Regulated Association of Protein Kinase B/Akt with Breast Tumor Kinase*

Received for publication, October 25, 2004, and in revised form, November 10, 2004
Published, JBC Papers in Press, November 10, 2004, DOI 10.1074/jbc.M412038200

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Increased protein-tyrosine kinase activity is a prognostic indicator of decreased disease-free survival in patients with advanced breast tumors. Breast tumor kinase (Brk) is a soluble protein-tyrosine kinase overexpressed in the majority of breast cancers and also in normal skin and gut epithelium, but not in normal breast epithelial cells. Herein, we show that Brk interacts with protein kinase B/Akt, a serine/threonine kinase involved in cell growth and survival. Epidermal growth factor (EGF) treatment of human keratinocytes or Brk-transfected COS-1 cells leads to the dissociation of the Brk-Akt complex, whereas a constitutively active Brk mutant containing a point mutation at Tyr-447 (YF-Brk) failed to dissociate from Akt upon EGF treatment. In addition, Brk-Akt dissociation was blocked by the inhibition of phosphatidylinositol 3-kinase. Similar to ectopic Brk, endogenous Brk in T47D breast cancer cells was less phosphorylated upon EGF treatment, but it remained constitutively associated with Akt in the presence of EGF. Overexpression of wild-type (wt)-Brk, kinase-inactive (KM)-Brk, or YF-Brk increased the Tyr phosphorylation of multiple signaling molecules including EGF receptor. However, only wt- and YF-Brk, but not KM-Brk, induced phosphorylation of Akt and inhibited the kinase activity of Akt in unstimulated cells. Similarly, overexpression of wt- or YF-, but not KM-Brk, blocked the phosphorylation of the forkhead transcription factor, a downstream Akt target. These results suggest that Brk may function as a signaling molecule whose kinase activity normally limits the activity of Akt in unstimulated cells. Additionally, these results suggest that in breast cancer cells Brk behaves similarly to a constitutively active Brk mutant (YF-Brk) and associates with tyrosine-phosphorylated proteins in deregulated signaling complexes. Together these data provide clues to the possible proto-oncogenic and oncogenic functions of Brk.

Breast tumor kinase (Brk<sup>1</sup>/protein-tyrosine kinase-6/Sik) is a nonreceptor tyrosine kinase that was identified from a human metastatic breast tumor (1). Brk expression is low or undetectable in normal mammary tissue and benign lesions; however, roughly 65% of breast tumors express appreciable Brk levels, and 27% of Brk-positive tumors overexpress Brk by 5-fold or more (2). Overexpression of Brk partially transforms human mammary epithelial cells (3), and Brk gene silencing inhibits breast cancer cell growth (4). Additionally, Brk has been shown to associate with the epidermal growth factor (EGF) receptor (EGFR) and confers increased sensitivity to the mitogenic effects of EGF (3). These results suggest that inappropriate overexpression of Brk may contribute to breast cancer development or progression toward a more malignant phenotype.

Brk is considered to be a member of a unique family of intracellular soluble Src-like tyrosine kinases that includes Srm, Frk, and Src42A/DarC41 (5). Brk family kinases are defined by a highly conserved exon structure that is distinct from other major tyrosine kinase families including c-Src (6). For example, Brk contains single SH3, SH2, and tyrosine kinase domains but shares only 46% amino acid identity with c-Src and lacks an N-terminal consensus sequence for myristoylation and membrane association. In contrast to c-Src, several Brk family kinases have been shown to have an inhibitory effect on Ras pathway signaling from receptor tyrosine kinases (5), and in mouse keratinocytes, the mouse homolog of Brk (Sik) functions as an intermediate in calcium-induced differentiation (7). Only a few substrates for Brk phosphorylation in vivo have been identified. Sam68 is a 68-kDa RNA-binding protein that can substitute for the human immunodeficiency virus Rev protein in mediating nuclear export of sequence-specific RNAs (8) as well as alter the fate of selected RNAs in the cytoplasm (9). Recently, the Sam68-like proteins, SLM-1 and SLM-2, have been shown to be Brk substrates (10). Similar to Sam68, phosphorylation of SLM-1 and SLM-2 by Brk leads to inhibition of their RNA binding function. Additionally, two related Brk substrate proteins of 45 and 50 kDa appear to be novel adaptor-like molecules with unknown function (11).

It is now well accepted that ligand-activated receptor protein-tyrosine kinases transduce their signals through association with a growing number of specific cytoplasmic target proteins, many of which contain SH2 and/or SH3 domains that bind to phosphotyrosine-containing and proline-rich sequences, respectively. Some receptor-associated proteins possess enzymatic activity, such as phospholipase C-γ1, whereas others lack...
apparent enzyme activity, such as Grb2, Nck, or Shc isoforms. Nonreceptor protein or lipid kinases, such as c-Src or PI3K, clearly rely on both the ability to form appropriate protein-protein complexes and kinase activity for proper function. Perhaps not surprising, given its SH2/SH3 domain structure, Brk has been shown to interact with EGFRs (3), and Brk overexpression in mammary epithelial cells resulted in enhanced phosphorylation of EGFR-related c-erbB3 receptors and potentiated c-erbB3-associated signaling to the PI3K/Akt pathway in response to EGF treatment of cells (12). Interestingly, however, the ability of Brk to increase cell growth in response to EGF was independent of its kinase activity (4), suggesting that Brk-induced changes in complex formation are sufficient to alter proliferative signaling.

Aside from these important initial studies (3, 11, 12), Brk function and interacting partners remain largely undefined. In this report, we sought to understand better the contribution of Brk kinase activity and protein interactions to altered signal transduction pathways. We found that independent of its kinase activity, Brk associates with Akt and other tyrosine-phosphorylated molecules in signaling complexes that dissociate upon stimulation of cells with EGF. In unstimulated cells, Brk kinase activity mediates Tyr phosphorylation and inhibition of Akt in the Brk-Akt complex and blocks downstream signaling upon stimulation of cells with EGF. In unstimulated cells, Brk phosphorylated molecules in signaling complexes that dissociate mediated by the C-terminal proline to alanine mutations at positions 424 and 427. We also included kinase-inactive KM-Akt and an Akt N-terminal deletion mutant (ΔN) lacking amino acids 4–129 of Akt, a region included kinase-inactive KM-Akt and an Akt N-terminal deletion mutant (ΔN) lacking amino acids 4–129 of Akt, a region containing the N-terminal pleckstrin homology (AH/PH) domain of Akt, located at amino acids 424–427, is also required for the presence of the Akt mutants was observed by Western blotting with anti-HA antibody. As seen in Fig. 1A, the p85 subunit of PI3K was weakly detectable relative to Akt in Brk immunoprecipitates from Brk-positive T47D cells, but not from Brk-null MDA-MB-468 cells (not shown). Additionally, the p85 subunit of PI3K was weakly detectable relative to Akt in Brk immunoprecipitates from Brk-positive T47D cells, but not from Brk-null MDA-MB-468 cells (not shown). The interaction between Akt and c-Src has been shown to require the PXXP domain in the C-terminal portion of Akt and the SH3 domain of c-Src. To determine whether the PXXP motif in Akt, located at amino acids 424–427, is also required for the Brk-Akt interaction, Brk was coexpressed with vector control, or HA-tagged wt-Akt, or PXXP Akt mutant containing proline to alanine mutations at positions 424 and 427. We also included kinase-inactive KM-Akt and an Akt N-terminal deletion mutant (ΔN) lacking amino acids 4–129 of Akt, a region that contains the N-terminal pleckstrin homology (AH/PH) domain of Akt. After immunoprecipitation with antibodies to Brk, the presence of the Akt mutants was observed by Western blotting with an anti-HA antibody. As seen in Fig. 1D, wt-Akt and both the PXXP and KM mutants of Akt were able to interact with wt-Brk, suggesting that Brk does not interact

**EXPERIMENTAL PROCEDURES**  

**Expression Vectors and Reagents—** cDNA constructs for HA-tagged wt-Akt and Akt mutants in the pCMV5 or pCDNA expression vectors were a kind gift from Dr. Yun Qiu (University of Maryland) and were originally described by P. Tsichlis (13, 14). pcCMV vectors containing wt and mutant Brk inserts were generously provided by Mark Crompton (Royal Holloway, University of London, Surrey). Expression vectors encoding wild-type and mutant FKHR molecules (15) were kindly provided by T. Unterman (University of Illinois). Brk antibodies and protein A/G Plus-agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HA-tagged monoclonal antibodies, total and phospho-Akt antibodies, PI3K p85 subunit, phospho-forkhead, total and phospho-GSK3 antibodies, and the Akt antibody conjugated to agarose beads. The resulting immune complexes were washed four times in immunoprecipitation buffer and subjected to **in vitro** Akt kinase assay using the Akt kinase assay kit (Cell Signaling Technologies, Beverly, MA) according to the manufacturer’s instructions, and subjected to SDS-PAGE and Western blotting.

**RESULTS**  

**Brk Associates with Akt in Immune Complexes—** In previous reports suggest that Brk can potentiate the PI3K/Akt signaling pathway via association with erbB family members (3, 12). To examine further how Brk may alter the function of the PI3K/Akt signaling pathway, we examined Brk immunoprecipitates for the presence of Akt molecules (Fig. 1). COS-1 cells were transfected with either RecMV-Brk or the RecMV parent vector as a control, and Brk was immunoprecipitated from whole cell lysates using Brk-specific antibodies. Brk immunoprecipitates were then subjected to Western blotting with Akt-specific antibodies (Fig. 1A). Endogenous Akt was present in Brk immunoprecipitates, but it was not found in vector or nonspecific IgG control immunoprecipitates. The specific interaction of Brk with Akt in COS-1 cells was confirmed by reverse immunoprecipitation of HA-tagged Akt followed by Western blotting with Brk-specific antibodies (Fig. 1B). Importantly, the Brk-Akt interaction between endogenously expressed molecules was also specifically detected in Akt-immunoprecipitates from Brk-positive T47D human breast cancer cells (Fig. 1C). Additionally, the p85 subunit of PI3K was weakly detectable relative to Akt in Brk immunoprecipitates from Brk-positive T47D cells, but not from Brk-null MDA-MB-468 cells (not shown).
Breast Tumor Kinase Associates with PKB/Akt

Fig. 1. Coimmunoprecipitation of Brk and Akt. A, Brk immunoprecipitation (IP) in COS cells. Expression vectors encoding Brk or parent vector (V) control DNA were transiently transfected into COS cells. Brk protein was immunoprecipitated using Brk-specific antibodies and protein G-agarose beads. Endogenous Akt was visualized by Western blotting in Brk immunoprecipitates and COS cell lysates using Akt-specific antibodies. Controls using nonspecific IgG and protein G-agarose beads (IgG) or lysates from vector-transfected cells demonstrated the specificity of the Brk-Akt interaction. B, Akt immunoprecipitation in COS cells. The above experiments in A were repeated after transient transfection of HA-tagged Akt and either control parent vector or Brk into COS cells. HA-tagged Akt was immunoprecipitated using an HA-specific monoclonal antibody, and Akt and Brk were visualized in immunoprecipitations by Western blotting (WB). C, Brk and Akt interact in T47D human breast cancer cells. Endogenous Akt was immunoprecipitated from Brk-positive T47D cell lysates using an Akt-specific antibody-bead conjugate, and Brk and Akt were visualized in immunoprecipitations by Western blotting. Controls using nonspecific antibodies and protein G-agarose beads (IgG) or lysates from vector-transfected cells demonstrated the specificity of the Brk-Akt interaction. D, Brk interacts with Akt PXXP motif mutant. Wt-Brk was expressed with either vector control, wt-, KM-, a PXXP mutant, or ΔN-Akt. Brk or IgG control immunoprecipitates were then Western blotted with anti-HA and anti-Brk antibodies. Lysates were also Western blotted with anti-HA and anti-Brk antibodies as controls for Akt and Brk expression.

with the PXXP domain of Akt in the same manner as c-Src and that Akt kinase activity is not required for this interaction. Furthermore, the interaction does not require the N terminus of Akt, although binding to this region is diminished relative to full-length Akt, suggesting that the AH/PH region of Akt may participate in the Brk interaction and that multiple regions of the full-length Akt molecule contribute to Brk interactions.

Wild-type and Kinase-dead Brk, but Not Constitutively Active Brk, Dissociate from Akt upon EGF Treatment—Akt association with protein kinase C (18) and with c-Src (19) increases in response to acute EGF treatment of epithelial cells. To examine the potential for EGF regulation of Brk-Akt complexes, we repeated the above coimmunoprecipitation experiments in serum-starved cells either left untreated or treated with EGF for 10 min. Because overexpression of Brk may alter its regulatory properties, we first examined human keratinocytes, which normally express Brk as a key kinase mediator of calcium-induced differentiation (7). Endogenous Akt was immunoprecipitated from serum-starved normal HaCat keratinocyte cells and probed using Brk-specific antibodies (Fig. 2A). Cell lysates served as loading controls and a measure of input protein relative to each immunoprecipitation reaction. Again, Brk was present in Akt immunoprecipitates from unstimulated HaCat cells but was not found in nonspecific IgG control immunoprecipitates. Interestingly, in contrast to studies with other signaling molecules (18, 19), EGF treatment resulted in the dissociation of Brk and Akt.

Dissociation of the Brk-Akt complex in response to EGFR activation suggested a role for the kinase activities of either Brk or Akt in this event (Fig. 2A). To address this question, COS-1 cells were transiently transfected with parent RcCMV control vector, or RcCMV expression vectors encoding either wt-Brk, a KM-Brk, or a mutant Brk with its regulatory C-terminal Tyr-447 mutated to Phe (YF-Brk). This mutation is analogous to the activating mutation in c-Src kinase (20–22) and creates a Brk protein with increased kinase activity (23), consistent with a role for Tyr-447 in Brk autoinhibition (23, 24).

After transient Brk expression in COS-1 cells, serum starvation (24 h), and EGF treatment (10 min), Akt immunoprecipitates were subjected to Western blotting with Brk-specific antibodies (Fig. 2B). The results clearly show that endogenous Akt associates with each Brk protein (wt, KM, and YF) in duplicate cultures of serum-starved COS-1 cells. Interestingly, as in human keratinocytes (Fig. 2A), both KM-Brk and wt-Brk dissociated entirely from Akt in EGF-stimulated cells, whereas YF-Brk did not fully dissociate with Akt after EGF treatment.

As an internal control for regulation of Akt protein-protein interactions, we were able to detect a similar weak interaction between endogenous Akt and c-Src molecules in serum-starved COS-1 cells (not shown). However, in contrast to the Brk-Akt interaction, the association between c-Src and Akt increased upon EGF treatment, as reported by Jiang and Qiu (19), indicating that Brk and c-Src are likely to have distinct functions in signaling complexes. These results suggest that Brk and Akt interact in unstimulated cells as part of a regulated signaling complex that undergoes EGF-induced dissociation. Interestingly, the kinase activity of Brk is not required for complex association or dissociation. Rather, Tyr-447, an autoinhibitory...
site on Brk (23), appears to be involved in Brk-Akt dissociation in response to EGF (Fig. 2 and see below).

Next, to determine whether Akt kinase activity is required for the regulated Brk-Akt interaction, the experiment shown in Fig. 2B was repeated, except each Brk construct was cotransfected with HA-tagged KM-Akt prior to serum starvation (24 h) and EGF treatment (10 min). HA-KM-Akt was immunoprecipitated with HA-specific monoclonal antibodies, and Brk was visualized by Western blotting using Brk-specific antibodies (Fig. 2C). Each Brk protein associated with HA-tagged KM-Akt, indicating that the kinase activities of either Brk or Akt are not required for this interaction. Rather, we routinely observed the strongest association between KM-Brk and Akt molecules relative to their active counterparts (Fig. 2 and see below). Similar to the interaction of wt-Akt with KM-Brk (Fig. 2B), KM-Brk dissociated entirely from KM-Akt upon EGF treatment of COS-1 cells (Fig. 2C). However, a much reduced but weak interaction between KM-Akt and wt-Brk was detected after EGF stimulation of starved cells, whereas YF-Brk failed to dissociate from KM-Akt as shown above for the interaction of YF-Brk with wt-Akt (Fig. 2B).

To examine whether activation of Akt by EGF-induced P13K activity is required for the disruption of the Brk-Akt complex, experiments were repeated in which wt-Brk was transiently transfected into COS-1 cells. Endogenous Akt was then immunoprecipitated from serum-starved cells and treated without or with EGF (10 min) in the presence or absence of LY294002 (Fig. 2D). Again, wt-Brk coimmunoprecipitated with endogenous Akt in starved, but not in EGF-stimulated cells, and Akt was present in each immunoprecipitation reaction. Surprisingly however, the P13K inhibitor LY294002 fully blocked Brk-Akt dissociation in the presence of EGF. Western blots of cell lysates using specific phospho- and total Akt antibodies showed that EGF treatment activated Akt well, whereas the LY inhibitor compound blocked activation of Akt by EGF (Fig. 2D, bottom panel). These results indicate that P13K activation, an upstream event leading to activation of Akt, can trigger the release of Brk/Akt complexes. Thus, although the kinase activity of Akt is not required (Fig. 2C), the Brk-Akt complex is predicted to dissociate upon stimulation of cells with growth factors that lead to activation of the P13K/Akt pathway.

Wt- and KM-Brk, but Not YF-Brk, Form Signaling Complexes That Dissociate upon EGF Treatment—The above results suggest that additional phosphoproteins may participate in the regulation of the Brk/Akt complex. We therefore examined Brk protein complexes using anti-phosphotyrosine antibodies (Fig. 3A). COS-1 cells were transiently transfected with either RC3V control vector or constructs encoding wt-Brk, KM-Brk, or YF-Brk. Transfected cells were serum-starved for 18–24 h and then treated without or with EGF for 10 min. Brk was immunoprecipitated using Brk-specific antibodies, and Western blots were probed with 4G10 monoclonal anti-phospho-Tyr antibodies. Although roughly equal amounts of each Brk protein were pulled down in the Brk immunoprecipitates (see uppermost panel), different sets of Tyr-phosphorylated proteins coimmunoprecipitated with each Brk protein, but not vector controls. At least one phosphoprotein of ~110 kDa (pp110) copurified predominantly with wt-Brk in unstimulated

FIG. 2. Brk and Akt dissociate in response to EGF treatment of keratinocyte and COS-1 cells. A, Brk and Akt interact in HaCat human keratinocyte cells. Brk-positive HaCat cells were plated, serum starved overnight, and treated without (−) or with (+) 100 ng/ml EGF for 10 min. Endogenous Akt was immunoprecipitated (IP) from cell lysates using an Akt-specific antibody-bead conjugate as in C, and endogenous Brk and Akt were visualized in immunoprecipitations by Western blotting. Controls using nonspecific antibodies and protein G-agarose beads (IgG) demonstrated the specificity of the Brk-Akt interaction. B, wt- and KM-, but not YF-Brk, expressed in COS cells dissociate from endogenous Akt. Triplicate COS cell cultures were transiently transfected with control vector or each Brk construct (KM, YF, or wt) and after 24 h to allow for Brk expression, cells were serum starved overnight. Cultures were untreated (−) in duplicate or treated (+) with 100 ng/ml EGF for 10 min, and endogenous Akt was immunoprecipitated from cell lysates using an Akt-specific antibody-bead conjugate. Brk and Akt in immunoprecipitations were visualized using specific antibodies. C, KM-Akt associates with wt-, KM-, and YF-Brk. Duplicate cultures of COS-1 cells were transfected with KM-Akt along with vector control (v), wt-, KM-, or YF-Brk. 24 h post-transfections the cells placed in serum-free medium overnight and then either left untreated (−) or treated (+) with 100 ng/ml EGF for 10 min. Akt immunoprecipitates were then Western blotted for with anti-Brk or anti-Akt antibodies. Nonspecific IgG antibodies served as a control for the specificity of the Brk-Akt interaction. D, inhibition of P13K blocks Brk-Akt complex dissociation. Wt-Brk was transiently transfected into COS-1 cells. After 24 h, cultures were placed in serum-free medium overnight and treated without (−) or with (+) 100 ng/ml EGF in the presence of absence of Me2SO vehicle or the PI3K inhibitor LY294002 (Ly294) at a final concentration of 10 nM in Me2SO. Endogenous Akt was immunoprecipitated using Akt-specific antibodies, and Brk and Akt were visualized in Akt immunoprecipitations by Western blotting using specific antibodies. Controls showing active phosphoserine 473 Akt and total Akt in whole cell lysates under the same conditions demonstrated the utility of the inhibitor.

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cells relative to vector controls. This band was greatly diminished upon EGF treatment. A faint band of 85–90 kDa behaved similarly. Surprisingly, Tyr-phosphorylated pp110 also copurified with KM-Brk in cell lysates from unstimulated, but not EGF-treated, COS-1 cells, although to a lesser extent relative to wt-Brk. Interestingly, numerous Tyr-phosphorylated proteins copurified with YF-Brk, and in contrast to both wt and KM-Brk, the appearance and intensity of YF-Brk-associated bands increased in response to EGF. Of note is the presence of major Tyr-phosphorylated proteins of ~110 and 85 kDa. In addition, phospho-Tyr blots revealed a phosphorylated band that comigrated with Akt (60 kDa) and a faint band representing autophosphorylated YF-Brk. Longer exposures of the same blot indicated that both phospho-Brk and the putative phospho-Akt band were also present in immunoprecipitates of wt-Brk, but not KM-Brk or vector controls from unstimulated cells (Fig. 3A, lower panels). Although Brk is present in each immunoprecipitation, Brk phosphorylation on tyrosine was undetectable in the presence of EGF, a condition that resulted in the dissociation of activated Akt (Figs. 1 and 2). Kamalati et al. (3) first reported Brk association with EGFR. Interestingly, we also observed increased Tyr-phosphorylated EGFR immuno-precipitated with each Brk protein in EGF-treated cells relative to unstimulated controls, indicating that EGFR was well activated under the same conditions that resulted in reduced Brk phosphorylation on tyrosine and Brk-Akt dissociation (Fig. 2). Western blot analysis with specific antibodies confirmed the identity of the indicated phosphoproteins in Brk immunoprecipitates (lower panel, and see Fig. 3, B and C). These results suggest that Brk overexpression can induce changes in Tyr phosphorylation independently of Brk kinase activity (pp110 and EGFR) but that phosphotyrosine-containing proteins are intensified in the presence of YF-Brk and enhanced further by EGF stimulation.

To confirm the presence of Tyr-phosphorylated Akt in cells expressing activated Brk, we repeated these experiments, but instead immunoprecipitated HA-tagged wt-Akt from Brk and HA-Akt cotransfected COS-1 cell lysates using HA-specific monoclonal antibodies and again immunoblotted with phospho-Tyr antibodies as in Fig. 3A (Fig. 3B). Equal amounts of HA-Akt were present in each immunoprecipitation reaction. C, YF-Brk induces Tyr phosphorylation of wt-Akt. COS-1 cells were transfected with either or YF-Brk or wt-Akt alone, or wt-Akt with either YF- or KM-Brk. After overnight serum starvation tyrosine-phosphorylated proteins were immunoprecipitated with 4G10 phosphotyrosine antibody. Immunoprecipitates were then Western blotted for the presence of HA-Akt with an anti-HA antibody. Lysates were Western blotted for total HA-Akt and Brk expression as controls for protein expression. V, vector.
trylated Akt. Furthermore, no increase in Tyr-phosphorylated Akt was observed in the presence of KM-Brk, relative to RC- CMV control, indicating that Brk-induced tyrosine phosphorylation of Akt is dependent on Brk kinase activity. Western blotting of total lysates with anti-HA and anti-Brk antibodies indicates that equal amounts of Akt and Brk were present in the immunoprecipitation reactions.

These results demonstrate that both wt- and KM-Brk participate in regulated signaling complexes that contain proteins phosphorylated on tyrosine (Fig. 3A) and that EGF treatment greatly diminishes Brk phosphorylation on tyrosine (addressed further below). In contrast to wt- and KM-Brk, highly active YF-Brk fails to dissociate from several phospho-Tyr-containing proteins, including Akt (Fig. 3B), whose Tyr phosphorylation actually increases upon EGF treatment, indicating that YF-Brk behaves in a “constitutive” manner in this assay and that EGF potentiates this activity. Qiu and Miller (23) reported the increased SH2 domain accessibility in constitutively active YF-Brk relative to wt-Brk. This may account for the heightened ability of YF-Brk to interact with Tyr-phosphorylated proteins relative to wt- and KM-Brk (Fig. 3A).

Brk and Akt Are Constitutively Associated in T47D Human Breast Cancer Cells—Of note is that Kamalati et al. (3) reported the constitutive association of Brk with EGFR in breast cancer cells and potentiation of EGFR signaling to PI3K and Akt in cells with greatly up-regulated EGFR (12). This suggests that Brk regulation may differ in breast cancer cells when it is overexpressed relative to normal cells that express Brk, such as human keratinocytes (Fig. 1D) or to COS-1 cells expressing wt-Brk molecules that undergo EGF-induced regulation. Therefore, we examined the regulation of Brk-Akt complexes in Brk-positive T47D human breast cancer cells (Fig. 4A). Endogenous Brk was immunoprecipitated from lysates of serum-starved Brk-positive T47D cells after acute treatment (10 min) without or with EGF (Fig. 4A). Again, Brk and Akt readily copurified in T47D cell lysates from unstimulated cells (Fig. 1C). Surprisingly, however, similar to the constitutive association of YF-Brk with Akt in COS-1 cells relative to wt- and KM-Brk (Figs. 2 and 3) and in contrast to our findings in keratinocytes, endogenous Brk and Akt molecules remained stably associated in Akt immunoprecipitates from EGFR-treated breast cancer cells. A time course (0–30 min) of EGF treatment indicated constitutive association of Brk and Akt in breast cancer cells (not shown). LY294002 preincubation had no effect on Brk-Akt association. As an independent control for EGFR signaling, we also detected increased mitogen-activated protein kinase activity and c-Src association with Akt in EGFR-treated T47D cells (not shown), consistent with the results of Jiang and Qiu (19). We and others have extensively characterized T47D breast cancer cell lines with regard to EGFR signaling (25–27).

These results may suggest that Brk is underphosphorylated at Tyr-447 or constitutively active in breast cancer cells. To assay Brk phosphorylation on tyrosine, we immunoprecipitated similarly treated lysates of T47D cells using the 4G10 anti-phosphotyrosine antibody conjugated to agarose beads. Brk was then visualized in Western blots with Brk-specific antibodies (Fig. 4A, bottom panel). As in COS-1 cells (Fig. 3A), Brk was phosphorylated on tyrosine in unstimulated T47D cells but was not immunoprecipitated by 4G10-agarose upon EGF treatment. LY294002 pretreatment had little effect on Brk dephosphorylation in response to EGF, as indicated by a faint band of Tyr-phosphorylated relative to the vehicle (Me2SO) pretreated condition (compare first and third lanes). Thus, endogenous Brk in T47D cells behaves like wt-Brk in COS-1 cells in that it is more phosphorylated in resting cells relative to EGF-treated cells. However, in contrast to wt-Brk in both COS-1 and HaCat cells, endogenous Brk in T47D cells fails to dissociate from Akt upon EGF treatment, suggesting that it may be partially active.

Remarkably, these results together with the finding that activated YF-Brk does not dissociate from multiple signaling molecules (Figs. 2 and 3) suggest that EGF treatment of either COS-1 or T47D cells does not lead to Brk activation per se, but instead diminishes global phosphorylation of Brk on tyrosine and may inactivate Brk, perhaps via autoinhibition (Figs. 3A and 4A). However, because Brk contains at least 9 tyrosine residues that are conserved among its family members, the degree of Brk phosphorylation on tyrosine in whole cell lysates may not accurately reflect Brk kinase activity. Additionally, phosphotyrosine antibodies may not recognize all of the regulated sites on Brk. Therefore, to test directly the ability of EGF to activate Brk, Brk kinase assays were performed in vitro to examine Brk auto-phosphorylation as a measure of its kinase activity (23). Brk was immunoprecipitated from whole cell lysates of serum-starved T47D cells untreated or treated with EGF for 10 min, and equal portions were either placed in vitro with 32P-labeled ATP or reserved for Western blotting (Fig. 4B). After 30 min, kinase reactions were stopped with gel loading buffer, boiled, and subjected to SDS-PAGE and autoradiography. Brk auto-phosphorylation in vitro was not increased further in response to EGF; quantification of the radiolabeled bands indicated that Brk from EGF-treated cells was 40% less phosphorylated relative to Brk from untreated controls. We were unable to detect an increase in Brk kinase activity after a time course of EGF treatment (not
with unstimulated cells (total Akt); EGF treatment increased the phosphorylation of Akt in Akt immunoprecipitations visualized by Western blotting using Akt-specific antibodies. Phosphorylated activated Akt underwent a gel up-shift in EGF-treated cells compared to untreated cells. After Akt immunoprecipitation from cell lysates, total and phospho-Akt in Akt immunoprecipitations were averaged (Fig. 5). Both YF- and wt-Brk, but not KM-Brk, inhibited Akt activity in immune complex kinase assays, suggesting that the kinase activity of Brk is required for Akt inhibition in resting cells. It is possible that substrate effects may produce an apparent inhibition of Akt in vitro if wt and YF-Brk compete with GSK3 for Akt binding. This is unlikely, however, because KM-Brk binds Akt well (Figs. 2A and 5A) but does not inhibit its ability to phosphorylate GSK3α/β in this assay (Fig. 5).

We next examined the serine/threonine phosphorylation state of Akt in Akt immune complexes from unstimulated and EGF-stimulated COS-1 cells overexpressing Brk proteins. COS-1 cells were transfected with control vectors or expression vectors encoding wt-, KM-, or YF-Brk as above (Fig. 5A), and Akt was immunoprecipitated using the Akt antibody-agarose conjugate. In vitro kinase assays were performed by incubation of Akt immunoprecipitates with ATP and purified recombinant GSK3α/β. After either 0 or 15-min reaction times, samples were subjected to Western blotting using phosphospecific GSK3α/β antibodies (Fig. 5A). Akt activity was consistently reduced in Brk-Akt complexes from COS-1 cells containing either wt- or YF-Brk relative to vector controls as measured by reduced GSK3α/β phosphorylation, whereas KM-Brk did not affect Akt activity in the Brk-Akt complex. To ensure that the Akt immune complexes contained Brk, blots were stripped and reprobed for Akt and Brk; roughly equal amounts of endogenous Akt were present in Akt immunoprecipitates, and this copurified with wt, KM, and YF-Brk. Brk expression did not appear to alter significantly Akt activity in whole cell lysates from the same experiment, as measured by phosphorylation of Akt Ser-473 (Fig. 5A, lower panels, and see Fig. 3B). In vitro Akt kinase assays were repeated in COS-1 cells after Brk transient transfection, and the results from multiple assays were averaged (Fig. 5B). Both YF- and wt-Brk, but not KM-Brk, inhibited Akt activity in immune complex kinase assays, suggesting that the kinase activity of Brk is required for Akt inhibition in resting cells. It is possible that substrate effects may produce an apparent inhibition of Akt in vitro if wt and YF-Brk compete with GSK3 for Akt binding. This is unlikely, however, because KM-Brk binds Akt well (Figs. 2A and 5A) but does not inhibit its ability to phosphorylate GSK3α/β in this assay (Fig. 5).

The Kinase Activity of Brk Inhibits Akt Activity in the Brk-Akt Complex—We were unable to detect gross effects of Brk overexpression on mitogen-activated protein kinase or Akt activities in whole cell lysates from several cell types, including COS-1, HeLa, human embryonic kidney 293, and various breast cancer cell lines (not shown and see Fig. 3B) possibly because basal kinase activities in unstimulated cells are low, and the Brk-Akt complex dissociates in growth factor-stimulated cells. Indeed, these signaling pathways may be independent after EGF-induced dissociation. However, the sensitivity of these assays for detection of subtle changes in kinase activities of resting cells may be limited in whole cell lysates (12). Therefore, to explore the functional significance of the Brk-Akt interaction, we performed in vitro Akt kinase assays on Brk-Akt complexes after immunoprecipitation of endogenous Akt (Fig. 5A). COS-1 cells were transfected with vector control, wt-Brk, KM-Brk, or YF-Brk, and after 18–24 h serum starvation endogenous Akt was immunoprecipitated using an Akt-antibody-agarose conjugate. In vitro kinase assays were performed by incubation of Akt immunoprecipitates with ATP and purified recombinant GSK3α/β. After either 0 or 15-min reaction times, samples were subjected to Western blotting using phosphospecific GSK3α/β antibodies (Fig. 5A). Akt activity was consistently reduced in Brk-Akt complexes from COS-1 cells containing either wt- or YF-Brk relative to vector controls as measured by reduced GSK3α/β phosphorylation, whereas KM-Brk did not affect Akt activity in the Brk-Akt complex. To ensure that the Akt immune complexes contained Brk, blots were stripped and reprobed for Akt and Brk; roughly equal amounts of endogenous Akt were present in Akt immunoprecipitates, and this copurified with wt, KM, and YF-Brk. Brk expression did not appear to alter significantly Akt activity in whole cell lysates from the same experiment, as measured by phosphorylation of Akt Ser-473 (Fig. 5A, lower panels, and see Fig. 3B). In vitro Akt kinase assays were repeated in COS-1 cells after Brk transient transfection, and the results from multiple assays were averaged (Fig. 5B). Both YF- and wt-Brk, but not KM-Brk, inhibited Akt activity in immune complex kinase assays, suggesting that the kinase activity of Brk is required for Akt inhibition in resting cells. It is possible that substrate effects may produce an apparent inhibition of Akt in vitro if wt and YF-Brk compete with GSK3 for Akt binding. This is unlikely, however, because KM-Brk binds Akt well (Figs. 2A and 5A) but does not inhibit its ability to phosphorylate GSK3α/β in this assay (Fig. 5).

We next examined the serine/threonine phosphorylation state of Akt in Akt immune complexes from unstimulated and EGF-stimulated COS-1 cells overexpressing Brk proteins. COS-1 cells were transfected with control vectors or expression vectors encoding wt-, KM-, or YF-Brk as above (Fig. 5A), and Akt was immunoprecipitated using the Akt antibody-agarose conjugate (Fig. 5C). There were no significant differences in the levels of Akt phosphoserine 473 or phosphothreonine 308 relative to total Akt in each immune complex isolated from unstimulated cells; slightly less of both phospho-Akt and total Akt was present in the YF-Brk condition. Brk expression could not overcome EGF-induced activation of Akt as measured by phosphorylation of either Ser-473 or Thr-308 (+EGF lanes). Similarly, Brk did not appreciably inhibit the high constitutive activity of myristoylated Akt (data not shown). However, Akt was consistently slightly less phosphorylated on Ser-473 in wt-Akt immunoprecipitates from EGF-treated YF-Brk-ex-
pressing COS-1 cells (Fig. 5C), a condition that resulted in Akt phosphorylation on tyrosine (Fig. 3). This result is consistent with the slightly greater inhibition of GSK3α/β phosphorylation in YF-Brk-Akt complexes relative to wt-Brk (Fig. 5A), inhibition of Brk-Akt complex dissociation in the presence of LY294002 (Fig. 2B), and incomplete dissociation of Akt from YF-Brk upon EGF stimulation of cells (Figs. 2 and 3).

Brk Inhibits Akt-dependent Phosphorylation of Forkhead—As an independent confirmation of the inhibitory activity of Brk toward Akt in the Brk/Akt complex, we assayed the phosphorylation of the FKHR as a downstream readout of Akt activity in intact cells (Fig. 6A). FKHR is a well characterized Akt substrate molecule whose phosphorylation on three residues (Thr-24, Ser-256, Ser-319) by Akt leads to its nuclear export and functional inhibition (28). COS-1 cells were transiently cotransfected with either wt-FKHR or phosphomutant T/S/S FKHR (lacking the Akt sites) and either wt-Brk or vector control. Whole cell lysates from “resting” (unstarved/unstimulated) COS-1 cells were then subjected to Western blotting for phospho-FKHR, total FKHR, or Brk. Resting COS-1 cells contained measurable levels of phospho-FKHR that were reduced to undetectable levels upon Brk expression, whereas total FKHR remained unchanged. Expression of the T/S/S mutant FKHR demonstrated the Akt specificity of the phosphorylated FKHR. To test the requirement of Brk kinase activity, a similar by separate set of experiments was conducted using KM- and YF-Brk (Fig. 6B). Under conditions of serum starvation, only minimal FKHR phosphorylation is apparent by Western blotting. Therefore, to increase the low basal levels of FKHR phosphorylation, after transient transfection, COS-1 cells were either serum starved or grown in medium containing 5% serum for 1–2 days prior to cell lysis and Western blotting (Fig. 6B). FKHR phosphorylation was undetectable in serum-starved cells, but it increased in cells growing in serum. Total FKHR levels were also increased in serum-grown cells relative to starved cells. KM-Brk had no effect on phospho-FKHR levels relative to vector controls, whereas YF-Brk diminished phospho-FKHR. Brk expression had little effect on total FKHR protein levels in serum-grown cells. These results suggest that Brk inhibits basal or “steady-state” Akt activity in cells growing in regular culture conditions (5% serum) by a mechanism that requires Brk kinase activity.

**DISCUSSION**

Herein, we sought to understand better aspects of Brk function in signal transduction which relate to its ability to form signaling complexes. In contrast to other well characterized signaling proteins that are known to undergo increased interaction with Akt in response to EGF, such as protein kinase C (18) and c-Src (19), we found that Brk participates in a regulated signaling complex with Akt and other signaling molecules, which is disrupted upon acute stimulation of cells with EGF (Figs. 3 and 4). The data presented here demonstrate that the Brk-Akt interaction involves Tyr phosphorylation of Akt (Fig. 3) and inhibition of Akt kinase activity (Fig. 5) and signaling to the FKHR transcription factor (Fig. 6). Furthermore, we show that Brk-Akt complexes dissociate in response to EGF stimulation in keratinocytes and COS-1 cells, but not T47D breast cancer cells (Fig. 2), eluding to possible mechanisms for the proto-oncogenic versus oncogenic function of Brk.

The data presented herein indicate that Brk expression inhibits the ability of Akt to phosphorylate downstream targets. Although this is contradictory to the properties of many Src family members, other Brk family members have been shown to inhibit signaling pathways, such as the Ras pathway (5, 29). Thus, this family of kinases may differ from traditional Src family members by limiting the magnitude of growth factor receptor inputs to multiple intracellular signaling pathways. Additionally, Brk and Brk family members are known to undergo subcellular redistribution in response to external stimuli (5, 30). Stable interaction of Brk with signaling complexes normally clustered at the cell membrane in unstimulated or resting cells may also serve to sequester Brk, thereby prohibiting its subcellular mobility and/or access to nuclear substrates (30).

With the exception of constitutively active YF-Brk, EGF stimulation of cells leads to the dissociation of multiprotein complexes, and it also appears to reduce both global phosphorylation of wt-Brk on tyrosine (Figs. 3A and 4A) and Brk activity (Fig. 4B). Furthermore, phosphorylation of an autoinhibitory site, Brk Tyr-447, is required for EGF-induced complex dissociation (Figs. 2 and 3). Indeed, the association of constitutively active YF-Brk with several Tyr-phosphorylated proteins increased upon EGF treatment (Fig. 3A), reminiscent of Brk-induced potentiation of EGF signaling events (12). We also demonstrate that the P13K inhibitor blocked EGF-induced dissociation of wt-Brk (Fig. 2D), suggesting that growth factor-mediated activation of P13K signaling leads to ultimate dissociation of inhibitory signaling complexes that are dependent on phosphorylation of Brk Tyr-447 but independent of Akt kinase activity (Fig. 2). If Brk cytoplasmic or nuclear mobility (i.e. access to nuclear Sam68) is important for its transforming potential, the constitutive association of YF-Brk with signaling complexes and inhibition of Akt signaling by YF-Brk may explain its reduced transforming potential in soft agar assays (3).

Kamalati et al. (3) reported a constitutive interaction between Brk and EGFR in MCF-10A cells engineered to overexpress Brk. Similarly, we found that endogenous Brk is constitutively associated with Akt in T47D breast cancer cells but not in normal human keratinocytes or COS-1 cells. Brk is undetectable in normal breast epithelial cells but is clearly overexpressed in the majority of breast cancers (2). Thus, one possible explanation for these results is that Brk may be relatively “underphosphorylated” at Tyr-447 and thus partially active in breast cancer cells where it is overexpressed abnormally. Supporting this hypothesis, we routinely observe a high basal level of Brk kinase activity in breast cancer cell lysates (Fig. 4B). It is also possible that breast epithelial cells lack Brk-specific kinases predicted to “regulate negatively” conserved Tyr-447, by analogy to c-Src (31), or that overexpression or altered function of signaling molecules in breast cancer cells can overcome regulation at this site. Our results (Fig. 3A) clearly demonstrate a constitutive behavior with...
regard to the induction of increased Tyr-phosphorylated proteins that associate with YF-Brk relative to KM- and wt-Brk. Indeed, the transforming potential of Brk seems to be related to the regulation of Tyr-447 (3) and may involve the ability of this conserved site to interact with SH2 domains of other key signaling molecules (32). Our limited mapping experiments indicate that the N-terminal AH/PH domain of Akt may be involved but is not required for interaction with Brk (Fig. 1D). Similarly, mutation of the conserved proline-rich motif of Akt did not block the Brk-Akt interaction but prevented Akt binding to c-Src (19), suggesting that unlike c-Src, the SH3 domain of Brk is not required for the Brk-Akt interaction. Identification of sequences in Brk that are required for Akt interaction are the subject of a separate study.3

Regular negative regulators of Akt kinase activity have been described (18, 33, 34). For example, the C-terminal modulator protein (CTMP) was cloned from a HeLa cell library using a two-hybrid approach in which the C terminus of Akt served as bait (33). Notably, similar to Brk interaction with Akt, CTMP was associated with inactive Akt at the cell membrane in unstimulated cells. Upon activation of Akt by growth factors the CTMP-Akt interaction was disrupted. Additionally, overexpression of CTMP led to decreased phosphorylation of Akt in resting cells, primarily on Ser-473, but could not abolish Akt phosphorylation on Thr-308 or Ser-473 in stimulated cells. Maira et al. (33) suggest that CTMP may serve to prevent inappropriate Akt activation by direct binding to the Akt C terminus and thereby temper excess cell growth and proliferation. Our results suggest that Brk may act to inhibit Akt signaling by a similar mechanism. Additionally, atypical protein kinase C-6 interacts with Akt and limits its phosphorylation and activation by growth factors (18). Recently, the phosphorylation of Akt on Tyr-474 was found to be associated with reduction of Ser-473 phosphorylation and inhibition of Akt kinase activity (35); regulation of Akt and other “AGC” kinase family members at this conserved site is postulated to be an inhibitory input designed to limit the extent of Akt activation (36). Thus, Brk may regulate Akt by phosphorylation of Akt Tyr-474. We are currently exploring this exciting possibility and the functional significance of the Brk-Akt interaction in relevant models, such as differentiating keratinocyte cells (7).

Is Brk inhibition of Akt inconsistent with its potential role as an oncogene in the breast? This paradox may be explained in part by the contributions of Brk kinase activity relative to its ability to alter protein complexes (37). Kamalati et al. (12) found that Brk overexpression could increase the Tyr phosphorylation of c-erbB3 receptors and potentiate EGF signaling to PI3K and Akt in c-erbB3 immunoprecipitates of breast epithelial cells. Although KM-Brk was not tested in these studies, both wt- and KM-Brk lead to increased EGF-induced cell proliferation in overexpression studies from the same group (3, 4). Herein, we also found that Brk binds to activated EGF receptor independently of Brk kinase activity (Fig. 3A), although EGF does not appear to activate Brk appreciably (Fig. 4, A and B). In addition, stable expression of either wt- or KM-Brk increased HEla cell proliferation in the presence and absence of EGF.4 These results along with previously published studies suggest that Brk-induced changes in cell growth are related to altered protein complex formation rather than Brk-kinase activity (12, 37). Brk overexpression leads to the robust Tyr phosphorylation of multiple proteins that associate with Brk, including p85 and p110. That KM-Brk induced the Tyr phosphorylation of at least one associated protein relative to vector controls indicates that this property of Brk is also independent of its kinase activity. Thus, the proto-oncogenic form of Brk may normally inhibit or limit Akt activity or signaling to Ras (5, 29) in untransformed cells or during differentiation of skin or gut epithelial cells (7, 38) by a mechanism that requires its kinase activity (Figs. 5 and 6), whereas oncogenic forms of Brk may contribute to altered cell proliferation by mechanisms that primarily require its SH2/SH3 domain structure rather than its kinase activity (37).

Clearly however, with regard to cell transformation, aspects of both Brk kinase activity and Brk protein-protein interaction contribute to its ability to partially transform NIH3T3 cells, as measured by growth in soft agar (3); in these studies, colony formation clearly required Brk kinase activity, but constitutively active YF-Brk induced an intermediate number of colonies that was between that induced by KM- and wt-Brk. Thus, we conclude that although the details of Brk signaling are still largely undefined, its role in biology is likely to be determined by its relative kinase activity in coordination with its interaction with other proteins as part of regulated signaling complexes. Notably, early studies on the actions of several oncoproteins, including c-Myc, E2F, Ras, and viral oncoproteins such as E1A, demonstrated both apoptosis and increased cell proliferation (39). In this case, the transforming potential of these oncogenes is dependent on the presence of other cooperating molecules and is mediated by separable mechanisms from those inducing cell death (40). It will be exciting to discover signaling molecules that cooperate with Brk during the transformation of epithelial cells of the breast (3), gastrointestinal tract and colon (41), prostate (42), and/or head and neck squamous cell carcinomas (43) and whether these primarily co-opt the kinase activity or complex forming abilities of Brk.

Acknowledgments—We thank Dr. Mark Crompton, Dr. Yun Qiu, and Dr. Terry Unterman for the Brk cDNAs, the HA-tagged Akt constructs, and the FKHR expression vectors, respectively. We are grateful to Dr. Doug Yee for antibodies recognizing total FKHR.

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Footnotes:

3 C. A. Lange, unpublished results.
4 C. A. Lange and P. Zhang, unpublished results.
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Regulated Association of Protein Kinase B/Akt with Breast Tumor Kinase
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J. Biol. Chem. 2005, 280:1982-1991.
doi: 10.1074/jbc.M412038200 originally published online November 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M412038200

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