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

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Abstract

Breast tumor kinase (Brk), an 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 Y^{1105} 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 epidermal growth factor–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. [Cancer Res 2008;68(19):7779–87]

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. Breast tumor kinase (Brk) is an intracellular tyrosine kinase and possesses SH3, SH2, and kinase domains in a similar arrangement to that of Src (1). However, Brk lacks an NH_2 terminal myristoylation signal (1) and its genomic sequence is distinct from Src family kinases (2). Brk was identified from a human metastatic breast tumor (1), and subsequent analysis revealed Brk overexpression in about two thirds of primary breast tumors, with the highest level in advanced tumors (3–5). This Brk overexpression positively correlates with the expression of HER family receptors (4, 6). In normal tissues, the expression of Brk is restricted to differential epithelial cells of skin and gastrointestinal tract (7). In addition to breast tumors, elevated expression of Brk has also been detected in metastatic melanoma (8), colon tumors (7), T-cell lymphoma (9), and serous carcinoma of ovary (10). In prostate cancers, although the expression of Brk is not significantly elevated, Brk translocates from the nucleus to cytoplasm during the progression of tumors (11).

Consistent with its potential role in promoting tumorigenesis, Brk stimulates the proliferation of breast tumor cells and mediates epidermal growth factor (EGF)–induced mitogenic and migratory effects (5, 12, 13). The signaling mechanisms underlying the mitogenic function of Brk, however, have not been completely unraveled. Brk associates with EGF receptor after receptor activation and enhances EGF-induced ErbB3 phosphorylation, which subsequently leads to an increased recruitment of phosphatidylinositol 3-kinase and activation of Akt (14). Additionally, Brk phosphorylates the growth inhibitory protein Sam68 and triggers its nuclear export (15). 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 phosphorylation of paxillin, which leads to the activation of Rac1 via the adaptor protein CrkII (13). Recently, Brk was shown to mediate EGF-induced and HRG-induced activation of p38 mitogen-activated protein kinase (MAPK), which contributes in part to the proliferation and migration of breast cancer cells in response to these growth factors (5). It is unclear how Brk triggers p38 MAPK 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 (16) and was identified as a tyrosine-phosphorylated protein associated with p120RasGAP (p120) in v-Src transformed cells (17). The association of p190 with p120 is promoted by phosphorylation of p190 at Y^{1105} (18). Additionally, phosphorylation at Y^{1087} in p190 stabilizes its interaction with p120 (19). 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* (20). In contrast to this activation of RhoGAP, the RasGAP activity of p120 is reduced when associated with p190 (21). Thus, association of these two GTPase-activating proteins (GAP) facilitates a cross-talk between Rho and Ras. The two tyrosine

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kinases Src and Abl-related gene (Arg) have been reported to directly phosphorylate p190 at Y^{1105} (18, 22). Src promotes p190 phosphorylation in EGF-stimulated fibroblasts, thereby facilitating actin stress fiber disassembly (23). Intriguingly, both Src and Arg are required for integrin-dependent phosphorylation and activation of p190 in fibroblasts (24). 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 (25), which are all essential steps in cell locomotion, Arg acts through p190 to decrease cell contractility, thereby reducing motility (26). 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 Y^{1105} 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 Y^{1105} 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 DMEM supplemented with 10% FCS. Mouse embryonic fibroblasts (MEF) were cultured in DMEM with 15% FCS. T47D cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids (NEAA), and 5 μg/mL insulin. MDA-MB231 and MCF7 cells were maintained in DMEM-F12 with 10% FCS, 2 mmol/L L-glutamine, and 0.1 mmol/L NEAA. Transfection was performed using Lipofectamine 200 reagent or by calcium phosphate method. Recombinant retroviruses were generated according to procedures described previously (27).

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 932–1143) 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-specific or Src-specific small interfering RNA (siRNA; from National RNAi Core Facility) was used to knockdown Brk or Src, respectively. The target sequences of various siRNAs are Src GCGGCTCCCAGATTGTCAACAA, Brk-1 AGTCGCAGAATTACATC-CACC, and Brk-2 TACCTCTCCCATGACCACAAT. To generate recombinant lentivirus, 293FT cells were cotransfected 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, glutathione *S*-transferase (GST)–Brk SH3 domain was purified from *Escherichia 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 Ras binding domain (RBD) agarose (Upstate Biotechnology) and GST-rhotekin pull-down assays, respectively. Briefly, cells were lysed with buffer containing 25 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 10 mmol/L $MgCl_2$, 10% glycerol, 1 mmol/L EDTA, 25 mmol/L NaF, 1 mmol/L Na_3VO_4, 2 mmol/L phenylmethylsulfonyl fluoride, 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 (13).

Migration and invasion assays. Transwell migration and invasion assays using EGF as the chemoattractant were performed as described (13). 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 Hoechst 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 (13).

***In vitro* kinase assay.** One microgram of Brk purified from baculovirus expression system (13) was incubated at 37°C for 10 min in 30 μL of kinase buffer containing 50 mmol/L HEPES (pH 7.5), 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 1 mmol/L DTT, 10 μmol/L 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 mmol/L NaCl, 300 mmol/L sucrose, 10 mmol/L PIPES (pH 6.8), 3 mmol/L $MgCl_2$, and 0.5% Triton X-100 for 5 min. Cells were blocked with PBS supplemented with 10% goat serum, 1% bovine serum albumin (BSA) and 50 mmol/L NH_4Cl, and then incubated with 0.1 mmol/L 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 wk were stained and then counted by the ImageJ software.

Tumorigenesis in mice. Four-week-old BALB/c nude mice (n = 40; National Laboratory Animal Center) were acclimated for 1 to 2 wk. The mice were housed in specific pathogen-free conditions and injected s.c. 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 filamentous 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 (Supplementary Fig. S1*A*). 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. 1*A*). 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

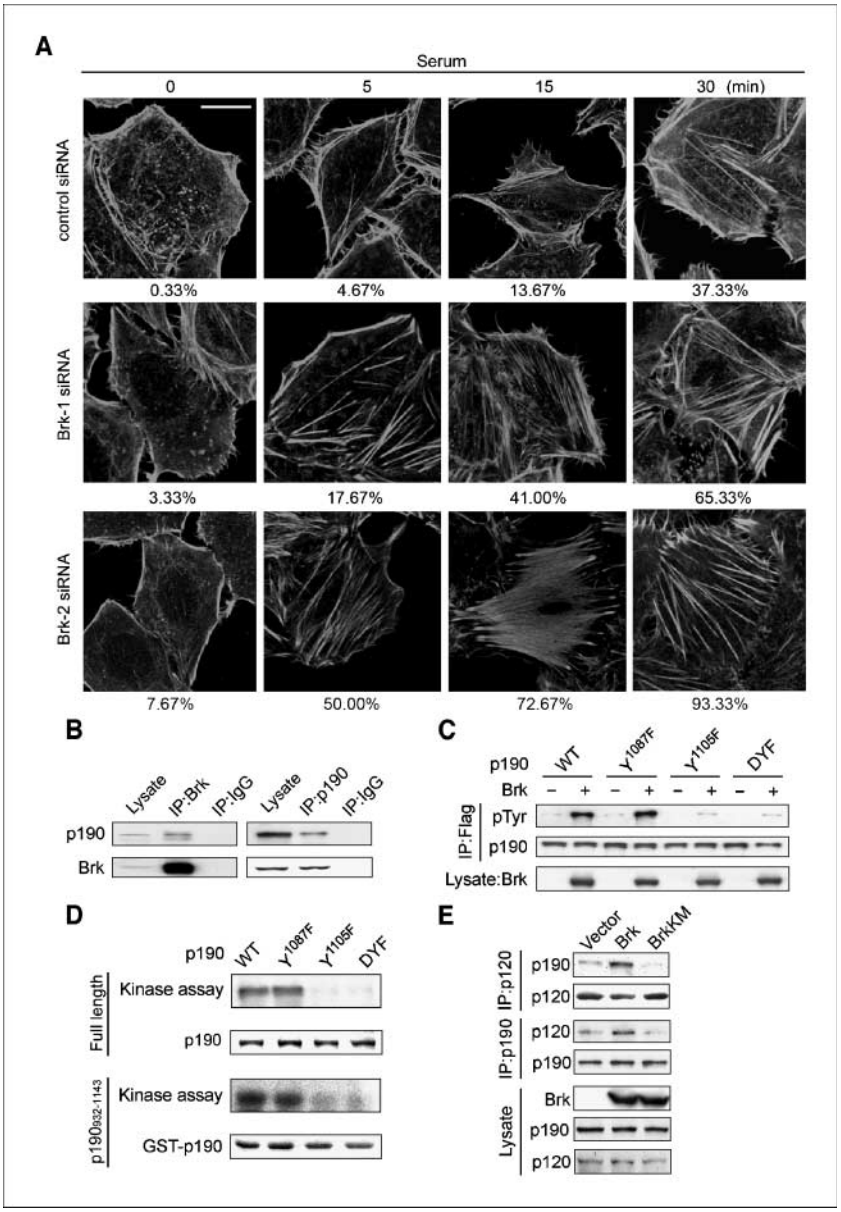

Figure 1. Brk regulates actin cytoskeletons and phosphorylates p190. *A,* HeLa cells expressing various siRNAs, as indicated, were serum-starved and then stimulated with serum for indicated time periods. Cells were fixed and stained with rhodamine-conjugated phalloidin and then examined by confocal microscopy. The percentage of cells with stress fibers seen in each condition is indicated on the bottom. *Bar,* 20 μm. *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. *C,* 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. *D,* Brk phosphorylates p190 *in vitro*. Various forms of full-length Flag-p190 immunoprecipitated from transfected cells or GST-p190 (932–1143) fragment purified from *E. coli* 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. *E,* 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.

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 (Supplementary Fig. S1*B*). Consistent with the efficiencies in down-regulating Brk, Brk-2 displayed a stronger inhibitory effect on spreading than Brk-1. These data showed the function of Brk in regulating actin cytoskeleton and promoting cell spreading.

Brk interacts with p190 and phosphorylates p190 at Y^{1105}. The inhibitory effect of Brk on actin stress fiber formation and stimulatory effect on cell spreading resemble those of Rho inactivation (28). In an attempt to characterize Brk-associated proteins, we noticed that a tyrosine phosphorylated protein with a molecular weight of >172 kDa was coprecipitated with Brk from lysate of cells overexpressing Flag-Brk (Supplementary Fig. S2). As the 190-kDa RhoGAP protein (p190) is heavily tyrosine phosphorylated in cells overexpressing certain tyrosine kinases and this phosphorylation promotes its Rho inactivating function (16), 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 (Supplementary Fig. S3). To show the association of endogenous Brk with endogenous p190, we generated a Brk-specific antibody that could immunoprecipitate endogenous Brk (Supplementary Fig. S4). With lysate of T47D cells, which express a high level of endogenous Brk (5), we showed that this anti-Brk antibody coprecipitated endogenous p190, whereas the anti-p190 antibody pulls down endogenous Brk (Fig. 1*B*). 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. 1*C*). To determine which tyrosine residue is involved in this

phosphorylation event, we mutated the Y^{1087} and/or Y^{1105} 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 (16). Whereas the Y^{1087} mutant was phosphorylated at a level similar to that of the wild-type protein, the Y^{1105} and $Y^{1087/1105}$ double mutant (DYF) were barely phosphorylated in cells overexpressing Brk (Fig. 1*C*). This result suggests Y^{1105} 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 (Supplementary Fig. S5). This purified Brk was used to phosphorylate full-length p190 or its mutants isolated from transfected cells by immunoprecipitation. This *in vitro* phosphorylation assay showed a specific phosphorylation of p190 at Y^{1105} by Brk (Fig. 1*D, top*). To rule out the possibility that this phosphorylation 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. 1*D, bottom*). Furthermore, in both kinase assays, the Y^{1087} mutant was phosphorylated at the same extent as the wild-type protein, whereas the Y^{1105} and DYF mutants were virtually refractory to be phosphorylated by Brk, thus confirming Y^{1105} as the major Brk phosphorylation site. Phosphorylation of p190 at Y^{1105} is known to promote its binding to p120 (16). Accordingly, an elevated association of p190 with p120 was observed in cell overexpressing Brk, as revealed by reciprocal immunoprecipitation analyses (Fig. 1*E*). Conversely, expression of BrkKM modestly reduced the complex formation between p190 and p120. Together, our results indicate that Brk can directly phosphorylate p190 at Y^{1105}, thereby facilitating the binding of p190 to p120.

Brk stimulates the functions of p190 to inactivate Rho and to activate Ras. Phosphorylation of 190 at Y^{1105} is known to stimulate its Rho-inactivating function (16). To examine the influence of Brk on this function of p190, GST-rhotekin pull-down assay was performed. Whereas 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. 2*A*). The Y^{1105}F 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. 2*A*). These data thus support that Brk stimulates the Rho inactivating function of p190 through phosphorylating p190 at Y^{1105}.

Complex formation between p190 and p120 was reported to down-regulate the RasGAP activity of p120 (21), leading to an elevation of Ras activity. Consistently, GST-RBD 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. 2*B*). In contrast to the wild-type p190, the p190 Y^{1105}F mutant neither induced Ras activation nor could it cooperate with Brk to affect Ras activity (Fig. 2*B*). These

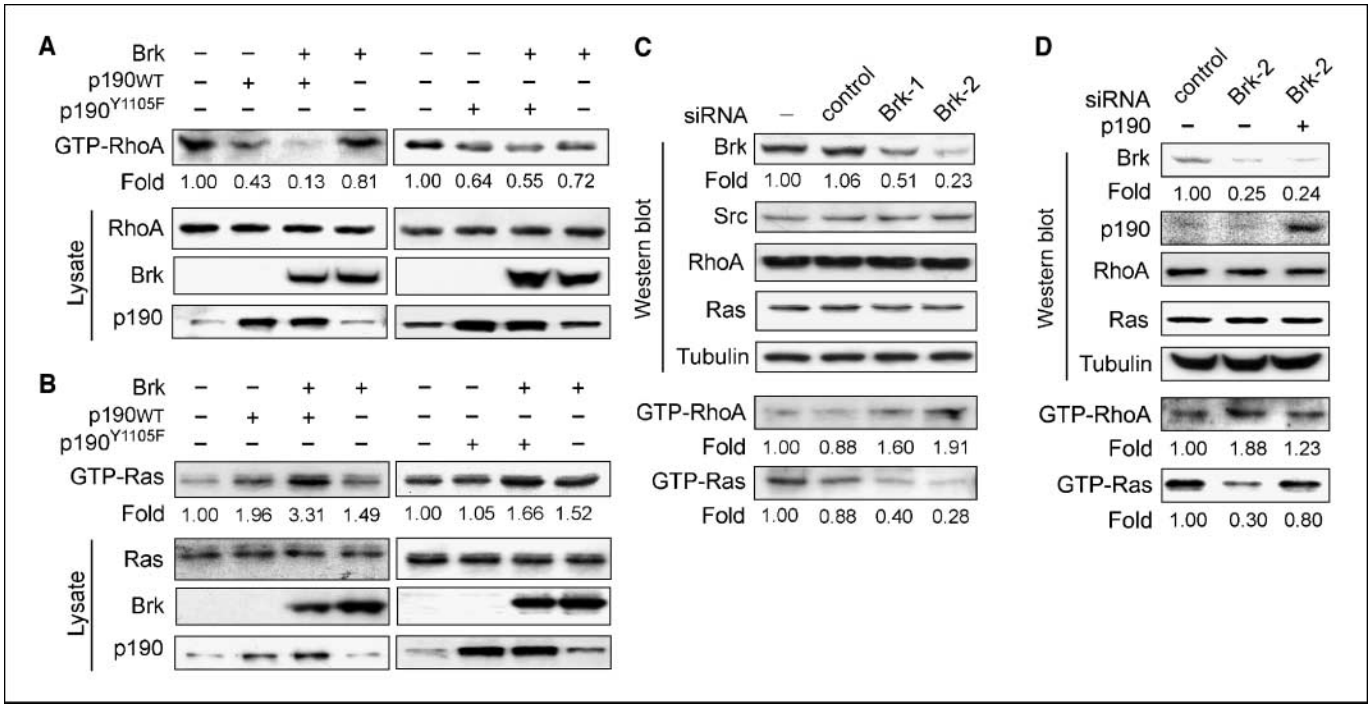

Figure 2. Brk synergizes with p190 to promote Rho inactivation and Ras activation. *A,* HeLa cells were transfected with Brk and/or various forms of p190. Cells were lysed for assaying GTP-bound RhoA, as described in Materials and Methods, or for Western blot with antibodies, as indicated. *B,* cell as in *A* were lysed for assaying GTP-bound Ras, as described in Materials and Methods, or for Western blot with antibodies, as indicated. The amounts of GTP-bound RhoA or 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. *C,* MCF7 cells stably expressing various siRNAs were lysed for Western blot with various antibodies or assayed for GTP-bound RhoA and GTP-bound Ras. 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 are quantified as in *A* and *B. D,* 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 as in *C*.

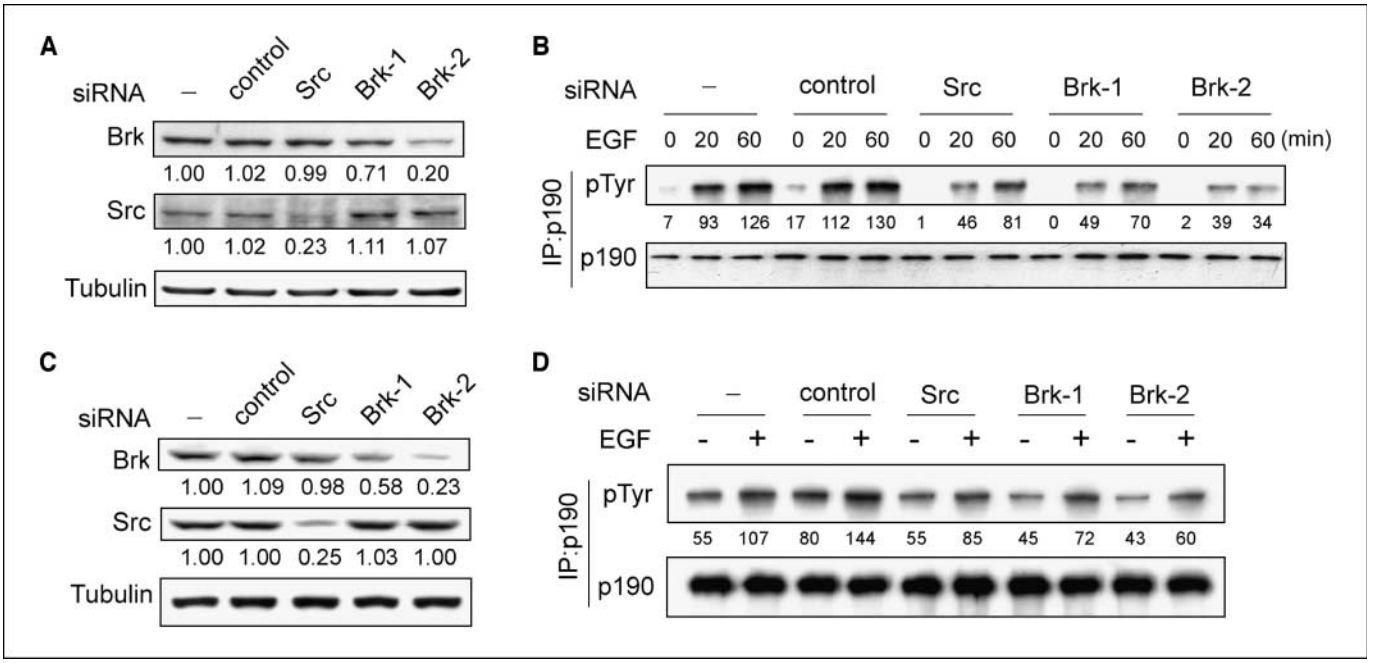

Figure 3. Brk mediates EGF-induced p190 phosphorylation in human cancer cells. A431 cells (*A*) or T47D cells (*C*) were infected with lentivirus carrying control siRNA, Src siRNA, or Brk siRNAs, as indicated. Infected cells were selected and then lysed for Western blot with antibodies as indicated. The numbers below blot images indicate the relative intensity of Brk or Src signal. A431 (*B*) or T47D (*D*) cells expressing various siRNAs were serum-starved and then stimulated with 15 ng/mL EGF for indicated time points (for A431 derivatives) or with 50 ng/mL EGF for 20 min (for T47D derivatives). 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.

data suggest that Brk-induced p190 Y^{1105} phosphorylation activates Ras by sequestrating p120.

Having shown 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 used the breast cancer cell line MCF7, which expresses a relatively high level of endogenous Brk (5). 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. 2*C*). 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. 2*C* and Supplementary Fig. S6*A*). 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. 2*D* and Supplementary Fig. S6*B*). 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 (18). Regardless of the underlying mechanism, our data showed 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 physiologic stimulus. Our previous study revealed that Brk kinase activity is activated by EGF signaling (13). Intriguingly, Src kinase can also induce p190 phosphorylation in EGF-stimulated cells (18, 23). 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 (13). We used Brk-specific and Src-specific siRNAs to down-regulate Brk and Src in this cell line, respectively. As shown in Fig. 3*A* and Supplementary Fig. S7A, the Brk-2 siRNA reduced Brk expression to ~20%, which was similar to the extent of Src down-regulation 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 down-regulation. 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, although Brk-2 and Src siRNA elicited a similar efficiency of down-regulating their cognate kinase, Brk-2 induced a more profound effect on inhibiting EGF-triggered p190 tyrosine phosphorylation (Fig. 3*B* and Supplementary Fig. S7*B*). To test whether Brk mediates EGF-induced p190 tyrosine phosphorylation in other cell systems, we used the breast carcinoma cell line T47D. Again, the efficiency of Brk-2 siRNA to reduce Brk level was comparable with that of Src siRNA to down-regulate Src (Fig. 3*C* and Supplementary Fig. S7*C*). 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 more than that of Src siRNA (Fig. 3*D* and Supplementary Fig. S7*D*). As Brk does not affect Src activity (13), 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

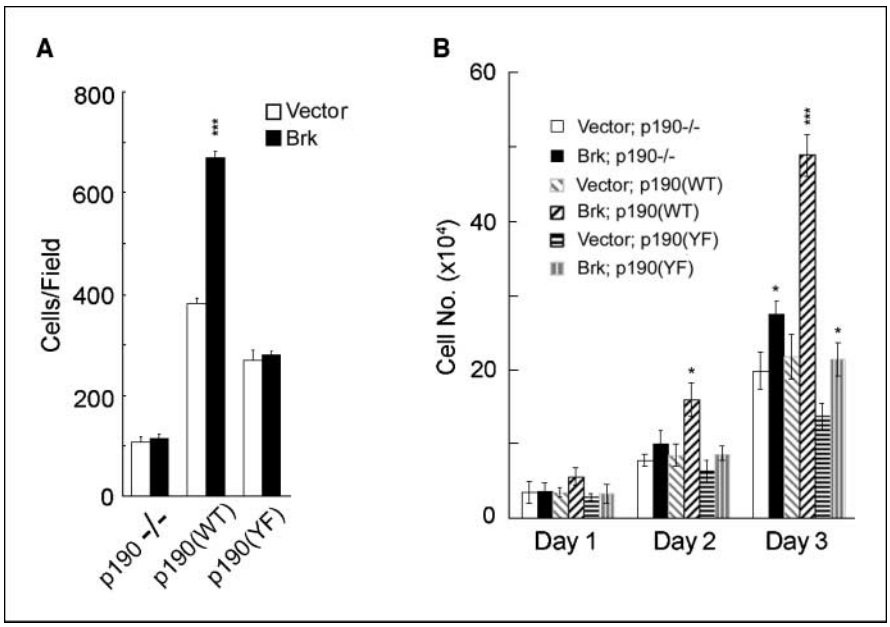

Figure 4. p190 phosphorylation mediates the proliferative and migratory effects of Brk. *A,* p190-null MEFs stably expressing Brk and/or p190 wild-type or mutant were serum-starved for 6 h and then plated onto Transwell chambers for assaying EGF-induced migration, as described in Materials and Methods. *B,* cells, as in *A,* were plated at a density of 5,000 cells per 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 ± SD (*, $P < 0.05$; ***, $P < 0.0005$, compared with cells carrying control vector; $n \geq 4$).

of Brk, Brk could play a similar or even greater role in this phosphorylation event 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 Y^{1105} 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 ($Y^{1105}F$) 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 $Y^{1105}F$ (YF) mutant were expressed at a comparable level, and the three Brk-expressing MEFs contained similar amounts of Brk (Supplementary Fig. S8). 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. 4*A*). However, this migration-promoting function of Brk was completely abrogated in cells lacking p190 or expressing the p190 YF mutant. These results thus showed an essential role of p190 Y^{1105} 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

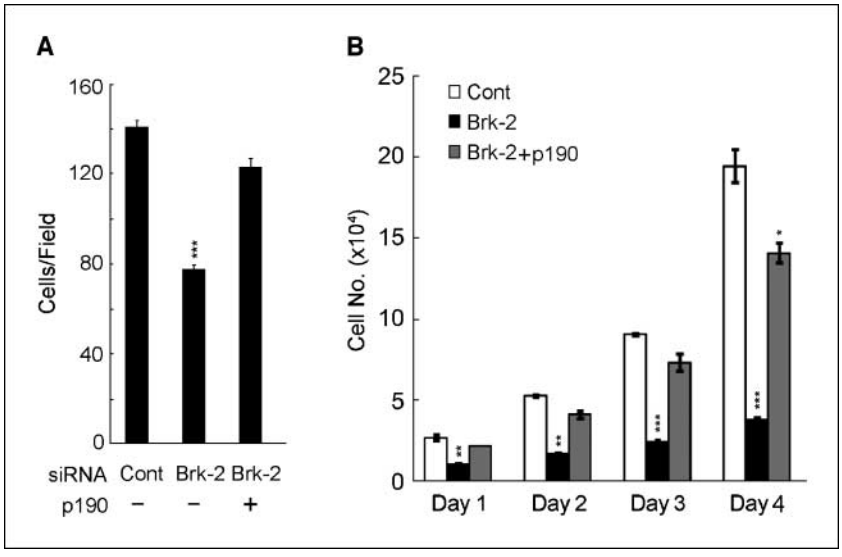

Figure 5. p190 overexpression partially rescues the migration and proliferation defects induced by Brk silencing. MCF7 cells stably expressing Brk-2 siRNA, as in Fig. 2*D*, were infected with lentivirus expressing p190 or a control vector (−). The infected cells were assayed for migration (*A*) and proliferation (*B*), as in Fig. 4. Data shown are means ± SD (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, compared with cells carrying control siRNA and control vector; $n \geq 4$).

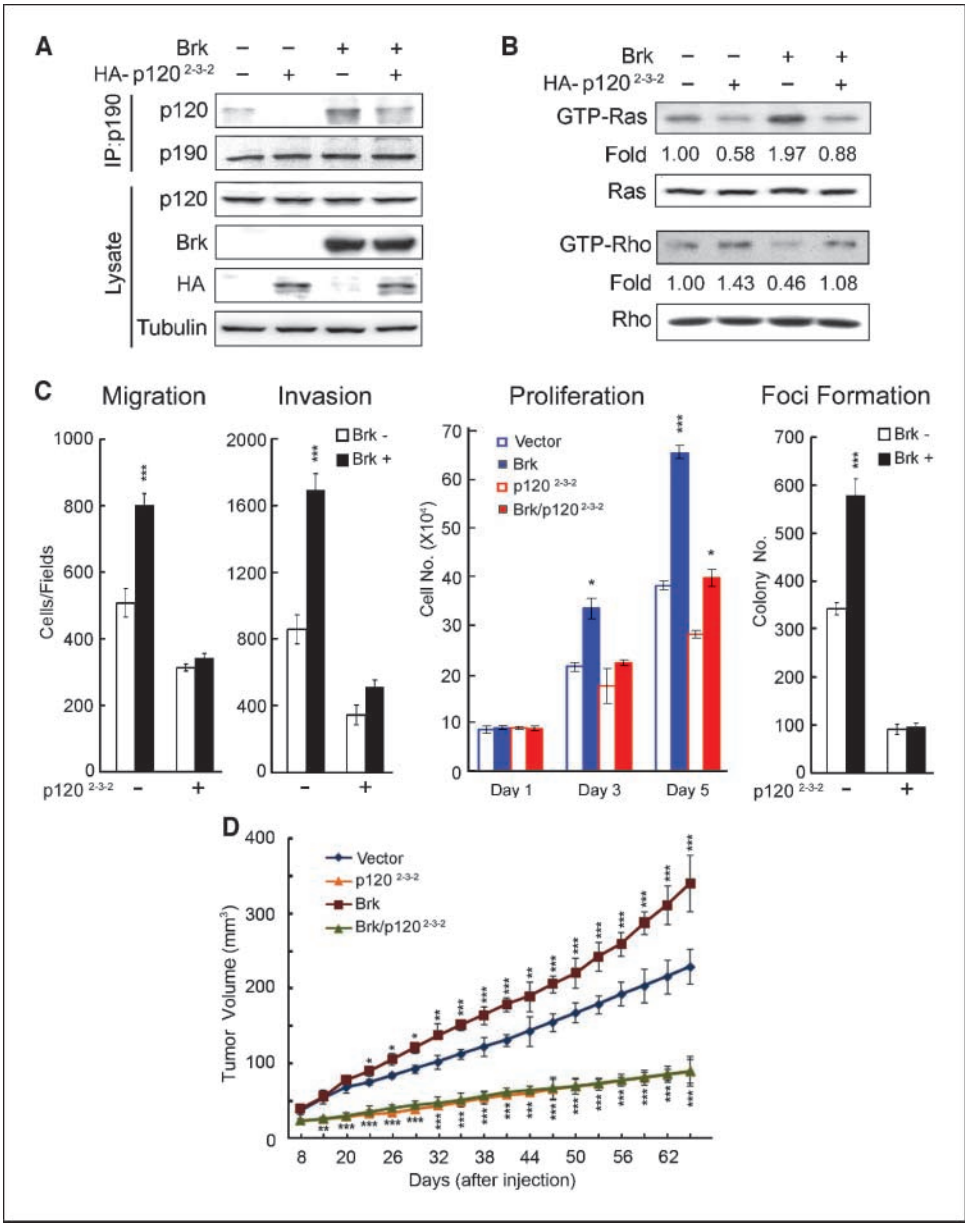

Figure 6. Disruption of p190/p120 complex blocks Brk-stimulated proliferation, migration, invasion, transformation, and tumorigenic functions. *A,* MDA-MB231 cells stably expressing Brk and/or $p120^{2-3-3}$ were lysed for immunoprecipitation with anti-p190. The immunoprecipitates and cell lysates were analyzed by Western blot with antibodies, as indicated. *B,* cells, as in *A,* were assayed for GTP-bound Rho and GST-bound Ras, as in Fig. 3. *C,* cells, as in *A*, were assayed for migration, invasion, proliferation, or soft agar colony formation, as described in Materials and Methods. Data shown are means ± SD (*, $P < 0.05$; ***, $P < 0.0005$, compared with cells carrying control vector; $n \geqq 4$). *D,* cells as in *A,* were injected s.c. into nude mice, as described in Materials and Methods. The tumor volume was measured every 3 d, beginning at day 8. Data shown are means ± SD (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$, compared with cells carrying control vector; $n \geqq 8$).

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. 4*B*). 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. 2*D*), we next investigated whether such overexpression could rescue the migration and proliferation defects induced by Brk silencing. Indeed, p190 overexpression greatly promoted migration and proliferation of MCF7 cells expressing Brk siRNA (Fig. 5*A* and *B*). 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 shown important roles of p190 Y^{1105} 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 (20). This construct was stably introduced to the breast cancer cell line MDA-MB231, which expresses a low level of endogenous Brk (13), 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. 6*A*). Consequently, the Brk-induced RhoA inactivation and Ras activation were both inhibited by the expression of $p120^{2-3-2}$ fragment (Fig. 6*B* and Supplementary Fig. S9). Consistent with our previous study, Brk overexpression in MDA-MB231 cells promoted tumor cell migration and invasion toward EGF (Fig. 6*C*). 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 and their ability to form colonies on soft agar, and again, these effects were greatly reduced in cells expressing $p120^{2\text{-}3\text{-}2}$ fragment (Fig. 6*C* and Supplementary Fig. S10). Finally, we evaluated the function of $p120^{2\text{-}3\text{-}2}$ fragment on Brk-induced tumor growth in a xenograft model, in which mice were injected s.c. with the MDA-MB231 derivatives. Whereas 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. 6*D* and Supplementary Fig. S11). 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 Y^{1105}, 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 Y^{1105} 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 promote its interaction with p120. In certain circumstances, Brk and these kinases seem to regulate p190 phosphorylation at different tissue locations or physiologic settings. For instance, Src, Fyn, and Arg are responsible for p190 phosphorylation during the development of brain (22, 29), wherein Brk expression has not been reported. Additionally, Src and Arg are required for p190 phosphorylation in response to integrin signaling (24, 26), which however does not induce the catalytic activity of Brk (13). Nevertheless, under EGF-treated conditions, Src similarly promotes p190 phosphorylation (18, 23). Using siRNAs to downregulate Src or Brk, we showed 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 compared with Src. As previous reports revealed a high correlation between Brk and ErbB2 overexpression in human breast tumors (4, 6), 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 Y^{1105} 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 cross-talk 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 (30), whereas overexpression of a chimera made of p190 RhoGAP domain and the COOH terminus of RhoA does not affect Ras-induced proliferation (31). Thus, p190 may possess complex and context-dependent effects on proliferation through its GAP domain-mediated Rho inactivation function and Y^{1105} 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 (13). 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, although 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 (Supplementary Fig. S12), consistent with an antagonistic cross-talk between RhoA and Rac1 (28). Similarly, paxillin phosphorylation was reported to result in both Rac1 activation (13) and RhoA suppression (32) at the leading edge of migrating cells, which are both required for the formation of membrane protrusions (32, 33). Consistently, inhibition of Brk blocks lamellipodia formation in response to the EGF migratory cue (13).

Whereas 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 (31). Similarly, Arg-induced p190 phosphorylation in fibroblasts reduces motility by decreasing RhoA-induced actomyosin contractility (26). One possibility is that, in our cell systems, actomyosin contractility can be generated by other compensating mechanisms, such as the Cdc42-MRCK pathway (34). 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.

Whereas 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 (35). Interestingly, increased level of p190-B was found in a subset of mutagen-induced mammary tumors (36) and inducible expression of p190-B in mammary gland during pregnancy results in hyperplastic lesions (37). 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 Y^{1105} in p190-B are not conserved. Accordingly, p190-B is not tyrosine phosphorylated in Src-transformed fibroblasts, where p190-A is heavily phosphorylated (38). Future study will determine whether p190-A is uniquely coupled to Brk signaling axis to promote breast malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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