho GTPases have been reported to be aberrantly expressed in human cancers or cancer-derived cell lines, such Rho subfamily members, Rac subfamily members, Cdc42 and Rnd3. Overexpression of RhoA has been observed in breast cancer, colon cancer, lung cancer (Fritz, Just et al. 1999; Fritz, Brachetti et al. 2002), gastric cancer (Pan, Bi et al. 2004), head and neck squamous cell carcinoma (Abraham, Kuriakose et al. 2001), bladder and testicular cancer (Kamai, Tsujii et al. 2003; Kamai, Yamanishi et al. 2004). *In vitro* studies have shown that elevated expression of RhoA promotes cell invasion by activating ROCK-actomyosin pathway (Yoshioka, Nakamori et al. 1999). The increased Rac activity has been reported to promote breast cancer development (Fritz, Just et al. 1999; Fritz, Brachetti et al. 2002). Rac1b, a highly active Rac1 splice variant, is found to be overexpressed in breast and colon cancer (Jordan, Brazao et al. 1999; Schnelzer, Prechtel et al. 2000). *In vitro* experiments also demonstrate that Rac1b is able to promote cell

transformation possibly through stimulating NFκB mediated survival signaling (Singh, Karnoub et al. 2004; Matos and Jordan 2005). In addition, elevated levels of hyperactive Rac3 are found in highly proliferative human breast cancer-derived cell-lines and tumor tissues. It has also been suggested that Rac3 promotes DNA synthesis of highly proliferative cells via activating its effector, PAK (Mira, Benard et al. 2000).

Alterations of Rho regulators in cancers

Mutations in or altered expression of the Rho GTPases regulators lead to deregulation of Rho GTPases signaling network. Tiam1, a RacGEF, is mutated in its N-terminal PH domain that affects its intracellular localization and is found in 10% of human renal-cell carcinomas, (Engers, Zwaka et al. 2000). Tiam1 protein levels are also found to be increased and correlated positively with disease prognosis in human prostate carcinomas (Engers, Mueller et al. 2006) and human breast tumors (Adam, Vadlamudi et al. 2001; Minard, Kim et al. 2004). In human glioma, Dock180, a RacGEF, is upregulated only in the invasive areas of the tumors, which is consistent with its ability to increase the invasion and migratory capacity of glioma (Jarzynka, Hu et al. 2007). LARG, leukemia-associated RhoGEF, is isolated from acute myeloid leukemia (AML). LARG gene is reported to fuse with mixed-lineage leukemia (MLL) gene resulting in LARG truncation. The MLL-LARG fusion retains LARG Rhode activity (Reuther, Lambert et al. 2001) and has been suggested to contribute to the development of leukemia by activating RhoA signaling pathways (Reuther, Lambert et al. 2001).

In addition to GEFs, many RhoGAPs alterations have also been found in human cancers. The genomic deletion of DLC-1 (a GAP for RhoA and Cdc42) is found in primary breast tumors (Yuan, Zhou et al. 2003), and the expression of DLC-2 is reported to be downregulated in hepatocellular carcinomas (Ching, Wong et al. 2003). Deletion or downregulation of DLC-1/2 leads to upregulation of RhoA activity and the downstream signaling to promote tumorigenesis. Furthermore, alterations in the expression of GDIs have been reported to promote developmental stages of cancer. RhoGDI (RhoGDI1 or RhoGDIα), which is able to bind RhoA, RhoB, Rac1, Rac2 and Cdc42 (Dovas and Couchman 2005), is overexpressed in invasive ovarian cancers (Jones, Krutzsch et al. 2002). RhoGDI may also influence breast cancer cell motility through enhanced transcriptional levels of estrogen receptors (ER) α and β (Su, Knoblauch et al. 2001).

Alterations of Rho GTPases effectors in cancers

Several Rho family effectors are proposed to alter Rho GTPases signaling and to promote tumorigenesis and tumor progression. The upregulation of PAK is reported in some breast cancers (Salh, Marotta et al. 2002). Overexpression of PAK induces anchorage-independent growth and abnormal mitotic spindles organization in epithelial breast cancer cells, MCF-7 (Vadlamudi, Adam et al. 2000). Furthermore, elevated expression levels of ROCK-I/II have been associated with higher stages of testicular cancer (Kamai, Yamanishi et al. 2004). It has been suggested that ROCK alters actomyosin activity thereby promoting testicular tumor cells migration and metastatic spreading.

4.3.3.2 Rho GTPases in tumorigenesis and tumor metastasis

Rho GTPases have been shown to regulate cell morphology and cytoskeleton organization, gene expression, cell proliferation and survival. These cellular functions are important in tumorigenesis. Several *in vitro* observations demonstrate that Rho GTPase signaling is required for Ras induced oncogenic transformation (Qiu, Chen et al. 1995; Zohn, Campbell et al. 1998). The constitutively active mutants of RhoA, Rac1 and Cdc42 contribute to Ras-induced morphological transformation, whereas dominant-negative mutants of these GTPases prevent such transformation event. The activated Rac1 has been shown to suppress high-intensity Ras signaling induced apoptosis via activation of NFκB (Joneson and Bar-Sagi 1999), thereby modulating the growth advantage for Ras transformed cells. Besides, the alterations of Rho GTPases signaling also correlate with human cancer formation. These findings suggest that Rho GTPases are involved in the tumorigenesis and tumor progression, which are further confirmed by mice tumor models. For instance, Tiam1 knockout mice are protected from initial development of Ras-induced skin cancers. Although the tumors in Tiam1 knockout mice are smaller and grew much slower than the tumors in wild-type mice, they exhibit a greater proportion of malignancy progression (Malliri, van der Kammen et al. 2002). These suggest that Tiam1-mediated Rac activity acts synergistically with Ras during tumor initiation to contribute to tumor progression of skin cancer. In addition, the similar experiments are performed using RhoB-deficient mice (Liu, Rane et al. 2001). Surprisingly, the number of Ras-induced skin tumor in RhoB-deficiency mice is more than that in wild-type

mice. This suggests that RhoB acts as a tumor suppressor likely via its function to stimulate apoptosis, thereby antagonizing the effects of Tiam1/Rac on Ras-induced tumorigensis.

Rho GTPases are also implicated in the promote tumor metastasis through several different mechanisms, including alteration of cell-cell or cell-matrix adhesion, loss of cell polarity, promotion of cell migration and tumor vascularization (Lozano, Betson et al. 2003). Once tumor is initiated, Rho GTPases can contribute to tumor development by stimulating cell growth and disrupting cell polarity. The elevated levels of Rnd3 have been shown to impair polarity, which leads to multilayering of epithelial cell via inhibiting RhoA downstream signaling (Guasch, Scambler et al. 1998; Hansen, Zegers et al. 2000). Inhibition of Rac1 leads to failure to asymmetrically deposit laminin and therefore causes the loss of cell polarity (O'Brien, Jou et al. 2001). The association of Rac1 with Par3 and Par6 is also involved in controlling epithelial cell polarity (Kim 2000). Thus, downregulation of certain Rho GTPases signaling results in loss of cell polarity. Besides, Rho GTPases also mediate the alteration of cell-cell or cell-matrix adhesions allowing tumor cells to become invasive.

Loss or deregulation of E-cadherin based cell–cell adhesions leads to EMT (Epithelial-mesenchymal transition) that allows stationary epithelial cells to become motile mesenchymal cell and that is also in correlation with progression of epithelial tumor (Cowin, Rowlands et al. 2005). Rho GTPases regulate both disassembly and stabilization of E-cadherin based adhesions and consequent alteration of cell-cell adhesion of tumor cells. The activation of

Tiam1/Rac signaling is required for the establishment and maintenance of E-cadherin based adhesions. *In vitro* and *in vivo* observations also show that loss of Tiam1 promotes EMT and the progression of mouse skin tumor (Malliri, van der Kammen et al. 2002; Malliri, van Es et al. 2004). However, it has also been found that active Rac1 not only promotes adherens junctions disassembly in keratinocytes (Braga, Betson et al. 2000), but also is required for Ras induced loss of adherens junctions (Quinlan 1999). How does Rac play opposing effects on regulating adherens junctions? In fact, Rac-induced cellular response is dependent on the components of ECM surrounding the cell. Changing ECM composition switches the effect of Rac1 from promoting to antagonizing cell-cell adherens junctions (Sander, van Delft et al. 1998). Invasive tumor cells require adaptation of cell–matrix adhesions to cross the tissue boundaries and spread to distal parts of the body. The ECM remodeling is essential for tumor cells to become locally invasive. RhoA and Rac1 modulate the degradation and remodeling of the ECM either by upregulating MMPs expression or by expressing TIMPs (Lozano, Betson et al. 2003). After EMT and adaptation of cell-ECM adhesion, tumor cells need to acquire migratory capacity to migrate towards distal parts of the body. It has been shown that RhoA and RhoC exert opposing functions in promoting migration of invasive breast carcinoma and colon carcinoma. While RhoA inhibits post-EMT migration, RhoC promotes it (Hakem, Sanchez-Sweatman et al. 2005; Bellovin, Simpson et al. 2006). In addition, a RhoC knockout mice also confirms that RhoC is not required for the initiation of tumor but rather for tumor progression and metastasis (Hakem, Sanchez-Sweatman et al. 2005). Another important function of RhoC is the induction of tumor vascularization by promoting the expression of angiogenic factors in inflammatory breast cancer

(van Golen, Bao et al. 2002), which is necessary for tumor cells entry into blood vessels and thereby metastasis.

5. The cross-talk between Rho and Ras

5.1 p120RasGAP

p120RasGAP (p120), encoded by *RASA1* gene, is the first protein to be identified as a RasGAP (Trahey and McCormick 1987; Gibbs, Schaber et al. 1988). p120 acts as a negative regulator of Ras proteins such as H-Ras, K-Ras, N-Ras, and R-Ras, but does not work on Rho/Rac/Rab proteins (Trahey and McCormick 1987; Gibbs, Schaber et al. 1988; Boguski and McCormick 1993). p120 contains two N-terminal SH2 domain flanking a SH3 domain, a PH domain, C2 domain, and a C-terminal RasGAP domain. The PH domain and C2 domain may be required for the Ca^{2+} and/or lipid binding and for its association with the inner leaflet of plasma membrane (Gawler, Zhang et al. 1995). The SH2 domains are required for p120 forming complex with activated EGF and PDGF receptors, which is responsible for the translocation of p120 from the cytosol to the plasma membrane after growth factor stimulation (Kazlauskas, Ellis et al. 1990; Margolis, Li et al. 1990; Moran, Polakis et al. 1991). In addition, the SH2 domains also mediate its association with p190RhoGAP (Moran, Koch et al. 1990; Settleman, Albright et al. 1992), and a highly tyrosine phosphorylated protein, p62Dok (Carpino, Wisniewski et al. 1997). Although a number of tyrosine kinases have been shown to

phosphorylate p120 in a variety of cells, the stoichiometry of such phosphorylation is generally low and none of these phosphorylation events has been demonstrated to alter the activity or localization of p120. In mice, p120 knockout results in early embryonic lethality associated with defective vasculogenesis and increased apoptotic cell death in developing brain (Henkemeyer, Rossi et al. 1995). In humans, inactivating mutations in *RASA1* coding region have been shown to cause a novel clinical disorder named capillary malformation-arteriovenous malformation (Eerola, Boon et al. 2003; Boon, Mulliken et al. 2005). These findings suggest important physiological functions for p120 in at least endothelial cells and neuronal cells.

5.2 p190RhoGAP

p190RhoGAP is the best-known p120 binding partner and is also a potent RhoA inhibitor (Roof, Haskell et al. 1998; Arthur, Petch et al. 2000). p190RhoGAP exists as two related proteins, namely p190RhoGAP-A (p190A) and p190RhoGAP-B (p190B), encoded in humans by the *GRLF1* and *ARHGAP5* genes, respectively. Both proteins share 51% identity and display similar domain structure, including an N-terminal GTPase domain followed by four FF domains (protein-protein interacting domains harboring two strictly conserved phenylalanine residues), a large middle domain, and the C-terminal RhoGAP catalytic domain. With the highly homologous and the ubiquitous expression pattern, the p190 isoforms not only act as a RhoA inhibitor, but also share several signaling pathways and interacting proteins, such as p120 (Burbelo, Miyamoto et al. 1995), Rnd1 and Rnd3 (Wennerberg, Forget et al. 2003) and TFII-I (Jiang, Sordella et al. 2005). For example, p190 proteins

sequester TFII-I in the cytoplasm via their FF domains, but upon PDGF receptor-mediated phosphorylation of an FF domain, TFII-I is released from p190 and translocates into the nucleus where it activates transcription of serum-inducible genes, such as *c-fos* (Jiang, Sordella et al. 2005). p190RhoGAP proteins have been reported to be recruited to integrin clustering site and is implicated in integrin signaling (Burbelo, Miyamoto et al. 1995). Furthermore, their GAP activity negatively regulates RhoGTPases leading to cytoskeletal disassembly and consequently cell motility and invasion (Keely, Parise et al. 1998). Despite these structural and functional similarities between the two p190 isoforms, they are different in some aspects. p190A is characterized to be phosphorylated by several tyrosine kinases such as Src. In contrast, p190B is not abundantly phosphorylated in Src-transformed Rat-1 fibroblasts (Matheson, Hu et al. 2006). p190B is necessary for mammary gland development by regulating ductal morphogenesis but not p190A (Chakravarty, Hadsell et al. 2003). In mouse, p190A or p190B knockout mice exhibit brain development defect leading to perinatal lethality. These facts indicate p190A and p190B are not functional redundant (Brouns, Matheson et al. 2000; Brouns, Matheson et al. 2001). In addition, p190B knockout mice cause 30% reduced in mass because of a defect in insulin/IGF-1 to CREB signaling that impacts on adipogenesis and consequent animal size , but these defects have not been reported in p190A knockout mice (Sordella, Classon et al. 2002; Sordella, Jiang et al. 2003).

5.3 The cross-talk between RhoA and Ras

Through a physical interaction with p120, p190RhoGAP plays an important

role in reciprocal regulation of Rho and Ras activities in response to extacellular stimulations elicited by integrin engagement and growth factors. The tyrosine phosphorylation of p190A, referred as p190 hereafter, is required for forming p190:120 complex and is induced by several tyrosine kinases, such as Src, Arg (Bradley, Hernandez et al. 2006), and Fyn (Wolf, Wilkes et al. 2001). Src is the best characterized p190 kinase, which phosphorylates p190 at Y1087 and Y1105 and consequent p190:p120 complex formation in response to EGF stimulation and/or integrin engagement (Chang, Gill et al. 1995; Roof, Haskell et al. 1998; Arthur, Petch et al. 2000). The phosphorylation of Y1105 is essential for the binding of p190 with the SH2 domain of p120, whereas Y1087 helps to stabilize this complex (Hu and Settleman 1997; Roof, Haskell et al. 1998). How does this complex inactivate RhoA? It is noticed that the two tyrosine residues are located out of the GAP domain (aa.1260-aa.1469) and that their phosphorylation does not affect the GAP activity of p190 (Haskell, Nickles et al. 2001). Accordingly, binding of p120 to tyrosine phosphorylated p190 does not alter the p190 activity *in vitro* (Hernandez, Settleman et al. 2004; Bradley, Hernandez et al. 2006). In fact, the complex formation between p190 and p120 is necessary for p190 membrane targeting, rather than GAP activity alteration (Bradley, Hernandez et al. 2006). On the other hand, p190:p120 complex does inactivate p120RasGAP leading to Ras activation (Moran, Polakis et al. 1991). In line with this, the tyrosine phosphorylation of p190 is the key for forming p190:p120 complex that inactivates p120RasGAP leading to Ras activation and targets p190RhoGAP onto plasma membrane where it downregulates RhoA.

Experimental rationale

Brk has been shown to mediate several signaling cascades that regulate a number of cellular processes, such as proliferation and migration, and is implicated to promote tumorigenesis. However, the molecular mechanisms that link Brk and metastatic malignancy are largely unknown. To address Brk-elicited signaling, identification of novel Brk substrates is significant. In the previously study, we characterized Brk is a paxillin kinase. Through phosphorylating paxillin, Brk mediates EGF induced Rac-dependent cell migration. Here, we identified p190RhoGAP is a direct Brk substrate. Brk induces p190RhoGAP tyrosine phosphorylation, which in turn regulates RhoA and Ras activities leading to the promotion of cell proliferation, migration, invasion and tumorigenesis.

Brk Phosphorylates p190RhoGAP to Regulate Rho and Ras and to Promote Breast Carcinoma Growth, Migration and Invasion

Introduction

Unraveling the signaling pathways responsible for the establishment of malignant phenotype in carcinoma cells is of crucial importance for the understanding of the pathology of cancer. Aberrant tyrosine kinase signaling has been shown to contribute to various steps of tumor development and progression. Brk is an intracellular tyrosine kinase and possesses SH3, SH2 and kinase domains in a similar arrangement to that of Src (Mitchell, Barker et al. 1994). However, Brk lacks an N-terminal myristoylation signal (Mitchell, Barker et al. 1994) and its genomic sequence is distinct from Src family kinases (Mitchell, Barker et al. 1997). Brk was identified from a human metastatic breast tumor (Mitchell, Barker et al. 1994), and subsequent analysis revealed Brk overexpression in ~2/3 of primary breast tumors, with the highest level in advanced tumors (Barker, Jackson et al. 1997; Born, Quintanilla-Fend et al. 2005; Ostrander, Daniel et al. 2007). This Brk overexpression positively correlates with the expression of HER family receptors (Born, Quintanilla-Fend et al. 2005; Aubele, Auer et al. 2007). In normal tissues, the expression of Brk is restricted to differential epithelial cells of skin and gastrointestinal tract (Llor, Serfas et al. 1999). In addition to breast tumors, elevated expression of Brk has also been detected in metastatic melanoma (Easty, Mitchell et al. 1997), colon tumors (Llor, Serfas et al. 1999), T-cell lymphoma (Kasprzycka, Majewski et al. 2006), and serous carcinoma of ovary (Schmandt, Bennett et al. 2006). In prostate cancers, although the expression of Brk is not significantly elevated, Brk translocates from the nucleus to cytoplasm during the progression of tumors (Derry, Prins et al. 2003).

Consistent with its potential role in promoting tumorigenesis, Brk stimulates the proliferation of breast tumor cells and mediates EGF-induced mitogenic and migratory effects (Kamalati, Jolin et al. 1996; Chen, Shen et al. 2004; Ostrander, Daniel et al. 2007). The signaling mechanisms underlying the mitogenic function of Brk, however, have not been completely unraveled. Brk associates with EGF receptor following receptor activation and enhances EGF-induced ErbB3 phosphorylation, which subsequently leads to an increased recruitment of PI 3-kinase and activation of Akt (Kamalati, Jolin et al. 2000). Additionally, Brk phosphorylates the growth-inhibitory protein Sam68 and triggers its nuclear export (Lukong, Larocque et al. 2005). Although these Brk-induced signaling events are implicated in cell proliferation, their significance in the tumor-promoting function of Brk remains elusive. In addition to stimulating cell growth, Brk is a potent inducer of migration and invasion. Our previous study revealed that this function of Brk is mediated in part by its phoshporylation of paxillin, which leads to the activation of Rac1 via the adaptor protein CrkII (Chen, Shen et al. 2004). Recently, Brk was shown to mediate EGF- and HRG-induced activation of p38 MAP kinase, which contributes in part to the proliferation and migration of breast cancer cells in response to these growth factors (Ostrander, Daniel et al. 2007). It is unclear how Brk triggers p38 MAP kinase activation. Identification of additional Brk substrates and interacting proteins would allow a further understanding of the functional mechanisms of Brk in tumorigenesis.

p190RhoGAP-A (p190) is a potent inhibitor of RhoA (Bernards and Settleman 2005), and was identified as a tyrosine phosphorylated protein associated with p120RasGAP (p120) in v-Src transformed cells (Ellis, Moran

et al. 1990). The association of p190 with p120 is promoted by phosphorylation of p190 at Y1105 (Roof, Haskell et al. 1998). Additionally, phosphorylation at Y1087 in p190 stabilizes its interaction with p120 (Hu and Settleman 1997). Although the formation of this RasGAP/RhoGAP complex does not directly affect the catalytic activity of p190, it promotes the recruitment of p190 to plasma membrane, which correlates with an increased activity of p190 to inhibit Rho in vivo (Bradley, Hernandez et al. 2006). In contrast to this activation of RhoGAP, the RasGAP activity of p120 is reduced when associated with p190 (Moran, Polakis et al. 1991). Thus, association of these two GAPs facilitates a crosstalk between Rho and Ras. The two tyrosine kinases Src and Arg (Abl-related gene) have been reported to directly phosphorylate p190 at Y1105 (Roof, Haskell et al. 1998; Hernandez, Settleman et al. 2004). Src promotes p190 phosphorylation in EGF-stimulated fibroblasts, thereby facilitating actin stress fiber disassembly (Chang, Gill et al. 1995). Intriguingly, both Src and Arg are required for integrin-dependent phosphorylation and activation of p190 in fibroblasts (Arthur, Petch et al. 2000). The effects of p190 activation on cell migration have been studied mainly in fibroblasts and seem to be complex. While p190 activity plays important roles in promoting cell spreading, membrane protrusion and cell polarity (Arthur and Burridge 2001), which are all essential steps in cell locomotion, Arg acts through p190 to decrease cell contractility, thereby reducing motility (Peacock, Miller et al. 2007). The influence of p190 on growth factor-induced migration has not been well studied.

In this study, we identified p190 as a substrate of Brk. Phosphorylation of p190 at Y1105 by Brk promotes the association of p190 with p120, leading to

Rho inactivation and Ras activation. In carcinoma cells expressing high levels of Brk, endogenous Brk contributes greatly to EGF-induced p190 phosphorylation. We present evidence showing that p190 Y1105 phosphorylation and consequent p190/p120 complex formation play crucial roles in the transformation and tumorigenic effects of Brk in breast cancers. Our findings reveal a previously unknown function of Brk in regulating both RhoA and Ras by phosphorylating p190, and provide mechanistic insights into the role of Brk in promoting tumor formation and progression.

Materials and Methods

Cell culture, transfection and retroviral infection

293T, HeLa, and A431 cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Mouse embryonic fibroblasts (MEFs) were cultured in DMEM medium with 15% FCS. T47D cells were cultured in RPMI1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mM nonessential amino acids (NEAA), and 5 μg/ml insulin. MDA-MB231 and MCF7 cells were maintained in DMEM-F12 medium with 10% FCS, 2 mM L-glutamine, and 0.1 mM NEAA. Transfection was performed using Lipofectamine 200 reagent or by calcium phosphate method. Recombinant retroviruses were generated according to procedures described previously (Tsai, Su et al. 2000).

Plasmid constructions

The coding region of p190 was excised from pKH3p190RhoGAP (provided by Sarah Parsons), and then subcloned to pBabe-Hygro and pCMV-Taq-2B to generate retroviral and mammalian expression vectors for p190, respectively. Various p190 mutants were constructed by in vitro mutagenesis using the QuickChange site-directed mutagenesis kit (Strategene). The plasmid pGEX-p190 fragment (amino acids 932 to 1143) was constructed by cloning the corresponding fragment to pGEX-4T1. To generate lentiviral expression construct for the SH2-SH3-SH2 fragment of p120 ($p120^{2\text{-}3\text{-}2}$), cDNA fragment for HA-tagged $p120^{2\text{-}3\text{-}2}$ was excised from pKHA232 (provided by Anthony

Koleske) and then cloned to pLenti6-GM-V5 vector (Invitrogen).

RNA interference

Lentivirus carrying Brk- or Src-specific siRNA (from National RNAi Core Facility, Taiwan) was used to knockdown Brk or Src, respectively. The target sequences of various siRNAs are “Src: GCGGCTCCCAGATTGTCAACAA, Brk-1: AGTCGCAGAATTACATCCACC, and Brk-2: TACCTCTCCCATGACCACAAT. To generate recombinant lentivirus, 293FT cells were co-transfected with the package, envelop and siRNA expressing constructs. The virus-containing supernatant was harvested and then used to infect cells and infected cells were selected with puromycin.

Antibodies

Antibodies to tubulin, Ras, and phosphotyrosine (4G10) were purchased from Upstate Biotechnology, whereas antibodies to Brk, RhoA and p120 were from Santa Cruz Biotechnology. The antibody to p190 was from BD Transduction Laboratory, and the anti-Flag M2 antibody was from Sigma. To generate Brk antibody capable of immunoprecipitation, GST-Brk SH3 domain was purified from E coli using only the soluble fraction of bacterial lysate and glutathione-Sepharose beads. The purified fusion protein was used to immunize rabbit and the resulting antiserum was affinity purified.

Assay for GTP-bound Rho, Rac and Ras

The levels of GTP-bound Ras and GTP-bound Rho were detected with the Raf-1 RBD (Ras binding domain) agarose (Upstate Biotechnology) and GST-rhotekin pull down assays, respectively. Briefly, cells were lysed with buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10 mM $MgCl_2$, 10% glycerol, 1 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysates were incubated with Raf-1 RBD agarose for 30 min at 4°C or 50 μg of GST-rhotekin beads for 1 hr at 4°C. The beads were washed and analyzed by Western blot to detect the bound Ras or Rho. GTP-bound Rac was detected as previously described (Chen, Shen et al. 2004).

Migration and invasion assays

Transwell migration and invasion assays using EGF as the chemoattractant were performed as described (Chen, Shen et al. 2004). After incubation at 37°C for 7 hr (for migration assay) or 24 hr (for invasion assay), cells remaining on the upper side of membrane were removed with a cotton swab. Cells that had migrated to the lower membrane surface were fixed and stained with Hochest 33342.

Immunoprecipitations and GST fusion proteins

Immunoprecipitations using cell lysates containing equal amounts of proteins and purification of GST fusion proteins with glutathione-Sepharose

beads were performed as described (Chen, Shen et al. 2004).

In vitro kinase assay

1 μg of Brk purified from baculovirus expression system (Chen, Shen et al. 2004) was incubated at 37°C for 10 min in 30 μl of kinase buffer containing 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 10 μM ATP, 10 μCi [γ-^{32}P]ATP and 2 μg p190 or its mutants purified from transfected cells. Alternatively, 0.5 μg GST-p190 fragment purified from E. coli was used as a Brk substrate. Substrate phosphorylation was analyzed by autoradiography.

Immunofluorescence analysis

Cells were fixed with 3.7% paraformaldehyde and permeabilized with buffer containing 50 mM NaCl, 300 mM sucrose, 10 mM PIPES (pH 6.8), 3 mM $MgCl_2$, and 0.5% Triton X-100 for 5 min. Cells were blocked with phosphate buffered saline (PBS) supplemented with 10% goat serum, 1% BSA and 50 mM NH_4Cl, and then incubated with 0.1 mM rhodamine-conjugated phalloidin diluted in PBS containing 0.2% BSA and 5% goat serum. Cells were then washed, mounted and examined with a Carl Zeiss LSM510 confocal laser-scanning microscope with a ×63 objective lens.

Soft agar colony formation assay

2.5×10^3 MDA-MB231 cell derivatives were resuspended in 0.3% of top agar and spread onto 60-mm plates containing 0.5% of bottom agar. Colonies

formed after 4 weeks were stained and then counted by the ImageJ software.

Tumorigenesis in mice

4-week-old BALB/c nude mice (n=40; National Laboratory Animal Center, Taipei, Taiwan) were acclimated for 1-2 weeks. The mice were housed in specific pathogen-free conditions and injected subcutaneously with 1×10^7 MDA-MB231 cell derivatives (n=10 for each group) mixed with PBS and Matrigel (vol/vol=1:1). Tumor volumes were calculated using the equation: $\text{width}^2 \times \text{length} \times 0.5$.

Results

Brk inhibits stress fiber formation and promotes cell spreading

To investigate the role of Brk in regulating actin cytoskeletons, we examined the effect of endogenous Brk on F-actin organization in serum-stimulated conditions. Two Brk-specific siRNAs were stably introduced to HeLa cells, and the Brk-2 siRNA induced a more efficient depletion of endogenous Brk than the Brk-1 siRNA (Fig. 1A). Expression of either Brk siRNA accelerated stress fiber formation in response to serum stimulation, which was evident by the increase in percentage of cells with stress fibers (Fig. 1B). Furthermore, the Brk-2 siRNA elicited a more profound effect on stress fiber induction than the Brk-1 siRNA. This finding indicates that endogenous Brk inhibits stress fiber formation. As actin cytoskeleton reorganization plays an important role in cell spreading, we investigated the influence of Brk on cell spreading. Using the HeLa cell system described above, we observed a significant inhibition of cell spreading by either Brk-1 or Brk-2 siRNA, but not by control siRNA (Fig. 1C). Consistent with the efficiencies in downregulating Brk, Brk-2 displayed a stronger inhibitory effect on spreading than Brk-1. These data demonstrated the function of Brk in regulating actin cytoskeleton and promoting cell spreading.

Brk interacts with p190 and phosphorylates p190 at Y1105

The inhibitory effect of Brk on actin stress fiber formation and stimulatory

effect on cell spreading resemble those of Rho inactivation (Burridge and Wennerberg 2004). In an attempt to characterize Brk-associated proteins, we noticed that a tyrosine phosphorylated protein with a molecular weight >172 kDa was coprecipitated with Brk from lysate of cells overexpressing Flag-Brk (Fig. 2). As the 190kDa RhoGAP protein (p190) is heavily tyrosine phosphorylated in cells overexpressing certain tyrosine kinases and this phosphorylation promotes its Rho inactivating function (Bernards and Settleman 2005), we investigated whether Brk could associate with p190 to promote its tyrosine phosphorylation. Immunoprecipitation analysis showed that p190 coprecipitated with Brk from lysates of HeLa cells overexpressing Flag-Brk (Fig. 4A). To demonstrate the association of endogenous Brk with endogenous p190, we generated a Brk-specific antibody that could immunoprecipitate endogenous Brk (Fig. 3). With lysate of T47D cells which express a high level of endogenous Brk (Ostrander, Daniel et al. 2007), we demonstrated that this anti-Brk antibody coprecipitated endogenous p190, whereas the anti-p190 antibody pulls down endogenous Brk (Fig. 4B). This specific interaction between Brk and p190 prompted us to investigate whether p190 is a substrate of Brk. To this end, HeLa cells were cotransfected with Brk and p190, and the level of p190 tyrosine phosphorylation was examined by immunoprecipitation with the p190 antibody followed by Western blotting with the phosphotyrosine antibody. This analysis revealed a marked induction of p190 tyrosine phosphorylation by Brk overexpression (Fig. 6). To determine which tyrosine residue is involved in this phosphorylation event, we mutated the Y1087 and/or Y1105 residues, as phosphorylation on these two residues is known to promote the association of p190 with p120, thereby increasing the Rho inactivating function of p190 (Bernards and Settleman 2005). Whereas

the Y1087 mutant was phosphorylated at a level similar to that of the wild type protein, the Y1105 and Y1087/1105 double mutant (DYF) were barely phosphorylated in cells overexpressing Brk (Fig. 6A). This result suggests Y1105 as the major phosphorylation site for Brk. To determine whether Brk could phosphorylate p190 in vitro, we expressed recombinant Brk in baculovirus and purified it to near homogeneity (Fig. 5). This purified Brk was used to phosphorylate full-length p190 or its mutants isolated from transfected cells by immunoprecipitation. This in vitro phosphorylation assay demonstrated a specific phosphorylation of p190 at Y1105 by Brk (Fig. 6B, upper panel). To rule out the possibility that this phosphorylation was resulted from a tyrosine kinase coprecipitated with p190, we used bacterially expressed p190(932-1143) fragment as the substrate. As bacteria do not contain tyrosine kinase, tyrosine phosphorylation on p190 should be attributed to Brk. Again, Brk was capable of phosphorylating this purified p190 fragment (Fig. 6B, lower panel). Furthermore, in both kinase assays, the Y1087 mutant was phosphorylated at the same extent as the wild type protein, whereas the Y1105 and DYF mutants were virtually refractory to be phosphorylated by Brk, thus confirming Y1105 as the major Brk-phosphorylation site.

Phosphorylation of p190 at Y1105 is known to promote its binding to p120 (Bernards and Settleman 2005). Accordingly, an elevated association of p190 with p120 was observed in cell overexpressing Brk, as revealed by reciprocal immunoprecipitation analyses (Fig. 7). Conversely, expression of BrkKM modestly reduced the complex formation between p190 and p120. Together, our results indicate that Brk can directly phosphorylate p190 at Y1105, thereby facilitating the binding of p190 to p120.

Brk stimulates the functions of p190 to inactivate Rho and to activate Ras

Phosphorylation of p190 at Y1105 is known to stimulate its Rho inactivating function (Bernards and Settleman 2005). To examine the influence of Brk on this function of p190, GST-rhotekin pull down assay was performed. While transfection of Brk or p190 alone led to a moderate or significant reduction of active Rho, respectively, cotransfection of Brk and p190 resulted in a synergistic inhibition of Rho activity (Fig. 8, left panel). The Y1105F mutant of p190 still possessed the Rho inactivation function, consistent with its carrying an intact GAP domain. However, when this mutant was coexpessed with Brk, no synergistic reduction of GTP-bound Rho was observed (Fig. 8, right panel). These data thus support that Brk stimulates the Rho inactivating function of p190 through phosphorylating p190 at Y1105.

Complex formation between p190 and p120 was reported to downregulate the RasGAP activity of p120 (Moran, Polakis et al. 1991), leading to an elevation of Ras activity. Consistently, GST-RBD (Ras binding domain of Raf) assay for GTP-bound Ras revealed that overexpression of p190 stimulated Ras activity, presumably via the formation of p190/p120 complex. Expression of Brk alone modestly enhanced Ras activity, which may be resulted from a functional interaction of Brk with endogenous p190. Importantly, coexpression of Brk and p190 led to a further stimulation of Ras activity (Fig. 9, left panel). In contrast to the wild type p190, the p190 Y1105F mutant neither induced Ras activation, nor could it cooperate with Brk to affect Ras activity (Fig. 9, right panel). These data suggest that Brk-induced p190 Y1105 phosphorylation

activates Ras by sequestrating p120.

Having demonstrated the ability of overexpressed Brk to cooperate with p190 for Ras activation and Rho inactivation, we next determined whether endogenous Brk could regulate Rho and Ras. To this end, we utilized the breast cancer cell line MCF7, which expresses a relatively high level of endogenous Brk (Ostrander, Daniel et al. 2007). The two Brk-specific siRNAs were stably introduced to MCF7 cells, and again the Brk-2 siRNA elicited a more efficient depletion of endogenous Brk than the Brk-1 siRNA (Fig. 10). This Brk silencing induced an elevation of Rho and reduction of Ras activities, and the extent of Ras inactivation and Rho activation correlated with the knockdown efficiency of these siRNAs (Fig. 10). Intriguingly, overexpression of p190 in Brk-silencing MCF7 cells not only abrogated Rho activation effect induced by Brk siRNA but also partially rescued Ras activity (Fig. 11). Perhaps the overexpressed p190 could be phosphorylated by other tyrosine kinases in this cell system. Alternatively, other signals might promote the association of overexpressed p190 with p120 through a p190 tyrosine phosphorylation-independent manner (Roof, Haskell et al. 1998). Regardless of the underlying mechanism, our data demonstrated the ability of endogenous Brk to oppositely regulate Rho and Ras and suggested a critical role of p190 in mediating these activities of Brk in breast cancer cells.

Brk mediates EGF-induced p190 tyrosine phosphorylation

Next, we investigated whether Brk could mediate p190 phosphorylation in response to a physiological stimulus. Our previous study revealed that Brk

kinase activity is activated by EGF signaling (Chen, Shen et al. 2004). Intriguingly, Src kinase can also induce p190 phosphorylation in EGF-stimulated cells (Chang, Gill et al. 1995; Roof, Haskell et al. 1998). We thus determined the possible involvement of Brk in EGF-induced p190 phosphorylation. In addition, the relative contribution of Brk and Src to this phosphorylation event was also investigated in human cancer cell lines containing high levels of Brk, such as the epidermoid carcinoma cell line A431 (Chen, Shen et al. 2004). We utilized Brk-specific and Src-specific siRNAs to downregulate Brk and Src in this cell line, respectively. As shown in Fig. 12A, the Brk-2 siRNA reduced Brk expression to ~20%, which was similar to the extent of Src downregulation achieved by Src siRNA. The Brk-1 again showed a weaker effect on Brk depletion than Brk-2. Importantly, neither Brk-1 nor Brk-2 did nonspecifically affect the expression of Src, whereas Src siRNA did not cause Brk downregulation. When the parental A431 cells were stimulated with EGF, a marked induction of p190 tyrosine phosphorylation was observed. However, this EGF-induced phosphorylation was attenuated in cells expressing either Brk siRNA or Src siRNA. Notably, even though Brk-2 and Src siRNA elicited a similar efficiency of downregulating their cognate kinase, Brk-2 induced a more profound effect on inhibiting EGF-triggered p190 tyrosine phosphorylation (Fig. 12B). To test whether Brk mediates EGF-induced p190 tyrosine phosphorylation in other cell systems, we utilized the breast carcinoma cell line T47D. Again, the efficiency of Brk-2 siRNA to reduce Brk level was comparable to that of Src siRNA to downregulate Src (Fig. 13A). Similar to what was observed in A431 cells, each of the Brk or Src siRNA could attenuate EGF-induced p190 tyrosine phosphorylation in T47D cells, and the inhibitory effect of Brk-2 siRNA on this phosphorylation was slightly

greater than that of Src siRNA (Fig. 13B). As Brk does not affect Src activity (Chen, Shen et al. 2004), the reduction of p190 phosphorylation by Brk silencing should not be attributed to Src inactivation. Thus, these data indicate that both Brk and Src are capable of mediating EGF-induced p190 tyrosine phosphorylation. Furthermore, in certain cancer cells that express high levels of Brk, Brk could play a similar or even greater role in this phosphorylation event, as compared with Src.

p190 is critical for the migratory and mitogenic effects of Brk

Studies described above have identified Brk as a bona fide kinase for p190 Y1105 residue in EGF-stimulated cells. Through this phosphorylation and subsequent complex formation between p190 and p120, Brk elicited opposite effects on two GTPase proteins, Ras and Rho. Then, the next important question was whether this newly identified signaling pathway contributes to any biological functions of Brk, such as cell migration and proliferation. To evaluate the contribution of p190 tyrosine phosphorylation to the migration-promoting function of Brk, we reconstituted the expression of wild type p190 or its phosphorylation-defective (Y1105F) mutant in the MEFs derived from p190 null mice by retrovirus-mediated gene transfer. Subsequently, the resulting MEFs were infected with retrovirus carrying Brk or control vector and infected cells were selected. Notably, the p190 WT and Y1105F (YF) mutant were expressed at a comparable level, and the three Brk-expressing MEFs contained similar amounts of Brk (Fig. 14A). When these six populations of MEFs were assayed for EGF-induced chemotactic migration, we found that expression of Brk led to a marked promotion of

chemotactic migration in MEFs carrying p190 WT (Fig. 14B). However, this migration-promoting function of Brk was completely abrogated in cells lacking p190 or expressing the p190 YF mutant. These results thus demonstrated an essential role of p190 Y1105 phosphorylation in Brk-induced migration.

Elevated Ras activity is well known to enhance cell proliferation. The finding that Brk promotes Ras activation via p190 phosphorylation prompted us to investigate the contribution of this phosphorylation event to Brk-induced proliferation. The six MEFs described above were taken for assaying their growth. Brk elicited a potent mitogenic effect in cells expressing p190 WT. However, in p190-deficient cells or cells carrying p190 YF mutant, Brk only modestly enhanced cell proliferation (Fig. 14C). Thus, p190 phosphorylation is a major pathway through which Brk promotes cell proliferation.

As overexpression of p190 reversed at least partially the effects of Brk siRNA on Rho and Ras regulations in MCF7 cells (see Fig. 11), we next investigated whether such overexpression could rescue the migration and proliferation defects induced by Brk silencing. Indeed, p190 overexpression greatly promoted migration (Fig. 15A) and proliferation (Fig. 15B) of MCF7 cells expressing Brk siRNA. These data support a critical role of p190 in the migratory and mitogenic functions of Brk in breast cancer cells.

Disruption of the p190/p120 complex blocks the tumor promoting activities of Brk in breast cancer cells.

Having demonstrated important roles of p190 Y1105 phosphorylation in Brk-induced proliferation and migration, we next investigated whether this phosphorylation event contributes to the tumor promoting activities of Brk. Previous studies revealed that a p120 construct containing the SH2-SH3-SH2 domain ($p120^{2-3-2}$) acts in a dominant-negative fashion to block p120 binding to p190, thereby eliminating the biological consequences of p190 tyrosine phosphorylation (Bradley, Hernandez et al. 2006). This construct was stably introduced to the breast caner cell line MDA-MB231, which expresses a low level of endogenous Brk (Chen, Shen et al. 2004) thus allowing the assessment of Brk tumor promoting function by overexpression strategy. As expected, expression of the $p120^{2-3-2}$ fragment in MDA-MB231 cells disrupted Brk-induced p190/p120 complex (Fig. 16A). Consequently, the Brk-induced RhoA inactivation and Ras activation were both inhibited by the expression of $p120^{2-3-2}$ fragment (Fig. 16B). Consistent with our previous study, Brk overexpression in MDA-MB231 cells promoted tumor cell migration (Fig. 17A) and invasion (Fig. 17A) towards EGF. Importantly, these effects of Brk were abrogated by the expression of $p120^{2-3-2}$ fragment. Brk overexpression also increased the proliferation of MDA-MB231 cells (Fig. 18A) and their ability to form colonies on soft agar (Fig. 18B), and again these effects were greatly reduced in cells expressing $p120^{2-3-2}$ fragment. Finally, we evaluated the function of $p120^{2-3-2}$ fragment on Brk-induced tumor growth in a xenograft model in which mice were injected subcutaneously with the MDA-MB231 derivatives. While overexpression of Brk accelerated tumor growth in animal,

expression of the p120$^{2\text{-}3\text{-}2}$ fragment completely reversed this effect of Brk (Fig. 19). Thus, disruption of the p190/p120 complex blocked the stimulatory effects of Brk on proliferation, migration, invasion, transformation and tumorigenicity in breast cancer cells, indicating that p190 phosphorylation is an important mechanism through which Brk promotes tumor formation and progression.

Discussion

In this study, we identify p190 as a substrate of Brk. Through phosphorylating p190 at Y1105, Brk promotes the complex formation between p190 and p120, thereby stimulating the RhoGAP activity of p190 and inhibiting the RasGAP activity of p120. Thus, this Brk-elicited signaling pathway leads to opposite regulations of two GTPases, Ras and Rho. We present several lines of evidence indicating that p190 tyrosine phosphorylation contribute significantly to various functions of Brk in tumor promotion. First, the mitogenic and migratory effects of Brk are greatly impaired in cells lacking p190 or expressing a phosphorylation-defective p190 mutant. Second, overexpression of p190 in breast cancer cells partially rescues the inhibitory effects of Brk siRNA on proliferation and migration. Finally, disruption of the p190/p120 complex in breast carcinoma cells with a dominant-negative p120 fragment significantly diminishes the stimulatory effects of Brk on proliferation, migration, invasion, transformation and tumor growth. Thus, our study uncovers p190 Y1105 phosphorylation and its downstream events as an important mechanism by which Brk promotes breast tumor formation and progression. Targeting of this pathway might develop a novel therapeutic approach for the management of breast cancer.

We show that Brk is capable of mediating p190 tyrosine phosphorylation under EGF-stimulated conditions. Three other tyrosine kinases, i.e., Src, Fyn and Arg, are known to phosphorylate p190 and to promote its interaction with p120. In certain circumstances, Brk and these kinases seem to regulate p190 phosphorylation at different tissue locations or physiological settings. For

instance, Src, Fyn, and Arg are responsible for p190 phosphorylation during the development of brain (Brouns, Matheson et al. 2001; Hernandez, Settleman et al. 2004), where Brk expression has not been reported. Additionally, Src and Arg are required for p190 phosphorylation in response to integrin signaling (Arthur, Petch et al. 2000), which, however, does not induce the catalytic activity of Brk (Chen, Shen et al. 2004). Nevertheless, under EGF-treated conditions, Src similarly promotes p190 phosphorylation (Chang, Gill et al. 1995; Roof, Haskell et al. 1998). Using siRNAs to downregulate Src or Brk, we demonstrated that these two kinases play redundant roles in EGF-stimulated p190 phosphorylation. Furthermore, in certain cancer cells that express high levels of Brk, Brk contributes equally or even more significantly to this phosphorylation, as compared with Src. As previous reports revealed a high correlation between Brk and ErbB2 overexpression in human breast tumors (Born, Quintanilla-Fend et al. 2005; Aubele, Auer et al. 2007), we postulate that this coordinated expression of Brk and ErbB2 would lead to a synergistic induction of p190 phosphorylation, thereby conferring the proliferative and migratory/invasive advantages on these tumors.

Our study indicates that p190 Y1105 phosphorylation is important for mediating Brk-induced cell proliferation, as this effect of Brk is greatly impaired in cells lacking p190 or expressing a phosphorylation-defective p190 mutant. Although phosphorylation of p190 can coordinate a crosstalk between Rho and Ras, we postulate that the growth-stimulating signal transduced by p190 phosphorylation is predominantly mediated by Ras activation rather than Rho inactivation. Accordingly, overexpression of the RhoGAP domain of p190 inhibits, rather than stimulates, Ras-induced transformation (Wang, Nur et al.

1997), whereas overexpression of a chimera made of p190 RhoGAP domain and the C-terminus of RhoA does not affect Ras-induced proliferation (Kusama, Mukai et al. 2006). Thus, p190 may possess complex and context-dependent effects on proliferation through its GAP domain-mediated Rho inactivation function and Y1105 phosphorylation-mediated Ras activation function. In line with this notion, reconstitution of p190 in p190 null MEFs does not significantly affect proliferation, whereas overexpression of p190 in MCF7 cells partially rescues the proliferation-inhibitory effect of Brk siRNA. Additionally, it is worth noting that Brk can still elicit a weak mitogenic effect in cells lacking p190, suggesting the involvement of other pathways in Brk-induced proliferation.

Our previous study revealed phosphorylation of paxillin as one mechanism through which Brk promotes migration and invasion (Chen, Shen et al. 2004). In this study, we show that phosphorylation of p190 similarly contributes to these effects of Brk. Intriguingly, the migration-promoting function of Brk is completely lost in p190 null MEF, even though Brk should still be capable of phosphorylating paxillin in such cell. Our finding suggests that phosphorylation of paxillin alone is insufficient to promote migration. This notion is conceivable in viewing that the highly dynamic migration process requires the integration of multiple signals and molecules in a spatially and temporally coordinated fashion. Of note, Brk and p190 act in synergism to induce not only RhoA inactivation but Rac1 activation (Fig. 20), consistent with an antagonistic crosstalk between RhoA and Rac1 (Burridge and Wennerberg 2004). Similarly, paxillin phosphorylation was reported to result in both Rac1 activation (Chen, Shen et al. 2004) and RhoA suppression (Tsubouchi, Sakakura et al. 2002) at

the leading edge of migrating cells, which are both required for the formation of membrane protrusions (Tsubouchi, Sakakura et al. 2002; Raftopoulou and Hall 2004). Consistently, inhibition of Brk blocks lamellipodia formation in response to the EGF migratory cue (Chen, Shen et al. 2004).

While we report a positive role of p190 phosphorylation in cell locomotion, a previous study found that overexpression of a RhoA-p190GAP domain fusion protein in a pancreatic cell line inhibits EGF-induced invasion (Kusama, Mukai et al. 2006). Similarly, Arg-induced p190 phosphorylation in fibroblasts reduces motility by decreasing RhoA-induced actomyosin contractility (Peacock, Miller et al. 2007). One possibility is that in our cell systems, actomyosin contractility can be generated by other compensating mechanisms, such as the Cdc42-MRCK pathway (Wilkinson, Paterson et al. 2005). Alternatively, the Brk-induced p190 phosphorylation may be spatially restricted to the membrane vicinity, and therefore does not significantly affect the contractility of cell body.

While this study reveals that p190 (p190-A) acts downstream of Brk to promote breast tumor proliferation and migration, a highly related p190-B was reported essential for mammary gland development by regulating ductal morphogenesis (Chakravarty, Hadsell et al. 2003). Interestingly, increased level of p190-B was found in a subset of mutagen-induced mammary tumors (Chakravarty, Roy et al. 2000), and inducible expression of p190-B in mammary gland during pregnancy results in hyperplastic lesions (Vargo-Gogola, Heckman et al. 2006). Although these findings suggest a potential role of deregulated p190-B in mammary malignancy, p190-B is

probably not a substrate of Brk as the sequences flanking the residue equivalent to Y1105 in p190-B are not conserved. Accordingly, p190-B is not tyrosine phosphorylated in Src-transformed fibroblasts, where p190-A is heavily phosphorylated (Matheson, Hu et al. 2006). Future study will determine whether p190-A is uniquely coupled to Brk signaling axis to promote breast malignancy.

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Figure

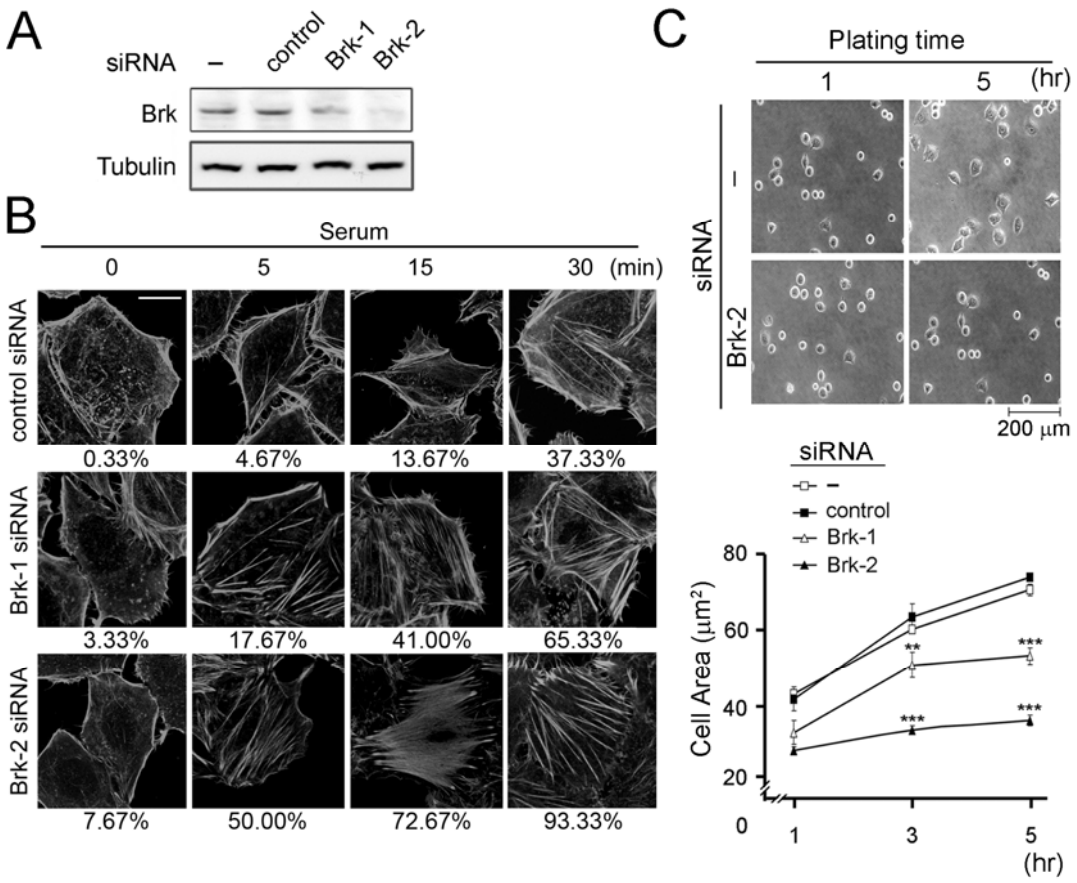

Fig. 1. Brk inhibits stress fiber formation and promotes cell spreading.

(A) HeLa cells were infected with lentivirus carrying various siRNAs as indicated and then selected with puromycin. Cells were lysed for Western blot with Brk or tubulin antibody as indicated. (B) HeLa cells expressing various siRNAs as indicated were serum-starved and then stimulated with serum for indicated time periods. Cells were fixed and stained with rhodamine-conjugated phalloidin, and then examined by confocal microscopy. The percentage of cells with stress fibers seen in each condition is indicated on the bottom. Bar, 20 μm. (C) Cells as in (A) were plated at a density of 1×10^5 cells/well in a 6-well plate, and representative

cell images at 1 and 5 hr are shown (left panel). Cell images at 1, 3 and 5 hr after plating were captured by phase contrast microcopy, and cell areas were analyzed by the Olympus Analysis LS Research software and plotted (lower panel). Data shown are means ± S.D. of 30 cells (**, $p<0.005$; ***; $p<0.0005$, as compared with parental HeLa cells).

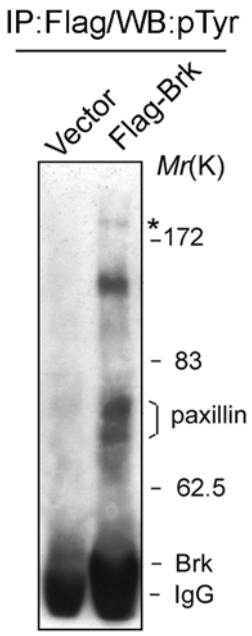

Fig. 2. Association of tyrosine phosphorylated proteins with Brk.

293T cells transfected with Flag-Brk were lysed for immunoprecipitation with M2 agarose beads. The bound proteins were analyzed by Western blot with the anti-phosphotyrosine antibody. The positions of Brk and paxillin are indicated, whereas asterisk marks the protein band larger than 172 kDa.

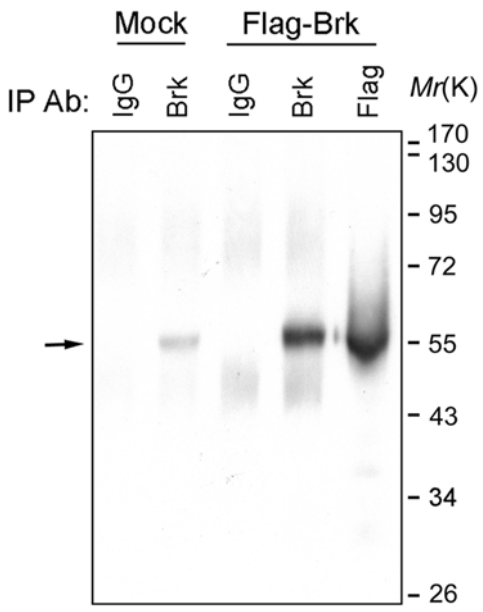

Fig. 3. Characterization of the anti-Brk antibody by immunoprecipitation analysis.

T47D cells mock transfected or transfected with Flag-Brk were lysed for immunoprecipitation analysis with anti-Brk antibody, M2 agarose beads (Flag), or a control antibody (IgG). The immunoprecipitates were analyzed by Western blot with anti-Brk antibody. Arrow marks the position of Brk.

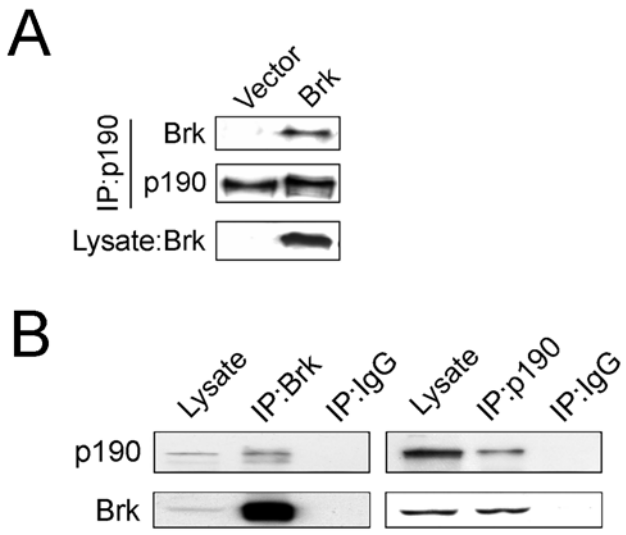

Fig. 4. Brk interacts with p190.

(A) HeLa cells transfected with control vector or Flag-Brk were lysed for immunoprecipitation with anti-p190. The immunoprecipitates and cell lysate were analyzed by Western blot with anti-p190 or anti-Brk as indicated. (B) Brk interacts with p190. T47D cells were lysed for immunoprecipitation with anti-p190, anti-Brk or a control antibody (IgG). Cell lysate and immunoprecipitates were analyzed by Western blot with antibodies as indicated.

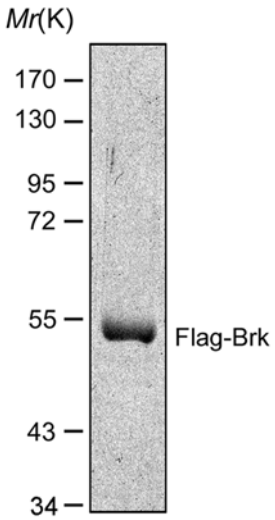

Fig. 5. Purity of recombinant Brk protein.

Flag-Brk immunoprecipitated from lysate of baculovirus by M2 agarose beads and then eluted by Flag peptide was run on SDS-PAGE and stained with Coomassie blue.

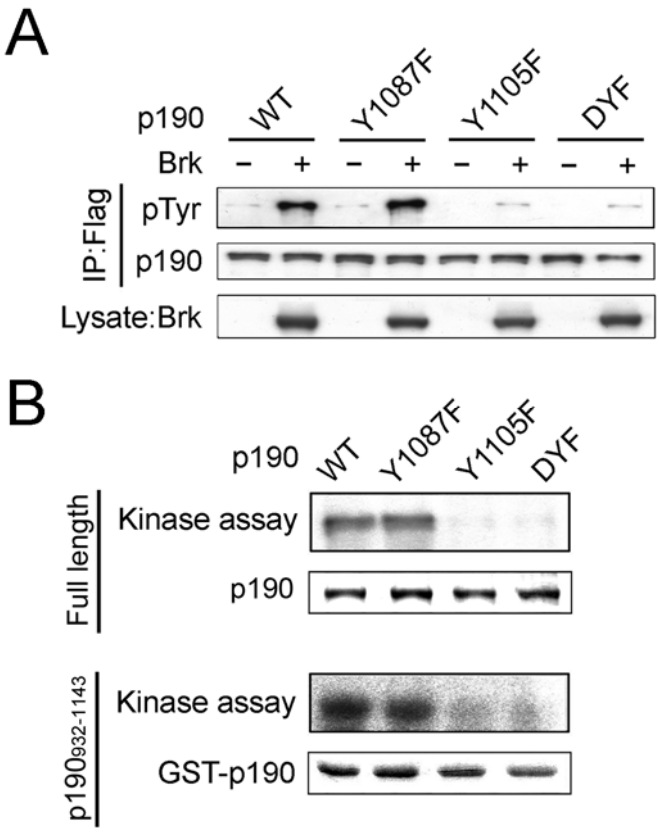

Fig. 6. Brk phosphorylates p190 *in vivo* and *in vitro*.

(A) Brk promotes p190 phosphorylation *in vivo*. 293T cells were transfected with Brk and/or various forms of Flag-p190 as indicated. Cells were lysed for immunoprecipitation with anti-Flag. The immunoprecipitates and cell lysates were analyzed by Western blot with anti-p190, anti-phosphotyrosine (pTyr) or anti-Brk antibody. (B) Brk phosphorylates p190 in *vitro*. Various forms of full-length Flag-p190 immunoprecipitated from transfected cells (upper panel) or GST-p190 (932-1143) fragment purified from E. coli (lower panel) were incubated with baculovirally purified Brk in an *in vitro* kinase reaction. The reaction products were analyzed by autoradiography to detect the phosphorylation of p190 (kinase assay), or by Western blotting with anti-p190 or anti-GST antibody.

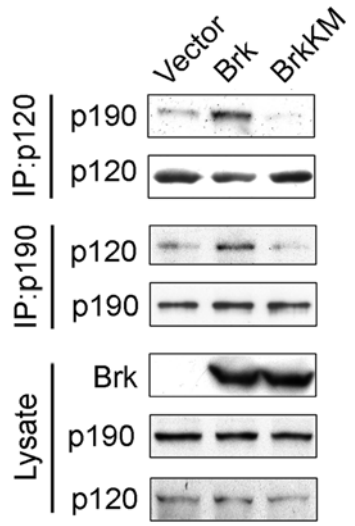

Fig. 7. Brk promotes the interaction of p190 with p120.

HeLa cells transfected with Brk or BrkKM were lysed for immunoprecipitation with anti-p120 or anti-p190. The immunoprecipitates and cell lysates were analyzed by Western blot with anti-p190, anti-p120 or anti-Brk as indicated.

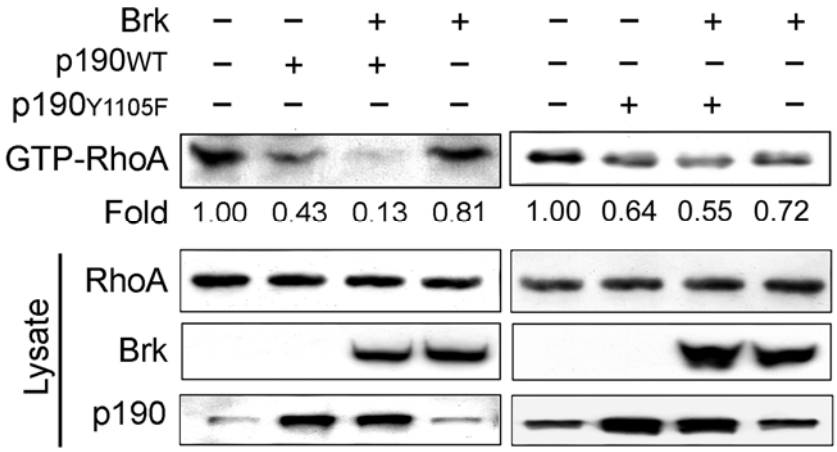

Fig. 8. Brk synergizes with p190 to promote Rho inactivation.

HeLa cells were transfected with Brk and/or various forms of p190. Cells were lysed for assaying GTP-bound RhoA by the GST-rhotekin beads, or for Western blot with antibodies as indicated. The amounts of GTP-bound RhoA were normalized by using those of total RhoA in cell lysates and are expressed as the fold of induction relative to cells transfected with the control vector.

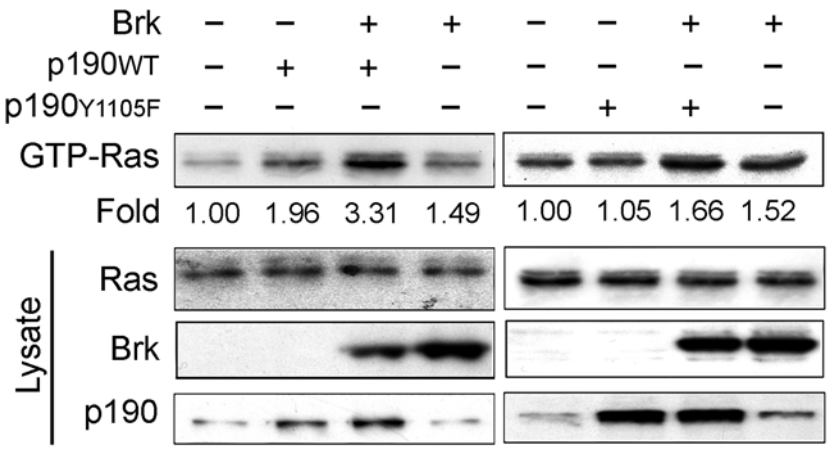

Fig. 9. Brk synergizes with p190 to promote Ras activation.

HeLa cells were transfected with Brk and/or various forms of p190. Cells were lysed for assaying GTP-bound Ras by Raf-1 RBD (Ras binding domain) agarose, or for Western blot with antibodies as indicated. The amounts of GTP-bound Ras were normalized by using those of total Ras in cell lysates and are expressed as the fold of induction relative to cells transfected with the control vector.

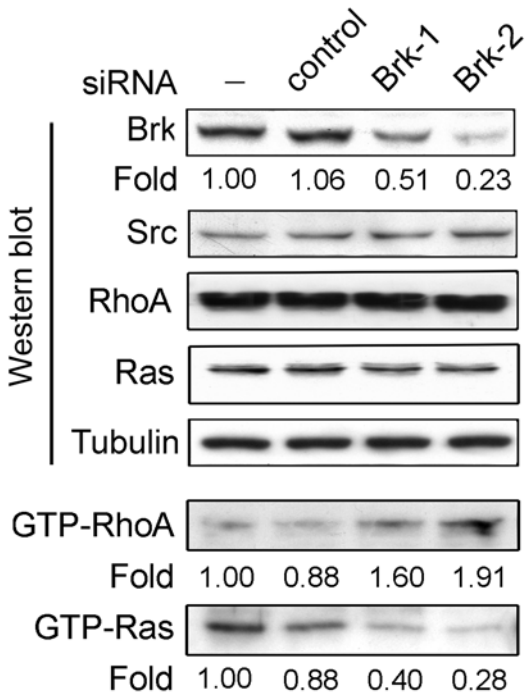

Fig. 10. Brk silencing induces an activation of Rho and an inhibition of Ras.

MCF7 cells stably expressing various siRNAs were lysed for Western blot with various antibodies, or assayed for GTP-bound Rho and GTP-bound Ras by GST-rhotekin and Raf-1 RBD agarose pull down assays, respectively. The intensities of Brk signal in siRNA expressing cells relative to that in control cells are indicated, and the relative amounts of GTP-bound RhoA and GTP-bound Ras were normalized by using those of total RhoA or Ras in cell lysates, respectively, and are expressed as the fold of induction relative to cells transfected with the control vector.

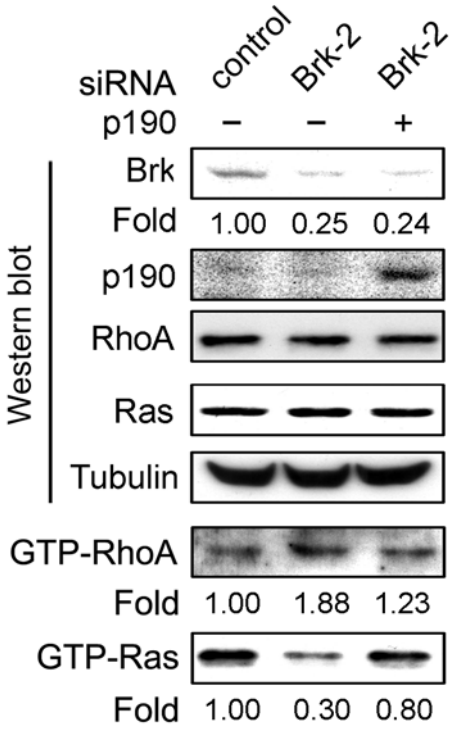

Fig. 11. p190 overexpression rescues the effects of Brk silencing on Rho and Ras regulation.

MCF7 cells stably expressing Brk-2 siRNA were transfected with p190. Cells were lysed for Western blot with various antibodies or assayed for GTP-bound RhoA and GTP-bound Ras by GST-rhotekin and Raf-1 RBD agarose pull down assays, respectively. The intensities of Brk signal in siRNA expressing cells relative to that in control cells are indicated, and the relative amounts of GTP-bound RhoA and GTP-bound Ras were normalized by using those of total RhoA or Ras in cell lysates, respectively, and are expressed as the fold of induction relative to cells transfected with the control vector.

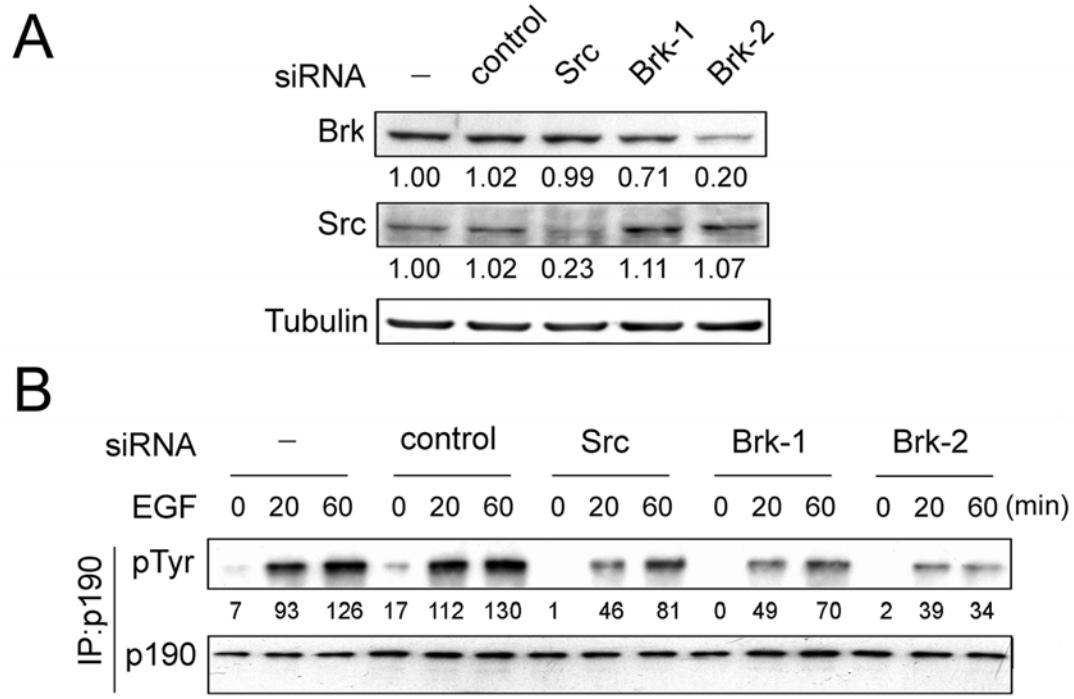

Fig. 12. Brk mediates EGF-induced p190 phosphorylation in human epidermoid carcinoma cell line A431.

(A) A431 cells were infected with lentivirus carrying control siRNA, Src siRNA or Brk siRNAs as indicated. Infected cells were selected and then lysed for Western blot with antibodies as indicated. The numbers below blot images indicate the relative intensity of Brk or Src signal. (B) Cells expressing various siRNAs were serum-starved and then stimulated with 15 ng/ml EGF for indicated time points. Cells were lysed for immunoprecipitation with anti-p190, followed by Western blot with anti-p190 or anti-phosphotyrosine (pTyr). The intensity of pTyr signal relative to that of p190 signal is indicated

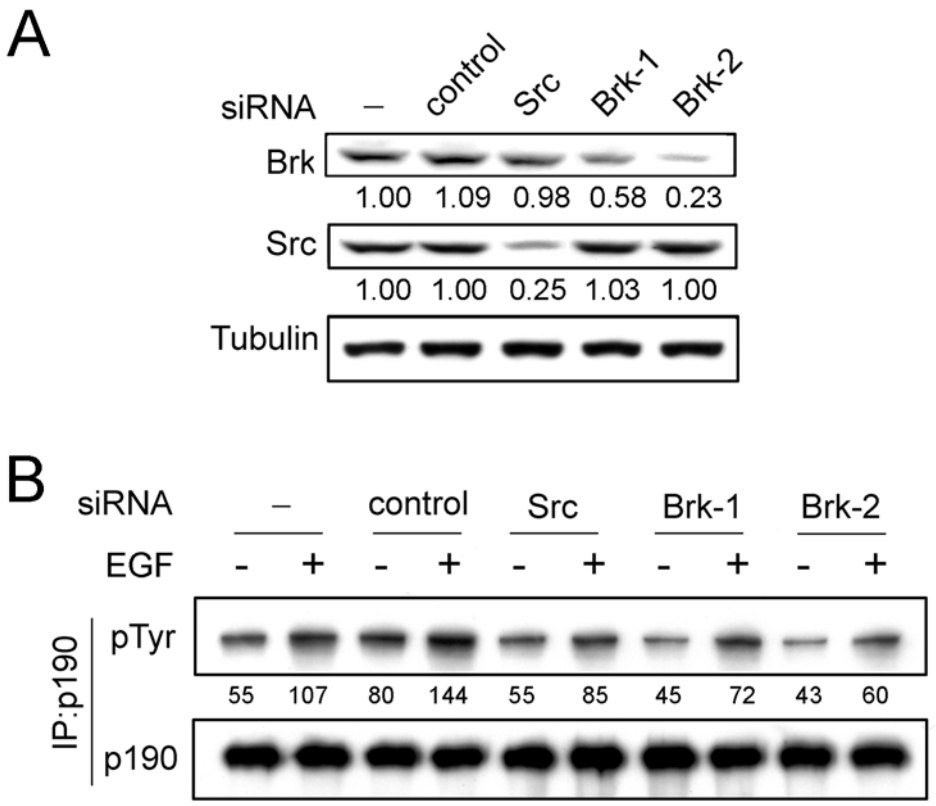

Fig. 13. Brk mediates EGF-induced p190 phosphorylation in breast carcinoma cell line T47D.

(A) T47D cells were infected with lentivirus carrying control siRNA, Src siRNA or Brk siRNAs as indicated. Infected cells were selected and then lysed for Western blot with antibodies as indicated. The numbers below blot images indicate the relative intensity of Brk or Src signal. (B) Cells expressing various siRNAs were serum-starved and then stimulated with 50 ng/ml EGF for 20 min. Cells were lysed for immunoprecipitation with anti-p190, followed by Western blot with anti-p190 or anti-phosphotyrosine (pTyr). The intensity of pTyr signal relative to that of p190 signal is indicated

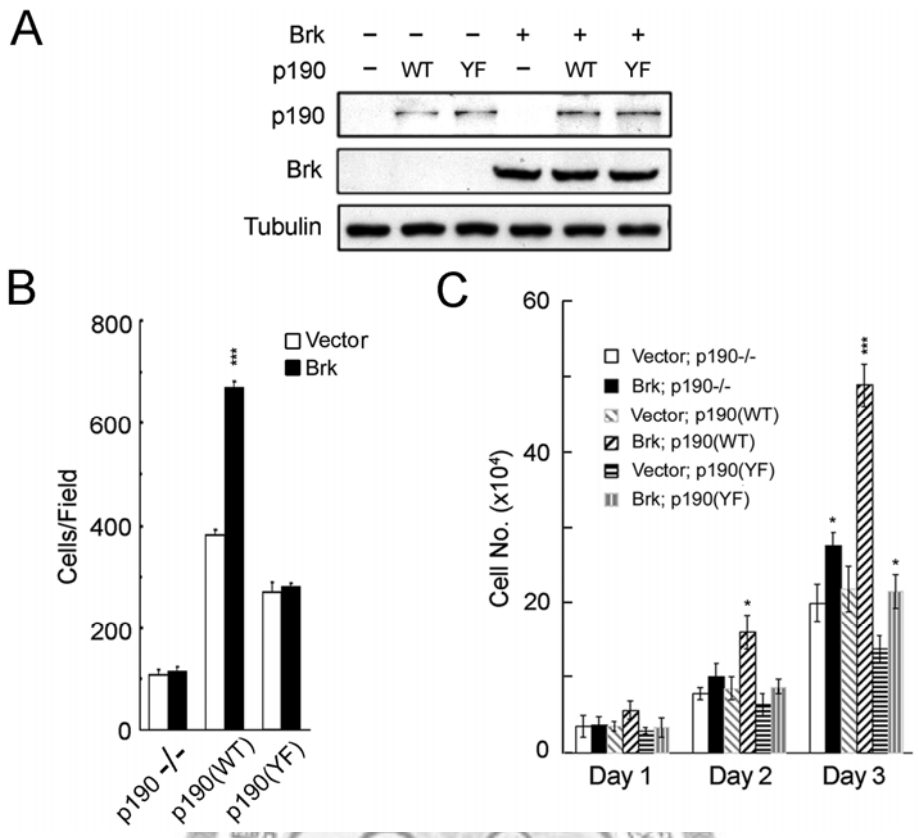

Fig. 14. The proliferative and migratory effects of Brk are mediated by p190 phosphorylation

(A) Stably expression of Brk and/or p190 wild type or mutant in p190 null MEFs. p190-/- MEFs were infected with retrovirus expressing p190 or its Y1105F mutant (YF) and then selected with hygromycin. The resulting three cell lines were infected with retrovirus carrying Brk or control vector and then selected with puromycin. Cells were lysed for Western blot with antibodies as indicated. (B) p190 null MEFs stably expressing Brk and/or p190 wild type or mutant were serum-starved for 6 hr and then plated onto Transwell chambers for assaying EGF-induced migration. (C) Cells were plated at a density of 5000 cells/well in 12-well plates, and then incubated in culture medium. Cell numbers at indicated days after plating were counted and plotted. Data shown are means ± S.D. (*$p<0.05$; *** $p<0.0005$, as compared with cells carrying control vector; $n \geqq 4$).

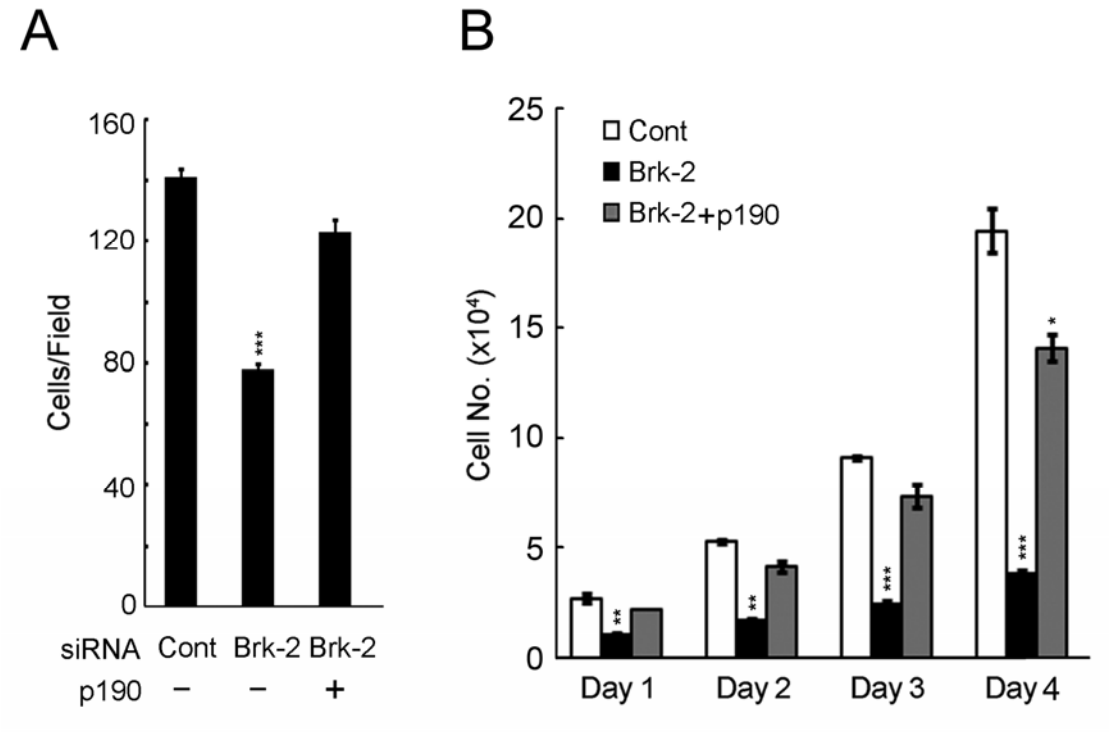

Fig. 15. p190 overexpression partially rescues the migration and proliferation defects induced by Brk silencing.

MCF7 cells stably expressing Brk-2 siRNA as in Fig. 11 were infected with lentivirus expressing p190 or a control vector (-). (A) The infected cells were serum-starved for 6 hr and then plated onto Transwell chambers for assaying EGF-induced migration. (B) Cells were plated at a density of 5000 cells/well in 12-well plates, and then incubated in culture medium. Cell numbers at indicated days after plating were counted and plotted. Data shown are means ± S.D. (*$p<0.05$; *** $p<0.0005$, as compared with cells carrying control vector; $n \geqq 4$).

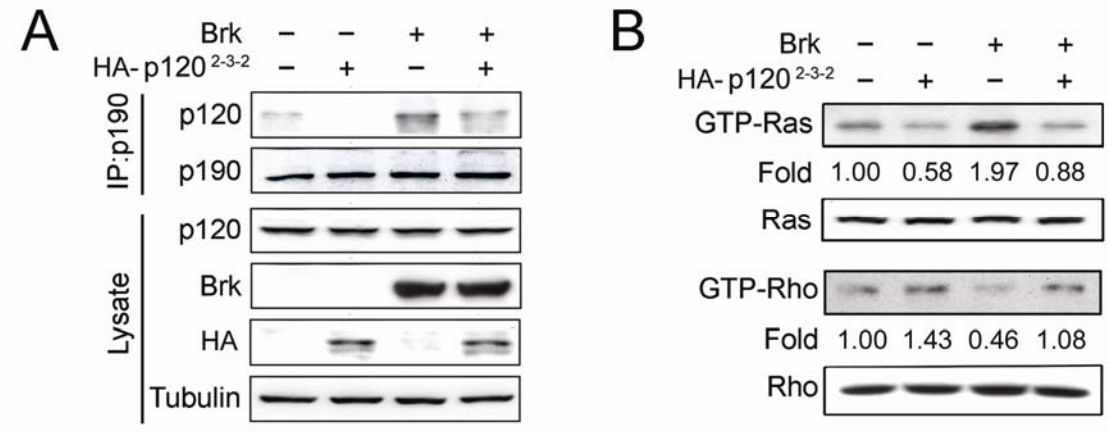

Fig. 16. Disruption of p190/p120 complex blocks Brk-induced RhoA inactivation and Ras activation.

(A) MDA-MB231 cells stably expressing Brk and/or $p120^{2-3-3}$ were lysed for immunoprecipitation with anti-p190. The immunoprecipitates and cell lysates were analyzed by Western blot with antibodies as indicated. (B) Cells were assayed for GTP-bound Rho and GST-bound Ras by GST-rhotekin and Raf-1 RBD agarose pull down assays, respectively. The relative amounts of GTP-bound RhoA and GTP-bound Ras were normalized by using those of total RhoA or Ras in cell lysates, respectively, and are expressed as the fold of induction relative to mock cells.

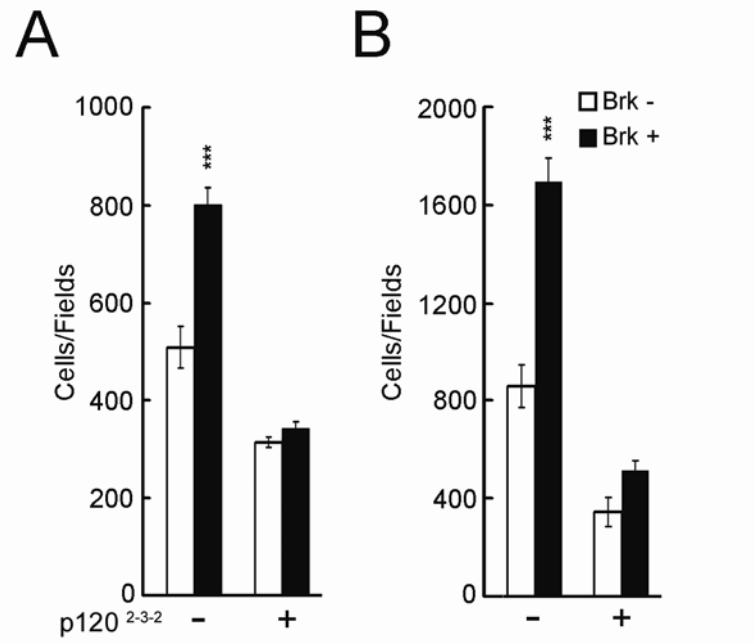

Fig. 17. Disruption of p190/p120 complex blocks Brk-induced cell migration and invasion.

(A) MDA-MB231 cells stably expressing Brk and/or $p120^{2\text{-}3\text{-}3}$ as in Fig.16 were serum-starved for 6 hr and then plated onto Transwell chambers for assaying EGF-induced migration. (B) Cells were plated at a density of 5000 cells/well in 12-well plates, and then incubated in culture medium. Cell numbers at indicated days after plating were counted and plotted. Data shown are means ± S.D. (*$p<0.05$; *** $p<0.0005$, as compared with cells carrying control vector; $n \geqq 4$).

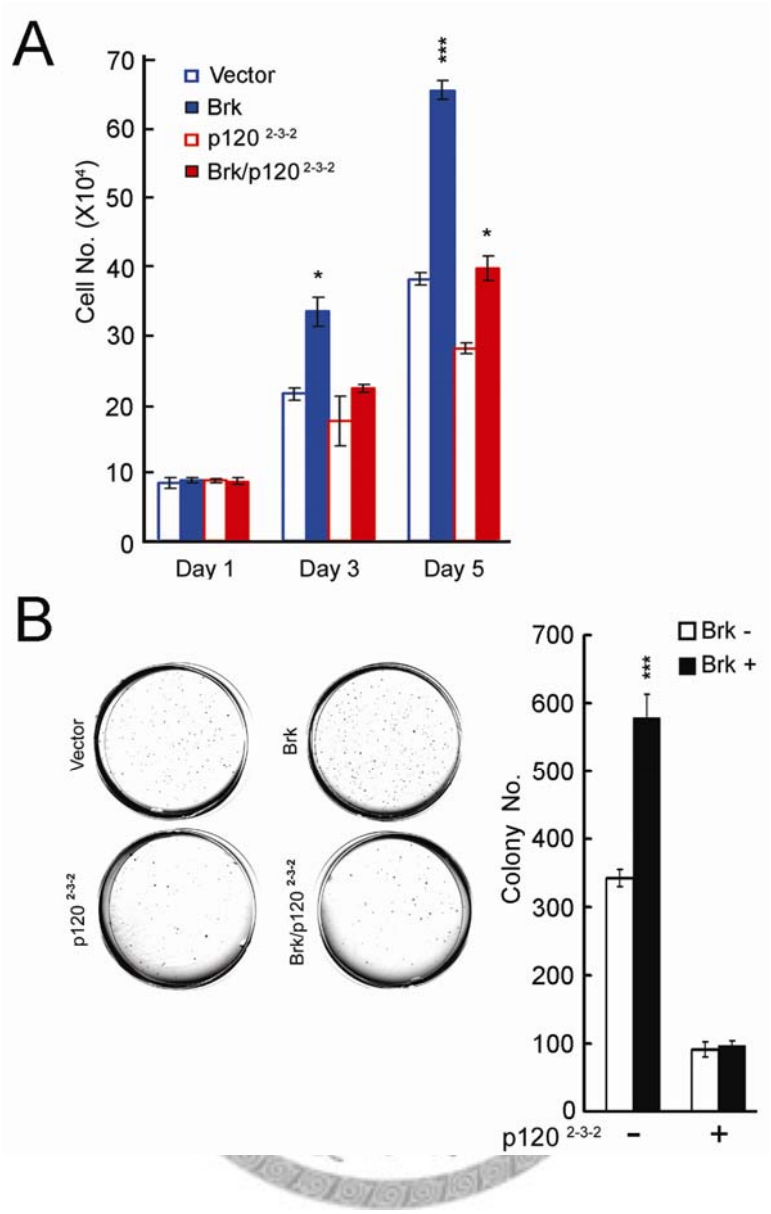

Fig. 18. Disruption of p190/p120 complex blocks Brk-induced cell proliferation and foci formation ability.

(A) MDA-MB231 cells stably expressing Brk and/or p120$^{2\text{-}3\text{-}3}$ as in Fig. 16 were plated at a density of 5000 cells/well in 12-well plates, and then incubated in culture medium. Cell numbers at indicated days after plating were counted and plotted. (B) Cells were diluted in 0.3% of top agar and spread onto 60-mm plates containing 0.5% of bottom agar. After 4 weeks, colonies were stained and counted. Data shown are means ± S.D. (*$p<0.05$; *** $p<0.0005$, as compared with cells carring control vector; $n \geqq 4$).

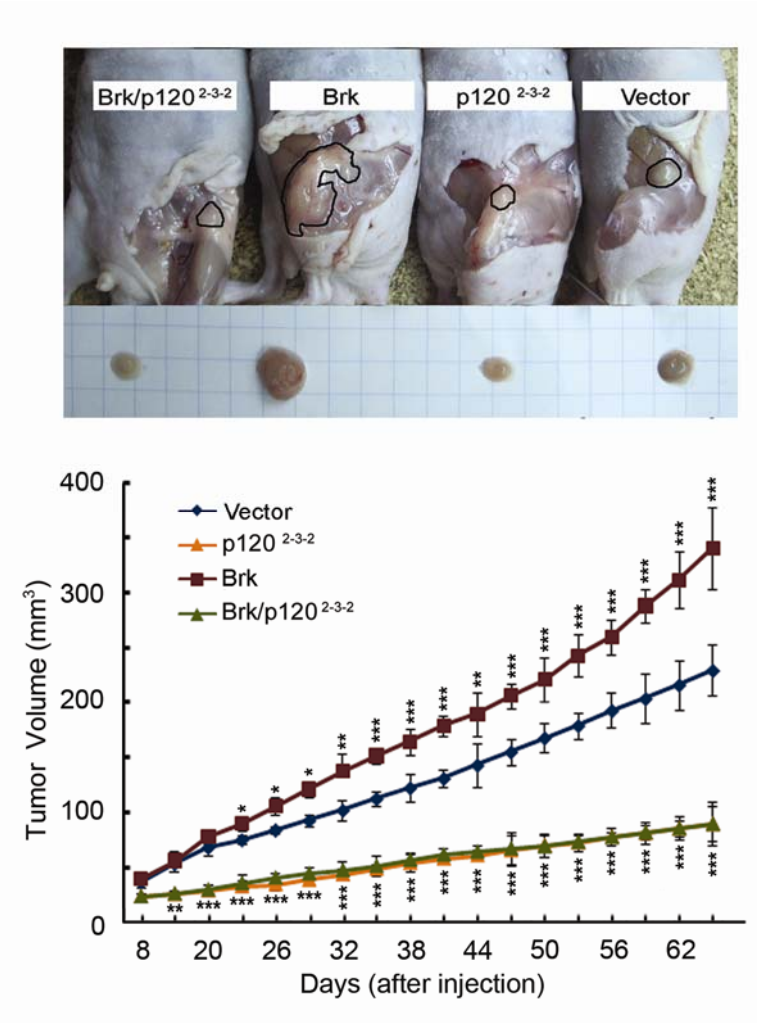

Fig.19. Disruption of p190/p120 complex blocks Brk-induced tumor growth.

MDA-MB231 cells stably expressing Brk and/or $p120^{2\text{-}3\text{-}3}$ as in Fig.11 were mixed with phosphate-buffered saline and Matrigel and then subcutaneously injected into mice. Tumors were measured with calipers, and tumor volumes were calculated using the equation: $width^2 \times length \times 0.5$. Data shown are means ± S.D. (*p<0.05; ***p<0.0005, as compared with cells carring control vector; n≧4).

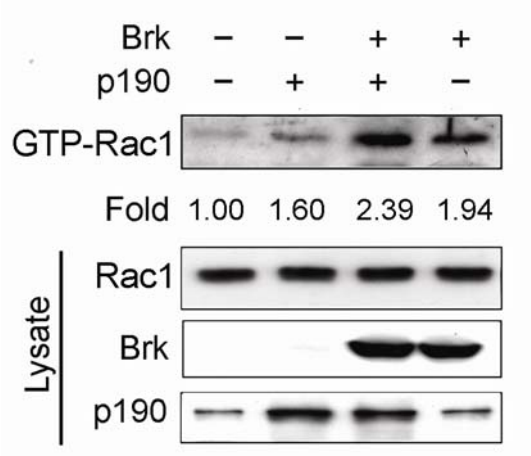

Fig. 20. Synergistic activation of Rac1 by Brk and p190.

HeLa cells were transfected with Brk and/or p190. Cells were lysed for assaying GTP-bound Rac1 by GST-PAK-CRIB pull-down assay, or for Western blot with antibodies as indicated. The amount of GTP-bound Rac was normalized by using that of total Rac in cell lysates, and is expressed as the fold of induction relative to cells transfected with the control vector.