Brk/PTK6 signaling in normal and cancer cell models

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Breast tumor kinase (Brk), also termed PTK6, is known to function in cell-type and context-dependent processes governing normal differentiation. However, in tumors in which Brk is overexpressed, this unusual soluble tyrosine kinase is emerging as a mediator of cancer cell phenotypes, including increased proliferation, survival, and migration. Nuclear and cytoplasmic substrates phosphorylated by Brk include a collection of regulatory RNA-binding proteins, adaptor molecules that link Brk to signaling pathways generally associated with the activation of growth factor receptors, and Signal Transducers and Activators of Transcription (STAT) molecules that are direct regulators of gene expression. Understanding Brk-dependent regulation of these key signaling pathways and how they influence cancer cell behavior is predicted to inform the development of improved ‘targeted’ cancer therapies and may provide insight into ways to avoid chemo-resistance to established treatments.

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**Current Opinion in Pharmacology** 2010, 10:662–669

This review comes from a themed issue on Endocrine and metabolic diseases

Edited by Gary Firestone

Available online 9th September 2010

1471-4892/$ – see front matter

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**DOI** 10.1016/j.coph.2010.08.007

**Brk identification and expression**

Breast tumor kinase, or Brk, was cloned from a metastatic breast tumor using a PCR-based screen for novel protein tyrosine kinases [1]. The identical protein tyrosine kinase 6 (PTK6) was independently cloned in melanoma cells as a partial sequence [2] as well as isolated as a full-length clone from normal tissues [3,4]. Brk is a novel non-receptor tyrosine kinase with limited homology to the c-src kinase family. The domain structure of Brk includes SH3 (src homology 3), SH2 (src homology 2), and protein kinase domains. However, Brk lacks a consensus sequence for myristoylation present in other src family members. The brk gene resides on chromosome 20q13.3–13.4 and is made up of 8 exons, which exhibit distinct boundaries relative to other family members, suggesting a functional divergence [5,6].

Brk, or the mouse homolog Sik, is normally expressed in the differentiating epithelial cells of the intestine, skin, prostate, and oral cavity [3,4,7,8] and has also been detected in lymphocytes [9]. Levels of Brk in the normal mammary gland and in the ovary are low to undetectable. However, Brk overexpression is common in malignancies arising from these tissue types [10,11]. For example, Brk has been detected in up to 85% of human breast tumors with staining intensities correlated to histological grade [12\(^*\)]. Brk was also cloned from melanoma cells [2] and has been detected in cultures of metastatic melanoma cells [13]. In tumors from tissues such as colon and prostate, where Brk is expressed normally, it has been shown to be overexpressed or mislocalized [7,14], perhaps indicating altered signaling in the malignant setting. Brk has not been shown to be mutated in tumors, but appears to be frequently upregulated at the transcriptional or post-transcriptional levels [5,15,16]. Recently, Xiang et al. [17] reported significant co-amplification of the region of chromosome 20 where the brk gene (Chr 2q13.3) is localized, and chromosome 17q21–22, the location of the erbB2 gene. In this study the brk gene was found amplified in 57 of 202 (28%) samples tested.

**Brk signaling**

**Substrates**

One key to understanding the function of Brk in normal tissue homeostasis and cancer is to identify the endogenous physiological substrates of Brk. To date a number of potential Brk substrates have been identified (Table 1; Figure 1). These include the RNA-binding proteins: Sam68, SLM-1, SLM-2, and PSF [18–21]; transcription factors: STAT3 and STAT5a/b [22,23]; and a variety of signaling molecules: p190RhoGAP, paxillin, Akt, IRS-4, BKS/STAP-2, and KAP3A [24\(^*\),25–29]. It is important to note that while Brk expression has been shown to induce tyrosine phosphorylation of each of these proteins, not all of these proteins have been shown to be bona fide Brk substrates in vitro.

Sam68 was one of the first Brk substrates identified and the most extensively studied. Derry et al. were the first to show that endogenous Brk and Sam68 co-localize in distinct nuclear dots, or Small Nuclear Bodies (SNBs), of cancer cell lines (Figure 2b). Expression of Brk was also found to negatively regulate the RNA-binding function of Sam68 [30]. Subsequent studies have confirmed that Brk expression negatively regulates Sam68 [20] and identified
the tyrosine residues phosphorylated by Brk [18]. Interestingly, a number of other RNA-binding factors have also been found to be Brk substrates [19,21]. Similar to Sam68, the Sam68-like mammalian proteins SLM-1 and SLM-2 are phosphorylated by Brk in vitro and Brk-induced phosphorylation negatively regulates SLM-1 and SLM-2 RNA-binding function (Figure 1a). While coexpression of SLM-1 and constitutively active Brk resulted in Brk nuclear localization [19], coexpression of Brk and the RNA-binding protein PSF, resulted in PSF cytoplasmic re-localization [21].

The tyrosine phosphorylation sites have been identified for a number of Brk substrates. Brk-induced phosphorylation of paxillin was mapped to Y31 and Y118 [24]. Phosphorylation at these two tyrosines has been shown to be phosphorylated by Brk are indicated in the column labeled ‘Phosphorylation sites’ and the references for the data summarized in the table are in the right hand column.

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Figure 1

Brk acts as a mediator for multiple signaling pathways. (a) Tyrosine phosphorylation by Brk decreases Sam68 (as well as SLM-1 and SLM-2, not shown) RNA-binding activity. (b) Brk phosphorylation enhances Stat3 and Stat5 transcriptional activity. (c) Brk signaling downstream of ErbB receptors activates multiple signaling pathways which can lead to changes in the gene programs expressed by cells.
to create binding sites for CrkII, leading to Rac activation [31,32]. In a separate study, Brk was shown to phosphor-
ylate Y1105 on p190RhoGAP-A (p190) [27]. Previous
studies have shown that phosphorylation of Tyr1105 in
p190 increases p190 association with p120RasGAP, lead-
ing to Rho inhibition and Ras activation [33,34]. Brk
specifically phosphorylates STAT3 and STAT5a/b on
tyrosines that are essential for transcriptional activation,
Y705 and Y694/699, respectively [22,23]. Overall, Brk-
induced phosphorylation of identified substrates has been
shown to promote mitogenic and cell migration pathways
(Figure 1).

ErbB family signaling
The first reports to investigate Brk-mediated signaling
events focused on the EGF pathway. Kamalati et al.
demonstrated an interaction between Brk and the ErbB
family member EGFR and found that exogenous expres-
sion of Brk in normal mammary epithelial cells enhanced
EGF-induced proliferation [35]. Subsequent studies have
found that Brk can also interact with additional
ErbB family members, ErbB2, ErbB3, and ErbB4
[17,36,37]. Brk overexpression in normal mammary epithe-

telial cells enhanced the interaction between ErbB3 and
PI3K, and lead to an increase in tyrosine phosphoryla-
tion of ErbB3 and PI3K/Akt signaling in response to EGF
[36]. Alternatively, Brk was found to enhance ERK1/2
activation in ErbB2 overexpressing cells [17].

Additional studies have also implicated Brk downstream
of ErbB signaling pathways (Figure 1c). Chen et al.
showed that EGF induced Brk autophosphorylation,
and identified paxillin as a Brk substrate. Brk-induced
paxillin phosphorylation promoted Rac activation and
cellular migration [24]. In support of this study, our
group has shown that Brk kinase activity is activated
by the ErbB receptor ligand, heregulin (HRG). We also
found that HRG-induced Rac activation is dependent on
Brk expression, and that Brk is required for HRG-
induced activation of p38 MAPK and ERK5 [12]. Other
proteins that have been shown to be phosphorylated by
Brk downstream of EGF are the RNA-binding proteins
Sam68 and PSF [21] and p190RhoGAP [27].

Brk and Akt
Kamalati et al. were the first to show that overexpression
of Brk could potentiate the activation of the PI3K/Akt
pathway [36]. Subsequently, our group has shown that
Brk and Akt interact in both breast cancer cell lines
(Figure 2a) and Cos-1 (African green monkey kidney)
cells [28]. Unexpectedly, we found that WT (wild-type)
but not kinase inactive (KM) Brk inhibited Akt kinase
activity in the purified Brk/Akt complex, and that this
intracellular interaction was tightly regulated by EGF
signaling. In Cos-1 cells, EGF stimulation resulted in the
dissociation of the Brk/Akt complex. EGF-induced dis-

cipation only occurred in the presence of WT or KM Brk,
but not when the constitutively active mutant (YF) of Brk
was expressed. Interestingly, the Brk/Akt complex did
not dissociate in response to EGF in T47D cells,
suggesting that in these breast cancer cells, Brk behaves
similarly to YF-Brk and may be constitutively active [28].
More recent studies in PTK6 knockout mice also suggest
that Brk inhibits Akt in differentiating murine intestinal
epithelial cells [38,39]. The small intestine of PTK6
mice displayed elevated activation of Akt, an expanded
zone of PCNA expression and increased BrdU incorpora-
tion [39]. Furthermore, when PTK6 mice were
challenged with gamma-irradiation, decreased apoptosis
and increased activation of Akt was observed in the small
intestine [38]. Together these studies suggest that Brk
signals differently during normal development relative to
cancer progression; Brk may function to limit Akt activity
in normal cells or when growth factors are low, but this
fails to occur in cancer cells, where Brk may somehow
amplify growth factor-dependent signaling inputs to PI3K/Akt pathway activation (Figure 2a).

**STAT family signaling**

Brk has also been shown to play a role in the activation of STAT signaling pathways. Liu et al. demonstrated that Brk expression induced tyrosine phosphorylation of STAT3 and showed that immunoprecipitated Brk phosphorylated purified GST-STAT3 [23]. Furthermore, Brk expression enhanced STAT3-dependent transcription. The adapter protein BKS/STAP-2 was the first Brk interacting protein identified and shown to be phosphorylated on tyrosine residues in Brk overexpressing cells [40]. More recently, STAP-2 Tyr-250 was identified as a Brk phosphorylation site [25]. Furthermore, STAP-2 Tyr-250 phosphorylation was found to be important for Brk-induced STAT3 transcriptional activation [25]. STAT5 has also been shown to be a direct substrate of Brk, and similar to STAT3, Brk-induced phosphorylation of STAT5 potentiated STAT5 transcriptional activity (Figure 1b) [22].

**Brk kinase activity**

Only a few studies have investigated the regulation of Brk kinase activity. As described above Brk kinase activity has been shown to be activated by EGF and heregulin-β1, peptide ligands that bind ErbB receptors [12,24*]. IGF1 has also been shown to enhance Brk kinase activity in MCF-7 cells [29]. In this study, IRS-4, which was also shown to be a substrate of Brk, enhanced IGF1-induced Brk autophosphorylation [29]. Similar to this study, but performed in T47D cells, we also observed an IGF-induced increase in Brk autophosphorylation, which peaked at 10 min. Additionally, we recently showed that HGF and MSP, peptide ligands for MET and RON receptors, respectively, activate Brk in both keratinocytes and breast cancer cells [51]. In addition to growth factor signaling, Brk has also been shown to be activated during keratinocyte differentiation. Two reports have shown that calcium rapidly activates the kinase activity of Brk/Sik in keratinocytes [41,42].

Structural and mutational studies on Brk have revealed that, while some similarities exist, key aspects of Brk kinase activity and substrate recognition are regulated differently than other Src family members. Brk kinase regulation is similar to other Src family kinases in that mutation of the C-terminal tyrosine (Y447 in Brk and Y527 in Src) to phenylalanine results in a constitutively active kinase [43]. One specific difference in the auto-regulation of kinase activity between Brk and c-Src occurs in the linker region. A conserved tryptophan in the linker region of c-Src is important for auto-inhibition of Src kinase activity. Unlike c-Src, when the conserved tryptophan (W184) in Brk is mutated, Brk kinase activity is completely abolished [44], suggesting that the linker region is important for Brk kinase activity, not auto-inhibition. Differences in Brk substrate binding have also been observed. It was found that the dissociation constant between a phosphorylated tyrosine peptide and the Brk SH2 domain is much weaker than other Src family SH2 domains [45]. It was also reported that the Brk SH3 domain plays an important role in substrate recognition [46]. Qiu et al. found that an SH3 binding substrate had a significantly lower binding affinity relative to a control substrate, while no difference was seen between an SH2 binding substrate and the control. The results of this study suggest that Brk SH3 domain interactions likely govern the specificity of Brk substrate phosphorylation [46].

**Brk-mediated biology**

Brk appears to participate in a variety of signaling pathways depending on the context in which it is expressed. For example, tissue type, hormonal milieu, physiologic status (i.e. normal or cancer) and the expression levels of fellow signaling molecules may dictate the specificity of Brk action. As a result, Brk has been shown to elicit a multitude of biological effects on cells both in culture and in mouse models. In normal or non-tumorigenic tissues, Brk has largely been shown to promote cellular differentiation, apoptosis, and more recently to mediate migration/wound healing [51]. However, in malignant settings Brk is reported to contribute to cancer progression by sensitizing cells to mitogenic signals and enhancing proliferation, anchorage-independent survival, and migration/invasion (see Table 1).

**Differentiation**

The major biological activity of Brk in the normal or non-malignant tissues of the skin and intestine, where it is most abundantly expressed, is to function as an inducer of cellular differentiation. The expression of Brk and mouse homolog, Sik, occurs at high levels in differentiated, nondividing epithelial cells [4,14]. Overexpression of Sik in mouse keratinocytes resulted in the upregulation of a known differentiation marker [41]. Likewise, differentiation of human keratinocytes induced Brk expression which is in turn required for increased expression of Keratin 10, a marker for differentiation [42]. Furthermore, Sik knockout mice display increased growth and proliferation in the small intestine, in addition to decreased expression of a well-characterized differentiation/maturity marker, I-FABP.

**Cell cycle progression**

Brk overexpression in cells derived from normal breast, MCF10-A and HB4a cells, results in increased sensitivity to the mitogenic effects of EGF [35*]. Similarly, HB4a cells displayed increased transformation potential in soft agar (anchorage-independent growth) assays when exogenous WT Brk was stably expressed, but not kinase-dead (KM) Brk [35*]. These data suggest that Brk expression in ‘normal’ cells of the breast confers a
proliferative and/or survival advantage. The requirement for Brk kinase activity in these events appears to be cell-type dependent; overexpression of either WT or KM Brk induced increased EGF-stimulated or serum-stimulated proliferation of T47D cells [47] and HeLa cells (Lange, unpublished results). Likewise, in T47D breast cancer cells, loss of Brk expression via transient or stable introduction of Brk-specific RNAi resulted in decreased proliferation [12*,47].

While the initial reports in immortalized mammary epithelial cells suggest Brk overexpression potentiates EGF-induced proliferation, we have found that in human mammary epithelial cells (HMECs) immortalized with hTERT and SV40 early region, Brk expression inhibits EGF-induced proliferation (Figure 3). Furthermore, the effect of Brk in these cells is dependent on Brk kinase activity. In contrast to wt and constitutively active (YF) Brk, expression of KM Brk failed to inhibit EGF-induced proliferation. One explanation for these results may be the relative expression of ErbB family members in the different cell models. As shown in Figure 3, the HMEC model expresses low levels of ErbB2, ErbB3, and ErbB4.

Recently, Brk has been shown to be co-amplified with ErbB2 in breast cancers [17]. Coexpression of Brk and ErbB2 in MCF10-A cells induces cell cycle progression by increasing Cyclin E and decreasing p27 levels. Together, Brk and ErbB2 reinitiate cell proliferation in MCF10-A 3D acinar structures leading to a transformed phenotype. Additionally, Brk expression decreases the efficacy of the ErbB2 kinase inhibitor, Lapatinib, indicating that Brk contributes to the development of resistance to current ‘receptor targeted’ therapeutic strategies. These data suggest that the activation of Brk downstream of ErbB family members [12*] should be evaluated as a potentially valuable drug target.

The participation of Brk in STAT pathways has also been demonstrated to stimulate cell growth. Liu et al. reported that Brk is able to induce transcriptional activation of STAT3 leading to a cooperative increase in cell proliferation [23]. Similarly, knockdown studies showed that Brk-induced activation of STAT5 is a proliferative signal in breast cancer cells [22].

Interestingly, Brk kinase activation in some non-breast cell models has been reported to decrease cell proliferation. In U2OS human osteosarcoma cells overexpression of Brk along with PSF, a Brk substrate, induces S-phase cell cycle arrest [21]. PSF phosphorylation by Brk alters its subcellular localization, decreases its RNA-binding ability, and promotes PSF induced cell cycle arrest [21]. Similarly, overexpression of the Brk substrate, Sam68, decreases exogenously expressed Brk-induced proliferation in astrocytes [18]. These data illustrate that Brk is able to participate in a variety of biological signals.

Figure 3

(a) Western blot analysis for the expression of Brk, ErbB1, ErbB2, ErbB3, ErbB4 and Actin. Total cell lysates were collected from asynchronous HeLa, T47D, and HMEC cell lines. Brk overexpressing HMECs were generated by infecting SV40-LT/hTERT immortalized parental HMECs (P) with retrovirus encoding wild-type (WT), kinase inactive (KM), or constitutively active (YF) Brk and then selecting for infected cells with zeomycin. Parental HMECs were also infected with retrovirus containing the vector control (V), pBabeZeo. (b) Brk kinase assay from HMEC cell lines described in (a). Brk was immunoprecipitated from HMECs with a Brk-specific antibody. Immunoprecipitated Brk was then incubated with recombinant Sam68 in the presence of ATP. Brk and Sam68 were then resolved by SDS-PAGE and western blotting for tyrosine phosphorylated proteins (4G10) was performed. The amount of Brk immunoprecipitated and present in the total cell lysate was determined by western blotting. (c) Brk expression inhibits EGF-induced proliferation in HMECs. Representative MTT assay from HMEC cell lines described in (a). Cells were plated at 1 x 10⁴ cells per well in a 24-well plate in the absence of growth factors. The following day (Day 0) cells were treated with 10 ng/mL EGF or left untreated. MTT readings were obtained on days 0, 3, and 6. All data points were divided by the Day 0 reading to give a fold increase. Error bars represent standard deviation.
that vary both with substrate (availability or location) and with cell-type (tissue or physiologic condition).

Migration
Accumulating evidence supports a direct involvement for Brk in the processes of migration and invasion that characterize metastatic breast malignancy. An early report by Chen et al. placed Brk upstream of paxillin, GrkII, and Rac1 to activate migration and invasion programs in skin (A431) and breast (MDA-MB231) cancer cell lines [24*]. We subsequently found that T47D breast cancer cells are dependent upon Brk expression for their migratory response to EGF and heregulin-beta1. Activated Brk downstream of ErbB receptors increased Rac1 activity, and stimulated increased p38 MAPK and Erk5 phosphorylation, leading to the elevation of cyclin D1 and activation of the MEF2 transcription factor. The Brk/Rac1/p38 MAPK pathway is required for EGF or Heregulin-beta1 induced breast cancer cell migration [12*]. In BT20 breast cancer cells expression of constitutively active Brk induced cells to migrate in transwell assays via phosphorylation of KAP3A, while KM Brk caused little more than control level migration [26]. Recently, Brk-induced phosphorylation of p190RhoGAP-A was found to play a role in EGF-induced motility and proliferation in mouse embryonic fibroblasts (MEF) cells and, migration, invasion, and proliferation in MDA-MB231 breast cancer cells [27]. Interestingly, HGF-induced migration of T47D and MDA-MB231 cells is dependent upon Brk and ERK5 complexes, but can occur independently of Brk kinase activity [51]. These data suggest that Brk employs a variety of signaling pathways and complex mechanisms to induce cell migration, but support the notion that in a subset of tumors, Brk may primarily act to promote cellular migration, invasive behavior and metastasis.

Angiogenesis
Wt Brk, but not KM Brk, has also been shown to promote angiogenesis in response to the chemokine, osteopontin (OPN). MDA-MB-231 cells respond to osteopontin by activating Brk, NF-kB, and ATF-4 signaling which leads to paracrine and juxtacrine signaling inducing VEGF dependent endothelial cell and breast cancer cell motility [48]. These data were validated in vivo using a matrigel plug assay which showed that OPN promoted angiogenesis and tumor cell proliferation in a VEGF dependent manner [48].

Apoptosis and autophagy
Brk kinase activity has been reported to play competing roles in cell death pathways, dependent upon the context of its expression. In breast cancer cells, oncogenic Brk functions to protect cells from autophagic induced death under anchorage-independent conditions [49], whereas in normal (non-malignant) tissues proto-oncogenic Brk sensitizes epithelial cells of the skin and intestine to apoptosis induced by serum deprivation and DNA damage [50]. These findings were confirmed in vivo in the intestine of Sik KO mice; following total body irradiation Brk expression was induced and subsequently promoted cellular apoptosis in wt but not KO mice [38*].

Summary
Together, the findings discussed herein suggest a divergent role for Brk as a regulatory proto-oncogene in selected tissues compared to the tumor-promoting activities that oncogenic Brk clearly displays in malignant settings, where it appears to promiscuously use growth factor (erbB, IGF, MET) receptor-directed pathways to contribute to disease progression. Although important roles for Brk kinase activity (i.e. Brk-mediated phosphorylation events) have been clearly shown to mediate effects on cell biology in some settings, scaffolding activities of Brk also appear to sufficiently regulate biology in other contexts. Going forward, it will be important to fully characterize the role of Brk substrate molecules and interacting proteins in order to discern the importance of Brk kinase activity relative to Brk domain (i.e. SH2 and SH3) structure as potential contributors to aberrant cancer cell biologies. Further studies on the regulation of Brk expression as early tumors progress may provide additional clues to how cancer cells adapt in order to evade host defenses and escape clinical interventions. Brk expression may inform therapy decisions. For example, Brk (as a mediator of apoptosis) may be a liability for cells exposed to radiation or classical chemotherapies aimed at inducing DNA damage [50]. On the other hand, Brk (as a downstream effector of erbB2) may provide tumors with an advantage over newer ‘targeted’ therapies (i.e. herceptin and other antibodies) aimed at the level of cell surface growth factor receptors [17]. Thus, cell (tumor type and stage) specific mechanisms of Brk action are vital considerations as we continue to navigate the era of ‘rational drug design’ characterized by the use of combination and/or sequential delivery of cancer therapies.

Acknowledgements
We would like to thank Ming Qiu for technical support. This work was supported by NIH R01 CA107547-01 (CAL), ACS Research Scholar Award RSG TBE-107800 (CAL), and National Research Service Award 1F32CA112844-01A2 (JHO).

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest


Chen HY, Shen CH, Tsai YT, Lin FC, Huang YP, Chen RH: Brk activates rac1 and promotes cell migration and invasion by phosphorylating paxillin. Mol Cell Biol 2004, 24:10558-10572. Chen et al. were the first to show that Brk expression can enhance cellular migration and invasion. This group was also the first to demonstrate that paxillin is a Brk substrate and Brk-induced phosphorylation of paxillin promotes Rac activation.


This paper and Ref. [38] define a role for Brk in normal epithelial cell biology that is clearly different to what is observed in cancer models. The PTK6 knockout mice displayed increased proliferation and enhanced Akt activation in the small intestine. Loss of PTK6 also lead to a decrease in the expression of specific markers of differentiation. Furthermore, when the PTK6 knockout mice were challenged with irradiation, it was found that PTK6 expression enhanced DNA damage-induced apoptosis.


