

Interactions of STAP-2 with BRK and STAT3/5 in Breast Cancer Cells

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1. Introduction

A number of molecular markers have recently been identified that may have prognostic value in breast cancer, and among the most notable of these are the estrogen receptor (ER) and the progesterone receptor (PR). ER/PR-positive breast cancer patients (60–80%) are hormone-responsive and therefore have a significantly better prognosis compared with ER/PR-negative patients (Clark and McGuire, 1983; Early Breast Cancer Trialists' Collaborative Group, 2005; Thorpe, 1988). However, approximately 50% of patients with advanced disease do not respond to endocrine therapy (Normanno et al., 2005).

Another well-known prognostic marker is epidermal growth factor receptor-2 (ErbB2; also known as HER2), which is a member of the epidermal growth factor receptor (EGFR) family. In approximately 30% of human breast cancers, ErbB2 is present at levels significantly above those found in normal cells (Hynes and Stern, 1994; Stern, 2000; Yarden, 2001). Recent studies have indicated that ErbB2 plays important roles in malignant transformation and tumorigenesis (Hudziak et al., 1987; Slamon et al., 1987, 1989). Therefore, breast cancer tumors that involve large amounts of ErbB2 protein are correlated with poor clinical outcomes (Menard et al., 2001). Herceptin (also known as Trastuzumab), a humanized monoclonal antibody against the extracellular domain of ErbB2, is used to treat patients expressing high levels of ErbB2. Although Herceptin significantly decreases the rates of breast cancer recurrence and mortality (Piccart-Gebhart et al., 2005; Slamon et al., 2001; Ward et al., 2009), almost 50% of patients with the ErbB2-amplified tumors do not respond to this treatment and develop resistance to the drug (Slamon et al., 2006).

Conversely, tumors with neither hormone receptor expression nor ErbB2 amplification are classified as triple-negative (TN) tumors (Sorlie et al., 2003). TN tumors are an aggressive subtype of breast cancer that account for approximately 15% of breast cancer cases. The TN tumor shows a higher histologic grade, and a worse prognosis compared with that of hormone receptor-positive or ErbB2-positive tumors (Dent et al., 2007; Liedtke et al., 2008). Therapeutically, despite being highly chemosensitive, their progression-free time is generally short (Dent et al., 2007; Liedtke et al., 2008), and TN tumors develop resistance to endocrine therapy and Herceptin, illustrating the urgent need for novel therapeutic strategies. Recently, new potential therapeutic targets for this type of breast cancer have been discovered, including poly-(ADP-ribose)-polymerase 1

(Bryant et al., 2005; Evers et al., 2008; Tutt et al., 2009), vascular endothelial growth factor receptor (Mendel et al., 2003), EGFR (Corkery et al., 2009; Pal and Mortimer, 2009), SRC tyrosine kinase (Conlin and Seidman, 2008; Finn, R.S., 2007), mammalian target of rapamycin (Saal et al., 2005), heat shock protein 90 (Caldas-Lopes et al., 2009), breast tumor kinase (BRK) and signal transducing adaptor protein-2 (STAP-2) (Mitchel et al., 2000; Ikeda et al., 2009, 2010, 2011).

STAP-2 is a recently identified adaptor protein, which contains pleckstrin homology (PH) and Src homology 2 (SH2)-like domains, as well as a signal transducer and activator of transcription 3 (STAT3)-binding motif in its C-terminal region (Minoguchi et al., 2003). Importantly, human STAP-2 was originally identified as BKS and is a substrate for BRK, a non-receptor protein tyrosine kinase (PTK) known alternatively as protein tyrosine kinase 6 (PTK6) (Mitchell et al., 2000). BRK is highly expressed in human breast cancer cells (Barker et al., 1997). Recently, STAP-2, STAT3, and STAT5A/B have been identified as BRK substrates. However, the molecular mechanism by which the STAP-2-BRK-STAT3/5 axis participates in the tumorigenesis in breast cancer remains poorly characterized. In this review, we focus on the STAP-2-BRK-STAT3/5 axis as a potential therapeutic target and/or prognostic marker and demonstrate a functional link between STAP-2 and BRK/STAT3/5-mediated transcriptional activation and cell growth in human breast cancer cells.

2. STAP-2

2.1 Structure and expression

Human STAP-2 is the first identified substrate for the BRK non-receptor tyrosine kinase (Mitchell et al., 2000). We also cloned murine STAP-2 as a *c-fms* interacting protein (Minoguchi et al., 2003). PTKs play an important role in regulating cell growth, differentiation, and transformation. Activated receptor tyrosine kinases transphosphorylate several tyrosines in their cytoplasmic domains, providing recognition sites for various adaptor and effector proteins in multiple signal transduction pathways. Adaptor proteins often function as inter- or intra-molecular bridges and thereby play an important role in the assembly of larger protein complexes or in the stabilization of certain conformational states. They also utilize their functional domains, such as SH2 and SH3 domains, to mediate interactions that link various proteins involved in signal transduction.

STAP-1 was cloned as a c-kit-interacting protein and bears high sequence and structural similarity to its sister protein, STAP-2 (Masuhara et al., 2000) (Fig. 1A). Both STAP-1 and STAP-2 contain an N-terminal PH domain and a region weakly related to an SH2 domain (overall 33% amino acid identity). The N-terminal PH domains of the STAP proteins share 36% amino acid identity and 58% similarity. In the absence of stimulation, over-expressed STAP-2 protein localizes throughout the cytoplasm and nucleus in A431 epidermoid carcinoma cells, but translocates to the plasma membrane following stimulation of EGFR. A mutant STAP-2 lacking the N-terminal PH domain (Δ PH) fails to localize at the plasma membrane, demonstrating that the PH domain of STAP-2 is necessary and sufficient for plasma membrane recruitment by EGFR stimulation.

The central region of STAP-2 is distantly related to the SH2 domain. This region of STAP-2 shares 40% sequence identity with that of STAP-1 and 29% sequence identity with the SH2 domain of human phospholipase C- γ 2. However, STAP-2 has a C-terminal proline-rich

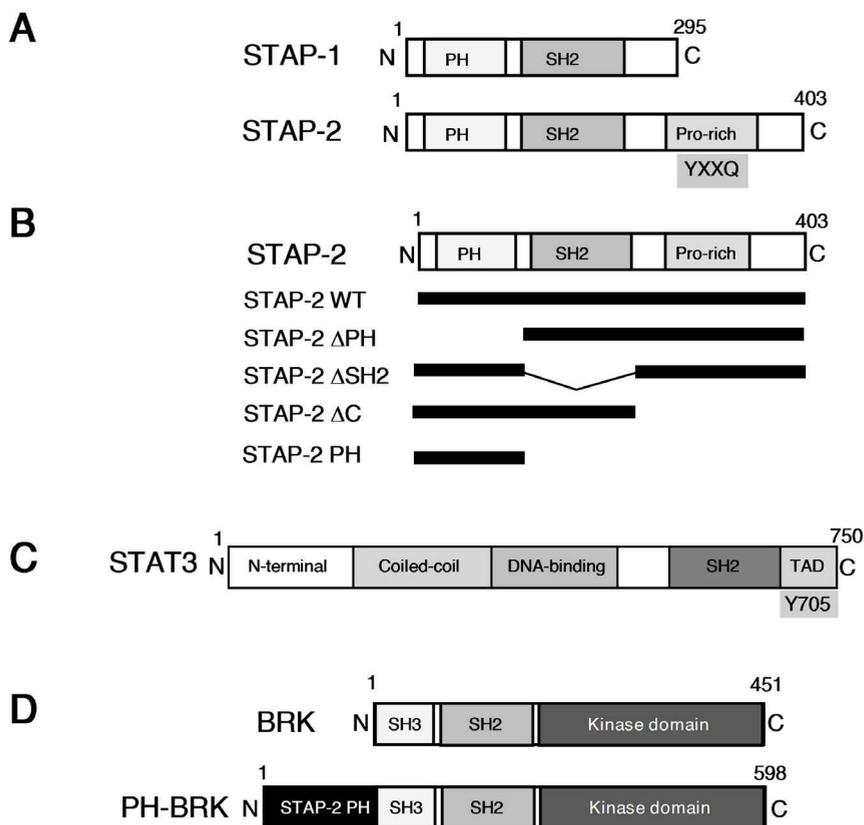


Fig. 1. Schematic diagrams of the domain structures of the STAP-1 (A), STAP-2 (A, B), STAT3 (C), BRK and the STAP-2 PH-BRK fusion protein (D).

region and a STAT3-binding motif, YXXQ, both of which are absent from STAP-1. Cytokine receptors commonly utilize a YXXQ motif in their cytoplasmic regions to recruit and activate STAT3 (Hirano et al., 2000). STAP-2 is expressed in a variety of tissues and cells such as lymphocytes, macrophages and hepatocytes (Minoguchi et al., 2003), and its abundant expression pattern (Fig. 2A) suggests that STAP-2 influences a variety of signaling or transcriptional molecules. STAP-1, however, shows a more restricted expression pattern, being located predominantly in hematopoietic cells (Masuhara et al., 2000). Notably, STAP-2 is constitutively expressed in macrophages, and the 5' region of the STAP-2 genomic sequence contains several potential binding sites for c-Rel, AP-1, p65/NF- κ B and STATs. In the murine myeloid leukemia cell line, M1, STAP-2 mRNA expression is strongly induced by LIF in parallel with its differentiation into macrophages. These expression patterns of STAP-2 support a paradigm whereby STAP-2 in macrophages mediates signals for acute-phase responses after infection. Indeed, LPS- or IL-6-stimulated induction of acute phase protein genes was significantly decreased in STAP-2-deficient hepatocytes (Minoguchi et al., 2003).

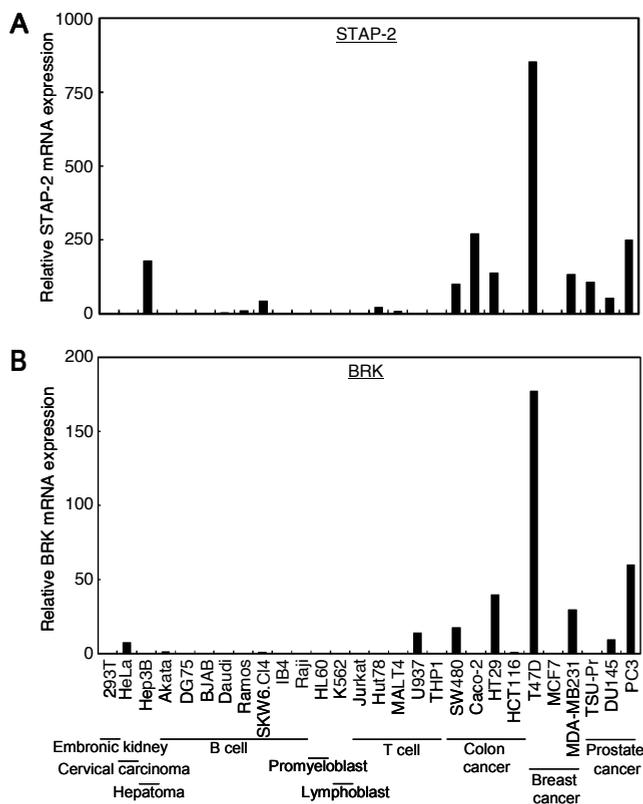


Fig. 2. Expression profile of STAP-2 and BRK. Expression of STAP-2 and BRK in a variety of human cell lines (A, B). Total RNA samples isolated from these cells were also subjected to quantitative real-time PCR analysis using *STAP-2* and *BRK* primers. Data represent the levels of these mRNA normalized to that of an *ACTIN* internal control and are expressed relative to the value of 293T samples. Shown is a representative experiment, which was repeated at least three times with similar results.

2.2 Interacting proteins

Recently, many STAP-2 binding partners have been identified. As summarized in Table 1, STAP-2 interacts with, and modulates the function of, several signaling molecules including STAT3/5- (Minoguchi et al., 2003; Sekine et al., 2005), Fc ϵ RI- (Yamamoto et al., 2003), M-CSFR/c-FMS- (Ikeda et al., 2007) and Toll-like receptor-mediated signals (Sekine et al., 2006). STAP-2 interacts with STAT3 through the C-terminal YXXQ motif and enhances STAT3 transcriptional activity. STAP-2 also interacts with STAT5 through its PH and SH2-like domains. It is noteworthy that thymocytes and peripheral T cells from STAP-2-deficient mice show enhanced IL-2- or TCR-dependent cell growth (Sekine et al., 2005). STAP-2 positively regulates LPS/TLR4-mediated signals in macrophages. STAP-2, particularly its SH2-like domain, binds to both MyD88 and IKK- α/β , but not to TRAF6 or IRAK1, and

forms a functional complex composed of MyD88-STAP-2-IKK- α/β . These interactions augment MyD88- and/or IKK- α/β -dependent signals, leading to enhancement of NF- κ B activity (Sekine et al., 2006).

Interacting protein	Domain or region	References
BRK	PH, (SH2-like)	Ikeda et al., 2009, 2010, 2011; Mitchel et al., 2000
STAT3	YXXQ motif, (SH2-like)	Ikeda et al., 2010; Minoguchi et al., 2003
STAT5A/B	PH, SH2-like	Sekine et al., 2005
c-Fms	PH, (SH2-like)	Ikeda et al., 2007
PLC γ 1/2	SH2-like, C-terminal	Yamamoto et al., 2003
MyD88	SH2-like	Sekine et al., 2006
IKK α/β	SH2-like	Sekine et al., 2006
LMP1	PH, SH2-like	Ikeda et al., 2008
TRAF1	Not determined	Ikeda et al., 2008
TRAF3	PH, SH2-like	Ikeda et al., 2008
FAK	SH2-like	Sekine et al., 2007
Cbl	PH, SH2-like	Sekine et al., 2007
Vav1	SH2-like, C-terminal	Sekine et al., 2009
Rac1	Not determined	Sekine et al., 2009

Table 1. STAP-2 interacting proteins.

M-CSFR/c-FMS directly interacts with the PH domain of STAP-2 independently following M-CSF-stimulation (Ikeda et al., 2007). STAP-2 regulates M-CSF-induced tyrosine phosphorylation of M-CSFR/c-FMS as well as the activation of Akt and extracellular signal regulated kinase. In addition, over-expression of STAP-2 results in the impairment of migration in response to M-CSF and of the wound-healing process in macrophages. This demonstrates that STAP-2 directly binds to M-CSFR/c-FMS and interferes with PI3K signaling, leading to macrophage motility. In T cells, STAP-2 enhances Cbl-dependent degradation of FAK and downregulates integrin/FAK-mediated cell adhesion to fibronectin (Sekine et al., 2007). Furthermore, STAP-2 constitutively interacts with, and enhances the tyrosine phosphorylation of, a GDP/GTP exchange factor, Vav1, and also binds to a small GTPase, Rac1 (Sekine et al., 2009). These interactions control chemokine-induced chemotaxis of T cells.

STAP-2 also interacts with Epstein-Barr virus (EBV)-derived latent membrane protein 1 (LMP1) and negatively regulates LMP1-induced NF- κ B activation (Ikeda et al., 2008). EBV is linked to the development of multiple malignancies, including post-transplant lymphoma, Hodgkin disease, and nasopharyngeal carcinoma (Thorley-Lawson, 2001). EBV-LMP1 is expressed in many EBV-associated tumor cells and is responsible for most of their altered cellular growth properties (Brinkmann and Schulz, 2006). STAP-2 associates with LMP1 through the PH and SH2-like domains, and this interaction occurs physiologically in EBV-positive human B cells. STAP-2 regulates LMP1-mediated NF- κ B signaling through direct or indirect interactions with TRAF3 and TRADD. Importantly, STAP-2 mRNA is induced by expression of LMP1 in human B cells, and transient expression of STAP-2 in EBV-positive human B cells decreases cell growth. These data suggest that STAP-2 responds to EBV infection and acts as an endogenous negative regulator of EBV-LMP1-mediated signaling through TRAF3 and TRADD.

3. BRK

3.1 Structure and expression

BRK was originally isolated from a metastatic breast carcinoma (Mitchell et al., 1994). An identical protein was independently cloned as a highly-expressed protein tyrosine kinase, PTK6, from human melanocytes (Lee et al., 1993). In addition, *Ptk6* (previously termed Sik), a cDNA for the mouse ortholog that has 80% amino acid identity to BRK/PTK6, was also cloned from mouse intestinal crypt cells (Siyanova et al., 1994). BRK displays approximately 56% homology to the kinase domain of c-Src and a similar domain arrangement (Serfas and Tyner, 2003). BRK is a 451 amino acid protein that contains an SH3 domain, an SH2 domain and a tyrosine kinase catalytic domain, but lacks an N-terminal myristoylation site for membrane targeting (Serfas and Tyner, 2003). BRK is expressed in many malignancies, such as colon and prostate tumors and metastatic melanomas (Derry et al., 2003; Easty et al., 1997; Llor et al., 1999; Schmandt et al., 2006). BRK expression is also detected in a large proportion of human mammary gland tumors, but is not expressed in normal mammary gland (Barker et al., 1997). In normal tissues, BRK expression is developmentally regulated and restricted to differentiating epithelial cells in a range of tissues including small intestine and colon (Vasioukhin et al., 1995; Haegebarth et al., 2005, 2006) (Fig. 2B).

3.2 Substrates, interacting proteins and activation

Several BRK-interacting proteins or substrates have been identified (Table 2). BRK substrates include RNA-binding proteins (Sam68 (Coyle et al., 2003; Derry et al., 2000; Lukong et al., 2005), SLM-1/2 (Haegebarth et al., 2004), and the polypyrimidine tract-binding protein-associated splicing factor (PSF) (Lukong et al., 2009)), transcription factors (STAT3 (Liu et al., 2006) and STAT5A/B (Weaver and Silva, 2007)), adaptor molecules (STAP-2) (Mitchell et al., 2000), and a variety of signaling molecules (paxillin (Chen et al., 2004), p190RhoGAP (Shen et al., 2008), kinesin-associated protein 3A (KAP3A) (Lukong and Richard, 2008), Akt (Zhang et al., 2005), β -catenin (Palka-Hamblin et al., 2010), and ARAP1 (Arf-GAP, Rho-GAP, ankyrin repeat and PH domain-containing protein 1; also known as centaurin δ -2) (Kang et al., 2010)). Although BRK expression is known to induce tyrosine phosphorylation in some of these, similar actions in others have yet to be confirmed.

Sam68 is the most extensively studied BRK substrate. BRK expression suppressed cell proliferation through EGFR-mediated phosphorylation of Sam68 in a human breast cancer cell line (Coyle et al., 2003; Derry et al., 2000; Lukong et al., 2005). Similarly, BRK phosphorylates the Sam68-like mammalian proteins, SLM-1 and SLM-2, and negatively regulates their RNA-binding functions (Haegebarth et al., 2004). Downstream of EGFR, PSF is a BRK substrate, and this tyrosine phosphorylation of PSF induces cytoplasmic relocation, impairment of its binding to polypyrimidine RNA, and cell cycle arrest (Lukong et al., 2009). Furthermore, BRK expression promotes cell migration and tumor invasion by phosphorylating the focal adhesion protein, paxillin, followed by activation of the small GTPase, Rac1, *via* the function of CrkII (Chen et al., 2004). BRK also phosphorylates p190RhoGAP-A (regulating the small GTPases, RhoA and Ras, and promoting breast malignancy) (Shen et al., 2008); a kinesin-2 subunit, KAP3A (promoting cell migration) (Lukong and Richard, 2008); and Akt (regulating basal Akt activity in normal cells) (Zhang et al., 2005).

Substrate	Function	Phosphorylation site	References
SAM68	Cell cycle	Y345, Y434, Y440	Coyle et al., 2003; Derry et al., 2000; Lukong et al., 2005
SLM-1/SLM-2	Not validated	Not determined	Haegebarth et al., 2004
PSF	Cell cycle	C-terminus	Lukong et al., 2009
STAT3	Cell cycle	Y705	Liu et al., 2006
STAT5A/B	Cell cycle	Y694/Y699	Weaver and Silva, 2007
STAP-2/BKS	Cell growth	Y250	Ikeda et al., 2009; Mitchel et al., 2000
Paxillin	Migration	Y31, Y118	Chen et al., 2004
p190RhoGAP	Migration	Y1109	Shen et al., 2008
KAP3A	Migration	C-terminus	Lukong and Richard, 2008
Akt	Intestinal epithelial cell differentiation	Not determined	Zhang et al., 2005
beta-catenin	Cell growth	Y64, Y142, Y331, Y333	Palka-Hamblin et al., 2010
ARAP1/centaurin-2	Regulation of EGFR internalization	Y231	Kang et al., 2010
BRK	Regulation of kinase activity	Y13, Y61, Y66, Y114, Y342, Y351	Qiu and Miller, 2002

Table 2. BRK substrates.

Mice deficient in the BRK murine ortholog, Sik, show increased cell proliferation, decreased apoptosis, and increased levels of activated Akt in the small intestine (Haegebarth et al., 2006), suggesting a role for BRK in differentiation of epithelial cells of the small intestine. This is further implied by the evidence that BRK directly phosphorylates and inhibits β -catenin, interrupting T-cell factor-mediated transcription in the intestine, and indicating that BRK may be a negative regulator of the Wnt-signaling pathway (Palka-Hamblin et al., 2010).

BRK may contribute to tumorigenesis by modulating EGF/EGFR signaling. It phosphorylates ARAP1, which results sequentially in inhibition of EGFR internalization, increased duration of EGF/EGFR signaling, and increased oncogenic capacity (Kang et al., 2010). Importantly, over-expression of BRK sensitizes human mammary epithelial cells to EGF and/or heregulin stimuli, and increases anchorage-independent growth (Kamalati et al., 1996, 2000), while down-regulation of BRK also influences EGF- and heregulin-induced cell proliferation. These observations suggest that BRK is involved in signaling induced by members of the EGFR family (Ostrander et al., 2007). Notably, BRK interacts with additional ErbB family members (ErbB2, ErbB3, and ErbB4) as well as EGFR (ErbB1) (Kamalati et al., 1996; Aubele et al., 2007; Xiang et al., 2008). BRK is co-amplified with ErbB2 to promote proliferation and confer resistance to lapatinib, an ErbB2 kinase inhibitor in breast cancer (Xiang et al., 2008), suggesting that BRK is a potential target in ErbB2-positive breast cancer. As mentioned above, BRK kinase activity is promoted by ligands for the ErbB receptor, such as EGF and heregulin. However, it is also activated by the expression of ErbB2 even in the absence of ligands, and by other stimuli such as IGF-1 (Qiu et al., 2005), calcium and ionomycin (Vasioukhin and Tyner, 1997; Wang et al., 2005), fetal bovine serum (Zhang et al., 2005), and osteopontin (Chakraborty et al., 2008). BRK activation occurs under the conditions already described, but also during keratinocyte differentiation, together with upregulation of expression (Wang et al., 2005).

The mechanism of regulation of BRK kinase activity is similar to SRC family kinases, with some notable differences. Structural studies reveal that BRK is autophosphorylated at Tyr-13, 61, 66, 114, 351 and 342 (Qiu and Miller, 2002). Tyr-342 (Y342 in BRK and Y416 in SRC) resides within the kinase activation loop, and phosphorylation of this residue increases kinase activity of wild-type (WT) BRK. However, mutation of Tyr-342 to alanine (Y342A) blocks activation of BRK (Qiu and Miller, 2002). In addition, mutation of the C-terminal

residue Tyr-447 (Y447 in BRK and Y527 in SRC) to Phe (Y447F) results in a constitutively active kinase (Kamalati et al., 1996). Trp-184 (W184 in BRK and W260 in SRC) lies within the SH2-kinase linker region and, in contrast to SRC family enzymes, has been shown to interact intramolecularly with residues in the BRK kinase domain. The conserved Trp184 to Ala (W184A) mutation completely inhibits kinase activity (Kim and Lee, 2005), as does the substitution of Lys-219 (K219 in BRK and K295 in SRC) with Met (K219M), which destroys the putative ATP binding site of BRK (Kamalati et al., 1996).

Unlike SRC family kinases, BRK lacks myristoylation signals for membrane targeting, allowing it some freedom in its subcellular localization and broadening its range of substrates and interacting proteins. Several studies demonstrate that the function of BRK varies between cell types and may be dependent on expression levels, kinase activity, interaction with substrates or other binding proteins and, significantly, on intracellular localization. Indeed, oncogenic functions of BRK are enhanced by targeting it to the plasma membrane but are abolished if it is modified to remain in the nucleus (Ie Kim and Lee, 2009). BRK expression is detected in both the cytoplasm and nucleus in the normal intestine, skin and oral epithelium, and in breast and colon tumors (Brauer and Tyner, 2010). In normal differentiated prostate epithelium and in well-differentiated prostate tumors, it is detected in the nucleus, but is absent from the nuclei of poorly-differentiated tumors, which may implicate it in differentiation of the prostate (Derry et al., 2003). At the present time, it is not clear how intracellular localization of BRK is regulated. Therefore, further studies are required to confirm the significance of BRK subcellular localization. Indeed, BRK does not contain any nuclear localization or export signal, suggesting that interaction with substrates such as STAP-2 or other interacting proteins may be important in regulating its distribution.

4. Signal transducer and activator of transcription (STAT) 3/5

The signal transducer and activator of transcription (STAT) family is known to mediate cell proliferation, differentiation and survival in immune responses, hematopoiesis, neurogenesis and many other biological processes (Darnell et al., 1994; Ihle, 1996; O'Shea, 1997). Seven different STAT genes have currently been identified in mammals. The encoded proteins vary in length between 750–850 amino acid residues and share 20–50% amino acid sequence identity. As shown in Fig. 1, notable features of STAT proteins are the STAT family DNA binding domain, an SH2 domain and a major tyrosine phosphorylation site at Y705 (STAT3). In general, STAT proteins bind as dimers to DNA target sites with a nine-base-pair (bp) consensus sequence, TTCCGGGAA, and binding constants in the nanomolar range. In unstimulated cells, STATs are present as monomers in the cytoplasm. STATs are activated by phosphorylation at tyrosine residues, which leads to the formation of dimers *via* reciprocal interactions between the SH2 domain of one monomer and the phosphorylated tyrosine of the other. Dimers then translocate to the nucleus where they recognize specific DNA-binding sites and induce target gene transcription. Tyrosine phosphorylation of STATs (Y705 in STAT3, and Y694/Y699 in STAT5A/B, respectively) is normally a transient and tightly regulated process. However, in tumor cells, constitutive activation of STATs is linked to persistent activity of tyrosine kinases, including Janus kinases, Src, EGFR, Bcr-Abl, and many others. These kinases might be activated by cytokines or by structural alterations. For instance, EGFR over-expression and activation may underlie STAT activation in breast, lung and head and neck cancer (Zhang et al., 2004). Constitutive activation or dysregulated expression of STATs is detected in primary tumors and cancer

cell lines, including leukemia, multiple myeloma, lymphomas, melanoma and cancers of the breast, skin, lung, ovaries, pancreas, prostate, kidney, thyroid, and head and neck (Desrivieres et al., 2006). Persistent signaling of specific STATs, particularly STAT3 and STAT5, has been demonstrated to directly contribute to oncogenesis by stimulating cell proliferation and preventing apoptosis (Bowman et al., 2000).

5. Interactions of STAP-2 with BRK and STAT3/5

5.1 BRK, STAP-2 and STAT3/5

STAP-2 is the first BRK interacting protein identified and shown to be phosphorylated. STAT3 and STAT5, which play crucial roles in cell proliferation and differentiation, are also believed to be activated by BRK (Liu et al., 2006; Weaver and Silva, 2007). Our previous studies demonstrate that STAP-2 interacts with and influences several signaling molecules, including STAT3 and STAT5. Furthermore, both STATs play fundamental roles in the normal growth and development of the mammary gland (Hennighausen et al., 1997), and are often over-expressed or constitutively-activated in breast cancer tumors (Bowman et al., 2000). It is therefore of key importance to clarify the interactions between BRK, STAP-2, and STAT3/5.

5.2 STAT3/5 activation by BRK and STAP-2

We attempted to elucidate the roles of STAP-2 in BRK-mediated transcriptional activation of STAT3/5 in breast cancer cells. Transient transfection experiments using expression of luciferase (Luc) reporter genes driven by STAT3 (STAT3-Luc) and STAT5 (STAT5-Luc) showed that STAP-2 enhances BRK-mediated activation of STAT3 and STAT5. We showed that co-expression of STAP-2 and BRK increases BRK-mediated STAT3/5 activation compared with BRK expression alone, and small-interfering RNA or short-hairpin RNA-mediated reduction of endogenous STAP-2 expression strongly decreases BRK-mediated STAT3/5 activation in T47D human breast cancer cells. To further clarify the molecular mechanisms underlying BRK/STAP-2-mediated STAT3/5 activation in breast cancer cells, we tested the effect of STAP-2 on BRK-mediated phosphorylation of STAT3/5, which is an important step for transcriptional activation. Phosphorylation of STAT3/5 is markedly enhanced in MCF7 human breast cancer cells overexpressing STAP-2. In addition, constitutive phosphorylation of STAT3/5 in control T47D cells is markedly reduced in STAP-2 knockdown cell clones. These results indicate that STAP-2 plays important roles in BRK-mediated tyrosine phosphorylation of STAT3/5 in breast cancer cells.

5.3 Molecular interactions among STAP-2, STAT3, STAT5 and BRK

We previously reported that STAP-2 binds to several functional molecules. For example, the SH2 domain of STAP-2 interacts with MyD88 and IKKs (Sekine et al., 2006), and the PH and SH2 domains of STAP-2 mediate its association with STAT5 (Sekine et al., 2005), LMP1 and TRAF3 (Ikeda et al., 2008). To assess the functional relationships among BRK, STAP-2 and STAT3/5, we determined the domains of STAP-2 responsible for BRK-mediated STAT3/5 activation. The BRK-mediated STAT3/5-Luc activity is significantly and dose-dependently enhanced by expression of STAP-2 WT and, to a slightly lesser degree, by expression of the mutant STAP-2 Δ SH2 and STAP-2 Δ C genes, which lack the SH2 domain and the C-terminal domain, respectively. Importantly, however, STAP-2 Δ PH (lacking the PH domain) does not elevate STAT3/5-Luc activity mediated by BRK. Similarly, enhanced phosphorylation of

STAT3/5 by BRK is observed in the presence of a construct encoding just the STAP-2 PH domain (STAP-2 PH). These findings indicate that the PH domain of STAP-2 plays an essential role in the BRK-mediated STAT3/5 activation. However, BRK-mediated STAT3/5 phosphorylation is incomplete in the presence of the STAP-2 PH domain compared to that induced by STAP-2 WT, indicating that other domains of STAP-2 may be required for full BRK-mediated STAT3/5 phosphorylation.

5.4 BRK kinase activity and intracellular localization

Aside from differences in phosphorylation profiles, further differences between STAP-2 WT and STAP-2 Δ PH were confirmed with confocal microscopy experiments. STAP-2 WT is distributed throughout the cytoplasm and nucleus, but STAP-2 Δ PH is located mainly in the cytoplasm. Interestingly, BRK distribution is largely consistent with that of STAP-2, being localized in the nucleus only in the presence of STAP-2 WT, and localizing entirely in the cytoplasm when the PH domain of STAP-2 is disrupted (i.e. in the presence of STAP-2 Δ PH). From this, we can conclude that STAP-2, *via* its PH domain, affects BRK distribution, probably because of its effects on the activation state of BRK. To clarify the effect of the STAP-2 PH domain on BRK activation, we used a STAP-2 PH-BRK fusion protein (PH-BRK), in which BRK is fused to the N terminus of STAP-2 PH. PH-BRK shows robust kinase activity compared with BRK WT and induces marked activation and tyrosine phosphorylation of STAT3 in breast cancer cells. Moreover, PH-BRK is mainly localized in the nucleus, although BRK is localized throughout the cytoplasm and nucleus. Therefore, the STAP-2 PH domain controls the kinase activity and localization of BRK and thus regulates BRK-mediated STAT3 activation. Similarly, PH-BRK also activates STAT5 (data not shown).

5.5 Cell growth

STAP-2 knockdown T47D clones grow more slowly than control T47D cells. Reducing the expression of BRK, STAT3, STAT5b, or STAP-2 expression in T47D cells using siRNA causes a significant decrease in cell growth, indicating that these proteins play important roles in T47D cell growth. It is noteworthy that growth is decreased to similar extents in these four T47D cell types. This may suggest that BRK-induced cell growth in T47D cells is largely dependent on STAT3/5 and STAP-2. Notably, the expression of several genes in the STAT3/5-mediated signaling pathway, including SOCS3, C/EBP δ , cyclin D1, and c-Myc, is also reduced by STAP-2 knockdown in T47D cells. Taken together, BRK/STAT3/5-mediated proliferation is a major mechanism for breast cancer cell growth, and STAP-2 plays essential roles in this process.

6. Conclusion

Activation of STAT3/5 by BRK is a critical event during the process of BRK-mediated tumorigenesis in breast cancer cells. Our manipulation of STAP-2 expression revealed essential roles of STAP-2 in this process through complex interactions between BRK, STAP-2 and STAT3/5. In particular, experiments using deletion mutants indicated that the PH domain of STAP-2 is involved in multiple processes including binding between BRK and STAP-2, activation and phosphorylation of STAT3/5, and activation of BRK. These findings suggest a model for how STAP-2 cooperates with BRK to enhance breast cancer growth. Our proposed mechanisms are now illustrated in Fig. 3. Taken together, STAP-2 plays crucial

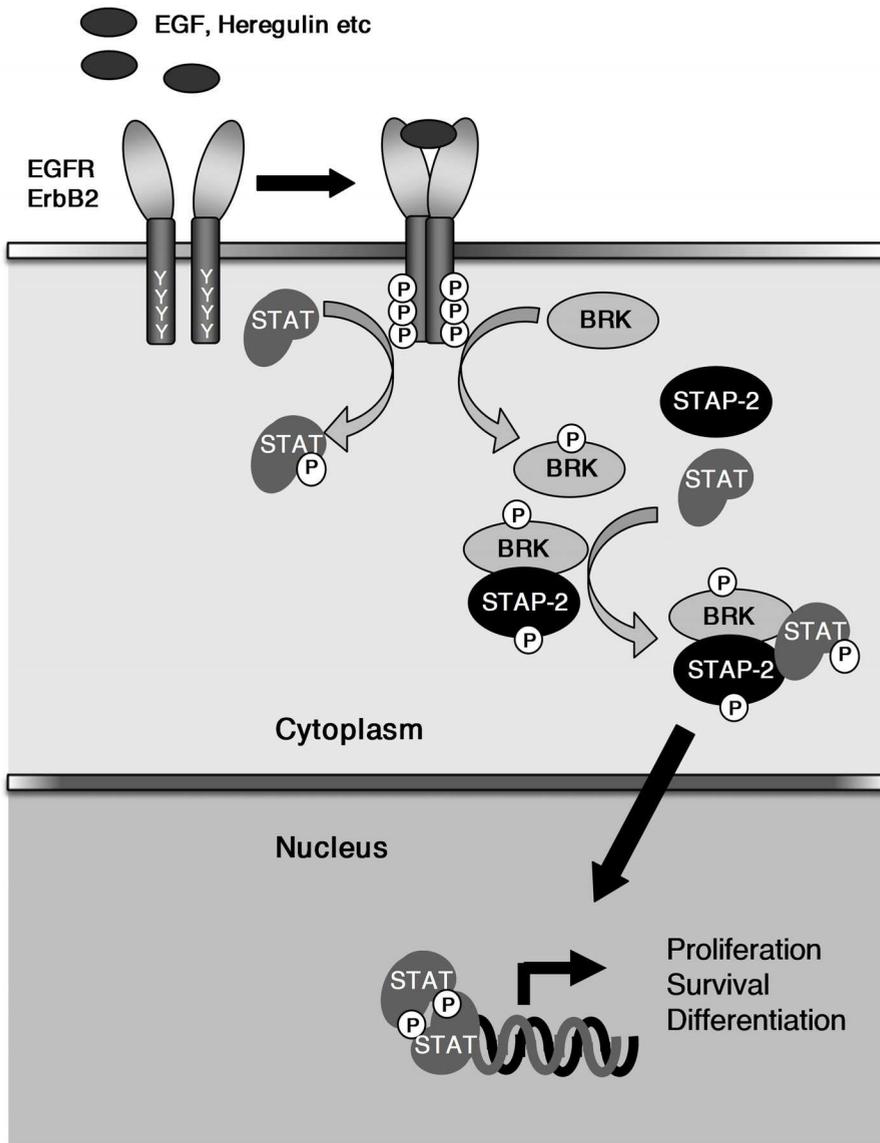


Fig. 3. Schematic showing the proposed function of STAP-2 in BRK-mediated STAT signaling. STAT activation occurs as shown *via* growth factor receptor signaling (EGFR), or nonreceptor tyrosine kinase signaling (BRK). BRK is not required when STATs bind directly to EGFR for activation (upper left), but BRK enhances activation of STATs phosphorylated by EGFR-activated BRK (upper right). In this pathway, STAP-2 can form complex with BRK and STAT, leading to activation of both BRK and STAT. Once activated, STATs dimerize and translocate to the nucleus where they activate the transcription of genes involved in proliferation, survival, and differentiation.

roles in both the BRK/STAT3 and BRK/STAT5 axes, which are major events for BRK-induced breast cancers. Our findings regarding BRK/STAP-2 interactions will therefore be helpful in developing breast cancer treatments. Furthermore, the synergistic effects of BRK and STAP-2 on STAT3/5 activation suggest that evaluating the expression of BRK together with STAP-2 may provide more useful prognostic scores for the outcomes of breast carcinomas than by measuring BRK expression alone.

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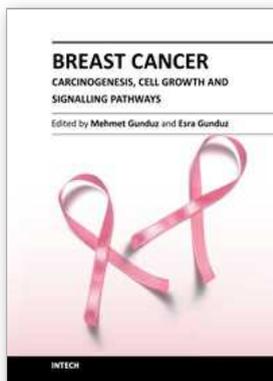
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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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