Tyrosine Phosphorylation of Sam68 by Breast Tumor Kinase Regulates Intranuclear Localization and Cell Cycle Progression □

The breast tumor kinase (BRK) is a growth promoting non-receptor tyrosine kinase overexpressed in the majority of human breast tumors. BRK is known to potentiate the epidermal growth factor (EGF) response in these cells. Although BRK is known to phosphorylate the RNA-binding protein Sam68, the specific tyrosines phosphorylated and the exact role of this phosphorylation remains unknown. Herein, we have generated Sam68 phospho-specific antibodies against C-terminal phosphorylated tyrosine residues within the Sam68 nuclear localization signal. We show that BRK phosphorylates Sam68 on all three tyrosines in the nuclear localization signal. By indirect immunofluorescence we observed that BRK and EGF treatment not only phosphorylates Sam68 but also induces its relocalization. Tyrosine 440 was identified as a principal modulator of Sam68 localization and this site was phosphorylated in response to EGF treatment in human breast tumor cell lines. Moreover, this phosphorylation event was inhibited by BRK small interfering RNA treatment, consistent with Sam68 being a physiological substrate of BRK downstream of the EGF receptor in breast cancer cells. Finally, we observed that Sam68 suppressed BRK-induced cell proliferation, suggesting that Sam68 does indeed contain anti-proliferative properties that may be neutralized in breast cancer cells by phosphorylation.

Sam68, initially identified as a c-Src-associated substrate during mitosis of 68 kDa (1, 2), has since been reported to be a substrate of other Src family tyrosine kinases as well as BRK tumor kinase (BRK) (3) and ZAP70 (4). Sam68 is a prototype of STAR proteins, so named for their implication in signal transduction and activation of RNA metabolism (5). STAR proteins contain the GSG (GRP33, Sam68, and GLD-1) or STAR domain, an evolutionarily conserved protein module initially identified by aligning the first three members of this family (6). Although the precise role of Sam68 is unknown, its multifunctionality is revealed by the presence of polyproline sequences, an RNA-binding K homology module shared by all GSG/STAR proteins and multiple potential tyrosine phosphorylation sites (5). The proline-rich sequences mediate interaction with SH3 domains of signaling molecules (7) and the WW domain containing proteins (8). The K homology domain of Sam68 has been shown to bind homopolymeric RNA poly(U), poly(A) (2, 9, 10), and synthetic RNA sequences with a core UAAA (11). However, its in vivo RNA targets have just begun to be identified (12).

Several reports have indicated that phosphorylation regulates key cellular roles of Sam68. Both tyrosine phosphorylation and SH3 binding severely hamper the RNA binding capability of Sam68 (13, 14). Sam68 was found to interact and colocalize with the splicing-associated factor YT512-B, to synergize with the human immunodeficiency virus Rev protein, enhancing export of unspliced viral RNA, and to increase protein expression from RNA containing the constitutive transport element of some retroviruses (3, 15–17). On the other hand, activation of the Ras-ERK pathway generates ERK-threonine-phosphorylated Sam68 that in turn enhances inclusion of exon v5 of the CD44 pre-RNA (18). Thus phosphorylation is an essential regulatory mechanism of the RNA-binding activity of Sam68.

Recently elevated phosphorylation of Sam68 and its correlation to increased acetylation was reported in breast cancer cell lines Hs578T, MDA-435, and MDA-468 (19), implying that tyrosine phosphorylation of Sam68 may be required for the invasiveness of these cancer cells. In fact, the involvement of Sam68 in tumor progression is supported by the finding that depletion of Sam68 is associated with neoplastic transformation (20). Consistent with this observation, it was demonstrated that overexpression of Sam68 blocks cell cycle progression (21). In contrast, overexpression of the RNA binding-defective splice variant (Sam68ΔKH), but not wild-type Sam68 was shown to suppress cell growth (22). Furthermore, the targeted disruption of Sam68 in DT20 cells was shown to cause growth retardation (23), which is inconsistent with earlier studies (20). Therefore, additional studies are required to define the role of Sam68 in cell cycle progression.

BRK is a non-receptor tyrosine kinase that belongs to the classified BRK family tyrosine kinases that include Frk, Srm, and Src42A (24). BRK contains an SH3, an SH2, a kinase domain, and a C-terminal regulatory tyrosine in a similar arrangement as Src family kinases, but lacks the myristoylation signal conserved within the Src family. We previously identified BRK as the first nuclear kinase that can phosphorylate Sam68 and negatively regulate its RNA-binding activity (3) and recently showed that both the Sam68-like proteins, SLM-1 and SLM-2, are also substrates of BRK (25). Other substrates of BRK comprise the adaptor proteins BKS (26) and Paxillin (27). Furthermore, BRK has been shown
to associate with the epidermal growth factor (EGF) receptor, GAP-associated p65 protein (28), erbB3/HER3 (29), and protein kinase B/Akt (29, 30). BRK is overexpressed in more than 65% of breast tumors (31). Small interfering RNA (siRNA) silencing of BRK was shown to specifically suppress BRK expression, resulting in significant inhibition of the proliferation of the T-47D breast cancer cell line (32). Besides, BRK was shown to potentiate the mitogenic effects of EGF stimulation (29, 33). These studies indicate that BRK indeed has growth promoting activities and is able to stimulate cell cycle progression. Wild-type BRK is known to reside throughout the cell and the overexpression of Sam68 promotes its nuclear localization (25). The nuclear expression of BRK occurs in normal prostate epithelial cells as well as in 70% of benign prostate hyperplasia, but not in high grade prostate intraepithelial neoplasia (34).

We have previously shown that both BRK and Sam68 reside in Sam68 nuclear bodies (SNBs), a novel nuclear structure that we discovered in 1999 (35). The C terminus of Sam68 is clustered with 16 tyrosine residues, all potential tyrosine phosphorylation sites. This region also harbors an unconventional nuclear localization signal (NLS) comprising the last 24 amino acids (420RPSLKAPPARPVKGAYREHPYGRY443) of the protein with tyrosine residues at positions 435, 440, and 443 (36). Mapping the phosphorylated tyrosines in the C-terminal of Sam68 is challenging, as the tyrosine residues are often located in clusters. Besides, traditional tryptic mapping is hampered by the scarcity of lysine and arginine residues in the C terminus. We have overcome this problem by generating Sam68 phospho-specific antibodies. We demonstrate that BRK phosphorylates Sam68 on all three tyrosines in the NLS. We show that tyrosine 440 dictates the localization of Sam68, as mutating it to phenylalanine completely blocks nuclear localization. Our data also identify Sam68 as a downstream substrate of BRK in breast cancer cells in response to EGF treatment. We also show that Sam68 inhibits cell cycle progression, whereas BRK has a stimulatory effect on cell growth. Co-infection of Sam68 and BRK in astrocytes prevents cell cycle progression as fewer cells were able to transverse the S phase of the cell cycle compared with mock-infected cells. We propose that phosphorylation of Sam68 may be a repressor of BRK-induced cell cycle progression.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Peptides comprising amino acids 432–443 containing 3 tyrosine residues in the C-terminal of Sam68 were synthesized at W. M. Keck Biotech Resource Center (New Haven, CT) and coupled to key-hole limpet hemocyanin (Sigma). Polyclonal antibodies were generated at Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA) by injecting New Zealand White rabbits with peptides. The following phospho-Sam68 antibodies were generated: anti-435pY (KGAY*REHPYGRY*), anti-440pY (KGAYREHPY*GRY), and anti-443pY (KGAYREH-PYGRY*). Polyclonal antibodies were affinity purified over the antigenic peptide coupled to Affi-Gel beads, eluted with 100 mM glycine, pH 2.5, and subjected to potentiate the mitogenic effects of EGF stimulation (29, 33). These studies indicate that BRK indeed has growth promoting activities and is able to stimulate cell cycle progression. Wild-type BRK is known to reside throughout the cell and the overexpression of Sam68 promotes its nuclear localization (25). The nuclear expression of BRK occurs in normal prostate epithelial cells as well as in 70% of benign prostate hyperplasia, but not in high grade prostate intraepithelial neoplasia (34).

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**Enzyme-linked Immunosorbent Assay (ELISA)**—ELISA plates (Costar, Cambridge, MA) were coated with the indicated quantity of peptide in 50 μL of 50 mM carbonate buffer, incubated at 37°C for 30 min, and blocked with blocking buffer (1% bovine serum albumin, 5% sucrose in phosphate-buffered saline (PBS)). Primary antibodies were diluted at 1:1000 in dilution buffer (1% bovine serum albumin, 0.5% ovalbumin, 10 mM Tris, pH 7.4, 150 mM NaCl) and added to the corresponding well followed by incubation at 37°C for 30 min. The plate was washed extensively with PBS containing 0.1% Tween. Goat anti-rabbit antibodies covalently coupled to horseradish peroxidase (Cappel Laboratories, Durham, NC) were incubated at 1:1000 in dilution buffer at 37°C for 15 min. The plate was washed and developed using BM Blue POD substrate (Roche Diagnostics) and quantitated using spectrophotometry at 405 nm.

**Expression Constructs**—Green fluorescent protein (GFP)-Sam68 was constructed by subcloning the EcoRI fragment of myc-Sam68 into pEGFP-C1 (35). GFP-Sam68 mutants were generated by PCR using the forward primer 5'-TCTCTGCTGAGGTTCTGTGACC-3' and the following reverse primers: GGTGAATTCCTTATTAGCTCC-CCTCACCTGCTGATCG (for Δ423–443); GTGAAATCTCCTTTTAA-TAACGTCATATGTTGTCTC (for 435F-Y); GTGAAATCTCCTTTT- TATAACGTCCATATGTTGTCTC (for 440F-Y), and GTGAAATCTCCTTTTTAATAACGTCCATATGTTGTCTC (for 443F-Y). The EcoRI site is underlined. Wild-type BRK and BRK-YF constructs in the vector pRCMV (33) were generously provided by Dr. Mark Crompton (School of Biological Sciences, Royal Holloway, University of London, London, United Kingdom). To introduce the myc epitope at the N terminus, the constructs were digested with Smal and Xbal to remove the BRK inserts. BRK inserts were then cloned into pcDNA3myc digested with EcoRI and primer A overhang containing a BamHI site (5'-GGAA-GATCTACCATGGTGTCTTGGGACA-3'). After digestion with BamHI, the fragment was cloned into the BglII site of pADTR5-K7-GFP (37). These plasmids express myc-BRK and myc-Sam68 under the regulation of a tetracycline-inducible promoter and a second cassette that constitutively expresses GFP. The latter cassette serves as a marker for transduction. Wild-type c-Src was a gift from Stéphane A. Laporte (McGill University, Montréal, Québec, Canada) and has been previously described (38). Recombinant adenoviruses were generated, purified, and titered as described previously (39).

**Cell Culture**—The following cell lines were obtained from the American Type Culture Collection: HeLa (CCL-2), MDA-231 (HTB-26), MDA-468 (HTB-132), and BT20 (HTB-19). The cells were maintained in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin (all from ICN, Costa Mesa, CA), and 10% bovine calf serum (Hyclone, Logan, UT). Cells were maintained at 37°C in 5% CO2.

**Protein Expression and Immunoprecipitation**—The day before transfection, HeLa cells were plated on glass coverslips at a density of 106 cells per 22-mm2 coverslip (Fisher Scientific). The cells were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol. For immunoprecipitations, cell lysates were incubated on ice with the primary antibody for 1 h. Then 30 μL of 50% protein A-Sepharose slurry was added and incubated at 4°C for 30 min with constant end-over-end mixing. The beads were washed twice with lysis buffer and once with PBS. Protein samples were analyzed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblotting was performed using the anti-Sam68 antibodies, anti-phospho-Sam68 antibodies, anti-BRK, and anti-myc (9E10) antibodies. Immunoreactive proteins were visualized using either goat anti-mouse or goat anti-rab-
bit antibodies conjugated to horseradish peroxidase (ICN Pharmaceuticals) and the chemiluminescence (ECL) detection kit (DuPont).

Dephosphorylation of BRK-phosphorylated GFP-Sam68 by Protein-tyrosine Phosphatase 1B (PTP1B) Immunoprecipitated phosphorylated GFP-Sam68 on beads was washed twice with lysis buffer and once with PBS, drained, and incubated with 20 μl of PTP1B buffer (10 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, pH 7.5) and 1 μg/ml of recombinant PTP1B (R&D Systems) at 37 °C for 30 min. The reaction was quenched by the addition of Laemmli sample buffer and subjected to SDS-gel electrophoresis.

Immunofluorescence on Cultured Cells and Tissue Array—HeLa cells were cultured directly on glass coverslips in a 6-well dish. Transfection of HeLa cells for immunofluorescence was achieved using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol using 2 μg of DNA. The cells were fixed with 1% paraformaldehyde in 1× PBS, pH 7.4, for 5 min and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. If the cells were to be visualized only for GFP, then the coverslips were mounted onto glass slides with glocerol containing 3 mg/ml 4,6-diamidino-2-phenylindole to stain the nuclei. If the cells required antibody staining, the permeabilized cells were first blocked with 10% calf serum (Hyclone) in PBS for 30 min. Goat anti-rabbit coupled to Alexa 488 (Molecular Probes) and goat anti-mouse coupled to Alexa 488 (Molecular Probes) were used as secondary antibodies (1:200) in PBS for 30 min. Goat anti-rabbit coupled to Alexa 488 (Molecular Probes) and goat anti-mouse coupled to Alexa 488 (Molecular Probes) were used as secondary antibodies. The cells were washed again, mounted onto glass slides, and visualized with a confocal microscope (Carl Zeiss, Thornwood, NY). LandMarkTM High Density Breast Tissue MicroArray on slides (catalog number 3190) was purchased from Ambion Inc. (Austin, TX). The tissue were permeabilized as described above and stained with the appropriate antibodies.

RNA Interference and EGF Stimulation—BRK siRNAs purchased from Dharmacon Research (Lafayette, CO) comprised a 19-base duplex RNA nucleotide sequences with two 3′ deoxythymidine residues (BRK siRNA, 5′-GGUGAAUUUCUGGAGACAACCTT-3′ and control, 5′-GGACACCAUGAAGUGCGTT-3′). Transfections were performed using Lipofectamine Plus (Invitrogen) twice at 24-h intervals as described (32). The cells were then stimulated at various time intervals with EGF at 100 ng/ml.

Cell Cycle Analysis of Astrocytes—Primary cultures of astrocytes were generated from newborn rat brains as described by McCarthy and de Vellis (40). Proliferating astrocytes were infected for 24 h with adenoviruses at a multiplicity of infection of 50 of the indicated adenovirus (AdGFP control, AdSam68, AdBRK) co-expressing the transgene and GFP from two different promoters as described previously (37, 41).

BRK regulates nuclear localization of Sam68

BRK-phosphorylated Sam68 is recognized by new anti-phospho-Sam68 antibodies—Sam68 is the first substrate identified for BRK (3). To determine whether tyrosines 435, 440, and 443 within the Sam68 NLS were phosphorylated by BRK, HeLa cells were transfected with Sam68 fused to the GFP to distinguish it in size from endogenous Sam68. An expression vector encoding GFP-Sam68 was transfected with or without constitutively active myc epitope-tagged BRK<sup>Y447F</sup> (BRK YF) (Fig. 1B). Cell lysates from the vector control (lane 1), BRK YF alone (lane 2), GFP-Sam68 alone (lane 3), or GFP-Sam68 plus BRK YF (lane 4) were separated by SDS-PAGE and immunoblotted with 4G10, 435pY, 440pY, 443pY, anti-GFP, anti-myc, and anti-actin antibodies. All phospho-specific antibodies including anti-phosphotyrosine 4G10 antibody, anti-435pY, -440pY, and -443pY antibodies recognized GFP-Sam68 when cotransfected with BRK YF (Fig. 1B, lane 4). When GFP-Sam68 was transfected without BRK YF, GFP-Sam68 was recognized weakly by 4G10, 435pY, 440pY, and 443pY antibodies (lane 3). These findings suggest that transfected GFP-Sam68 is phosphorylated endogenously at Tyr<sup>435</sup>, Tyr<sup>440</sup>, and Tyr<sup>443</sup> in HeLa cells and this background phosphorylation has been observed previously with overexpressed myc-Sam68 in HeLa cells (7). To further delineate the pattern of phosphorylation of the three N-terminal tyrosines by BRK we used an immunoprecipitation/imunoblotting strategy with the various Sam68 phospho-specific antibodies (Fig. 1C). We observed that Sam68 is simultaneously phosphorylated on Tyr<sup>435</sup>/Tyr<sup>440</sup> (Fig. 1C, lanes 4 and 10), Tyr<sup>440</sup>/Tyr<sup>443</sup> (Fig. 1C, lanes 6 and 12), and Tyr<sup>435</sup>/Tyr<sup>443</sup> (data not shown) and that this phosphorylation was increased with BRK. These data suggest that BRK fully phosphorylates Sam68 tyrosines 435, 440, and 443 within the cell.

To confirm that tyrosine residues are phosphorylated in GFP-Sam68, we subjected immunoprecipitates to dephosphorylation assays with recombinant PTP1B followed by immunoblotting with anti-Sam68 antibodies (Fig. 1D). In the presence of the PTPase, the phosphotyrosine content of phospho-GFP Sam68 was significantly reduced for each of the phospho–Sam68 antibodies (compare, lanes 2 and 4). These results demonstrate that tyrosines 435, 440, and 443 are indeed phosphorylated by BRK and confirm that we have generated phospho-Sam68 antibodies.

Phospho-Sam68 Antibodies Are Site-specific—To demonstrate site specificity of the phospho-Sam68 antibodies, we generated a C-terminal-truncated Sam68 protein that deletes the C-terminal 9 amino acids...
from the GFP-Sam68 fusion protein (Δ432–443). In addition, each tyrosine within the NLS was separately replaced with a phenylalanine and expressed as GFP fusion proteins. Expression vectors encoding the mutant GFP-Sam68 fusion proteins were cotransfected with the plasmid expressing BRK YF in HeLa cells. The cell lysates were separated by SDS-PAGE and immunoblotted with the indicated phospho-specific antibodies (Fig. 1E). Deletion of the C-terminal 9 amino acids completely abolished the recognition of all 3 Sam68 phospho-specific antibodies (Fig. 1E, lane 2). The GFP-Sam68 Y435F, Y440F, and Y443F proteins were not recognized by their respective antibodies (Fig. 1E, lanes 3–5). The fact that, for example, α-440pY recognized wild-type GFP-Sam68 (lane 1) as well as the Y435F (lane 3) and Y443F (lane 5) mutants demonstrates that the absence of phosphorylation at Tyr⁴³⁵ and Tyr⁴⁴³ does not influence the recognition of Tyr⁴⁴⁰ by the anti-440pY antibody. These results further show that the phospho-Sam68 antibodies are site-specific.

BRK Regulates Nuclear Distribution of SNBs—As shown in Fig. 1, B and C, BRK phosphorylates all 3 tyrosines within the Sam68 NLS. We therefore reasoned that distribution of SNBs, shown previously to be highly prevalent in cancer cells (35), may be regulated by phosphorylation. As such, the GFP-Sam68 chimeric protein was expressed either alone or in combination with vectors encoding constitutively active BRK YF (Fig. 2). GFP-Sam68 alone localized in the nucleoplasm and within 2–3 SNBs as described previously (35). These SNBs were not stained by phospho-Sam68 antibodies (Fig. 2A, top panels). Remarkably, co-transfection of BRK YF caused a dramatic relocalization and multiplication of GFP-Sam68 from ~3 to ~40 SNBs that we termed the multiple SNBs phenotype (or mSNBs) (Fig. 2A, middle panel). Each

FIGURE 1. Novel phospho-Sam68 antibodies are specific. A, schematic representation of full-length Sam68 showing the C-terminal peptide within the NLS used to generate Sam68 phospho-specific antibodies. The backbone peptide (435Y) contains three tyrosine residues. Synthetic peptides containing phosphorylated tyrosines at positions 435 (435pY), 440 (440pY), and 443 (443pY) are shown and the tyrosine residues are underlined. B, phosphorylation of Sam68 Tyr⁴³⁵, Tyr⁴⁴⁰, and Tyr⁴⁴³ by BRK. HeLa cells were transfected with an empty vector (lane 1), active BRK Y-F (lane 2), GFP-Sam68 (lane 3), or co-transfected with GFP-Sam68 and BRK YF (lane 5). Tyrosine phosphorylation of GFP-Sam68 was detectable with novel Sam68 phosphospecific antibodies α-435pY, α-440pY, and α-443pY as well as the general anti-phosphotyrosine antibody 4G10 in Western blot analysis. Controls depicting the expression of transfected proteins are detected by antibodies against GFP and myc are represented. C, essentially as indicated above in B, except that Sam68 was immunoprecipitated from HeLa cell lysates using anti-Sam68 antibody and each of Sam68 phosphospecific antibodies, α-435pY, α-440pY, and α-443pY. The proteins were separated by SDS-PAGE and immunoblotted with α-440pY. D, tyrosine dephosphorylation of Sam68 by recombinant PTP1B. HeLa cells transfected with GFP-Sam68 in the absence (lanes 1, 3, and 5) or presence of active myc-BRK YF (lanes 2, 4, and 6) were lysed and immunoprecipitated with α-Sam68 antibodies and the immunoprecipitates were split equally for analysis. Lanes 1 and 2, immunoprecipitates were resolved on SDS-PAGE and transferred to nitrocellulose membranes and immunoblotted with α-Sam68 antibodies (top panel, lanes 1 and 2) or with α-435pY, α-440pY, and α-443pY (bottom panel, lanes 1 and 2). Lanes 3 and 4, immunoprecipitates were incubated with 1 μg/ml recombinant PTPase (PTP1B), subjected to SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with α-Sam68 antibodies or α-440pY. Expressed constructs in the total cell lysates were detected with antibodies recognizing the epitope tags. E, phospho-Sam68 antibodies are site-specific. Expression vectors encoding mutant GFP-Sam68 fusion proteins were cotransfected with the plasmid expressing myc-BRK YF in HeLa cells. Total cell lysates were divided equally, separated by SDS-PAGE, and immunoblotted with the indicated phospho-Sam68 antibodies as well as antiphosphotyrosine antibodies. 4G10. Anti-GFP antibodies were used to demonstrate equal loading of the expressed Sam68 mutants and immunoblotting with anti-actin antibodies served as a loading control.
nuclear focus was indeed recognized with the anti-440pY antibodies demonstrating the phosphorylation of Sam68 in mSNBs. As demonstrated recently, activated BRK YF has a predominantly nuclear localization (25), however, cotransfection with GFP-Sam68 caused its redistribution within mSNBs (Fig. 2B).

Tyr440 is Essential for Nuclear Localization of Sam68—We have established that BRK phosphorylates Sam68 in the NLS resulting in the induction of mSNBs. Thus, we set out to investigate whether one or all of the NLS tyrosines are essential in localization and generation of mSNBs. The localization of GFP-Sam68 mutants Y435F, Y440F, Y443F, and Sam68 Δ432–443 was therefore examined in HeLa cells in the presence or absence of BRK (Fig. 3A). Sam68 Δ432–443 was expressed in the cytoplasm and also in the perinuclear compartment, as expected with the removal of the NLS (Fig. 3A). Substitution of Y440F caused the mutant GFP-Sam68 Y440F to localize within the cytoplasm, whereas the substitution of Sam68 Y435F and Y443F had no effect on the nuclear localization of Sam68 (Fig. 3A). These data identify Tyr440, and neither Tyr435 nor Tyr443, as critical residues for the nuclear localization of Sam68. BRK, however, appears to alter the cytoplasmic pattern of GFP-Sam68 Y440F and Sam68 Δ432–443 causing the appearance of multiply cytoplasmic foci (Fig. 3A, right panel). Moreover, the expression of BRK YF caused the relocalization of Y435F and Y443F GFP-Sam68 to multiple SNBs, further demonstrating that GFP-Sam68 harboring amino acid substitutions of Tyr435 and Tyr443 behave like wild-type GFP-Sam68. To rule out the possibility that tyrosine-phosphorylated Sam68 form foci, we co-expressed GFP-Sam68 with active c-Src and we did not observe a redistribution of SNBs, suggesting that BRK has a unique ability to redistribute Sam68 (Fig. 3B).

Sam68 Is Phosphorylated in Breast Cancer Cell Lines—Thus far we have performed experiments with active BRK YF and overexpression. We wished to reproduce our findings with cell lines and tumor tissues endogenously expressing BRK and Sam68. Phosphorylation of endogenous Sam68 in breast cancer cell lines Hs578T, MDA-435, and MDA-468 has been recently reported (19). As the highest level of Sam68 phosphorylation was observed in MDA-468, we therefore surveyed this cell line and breast tumor cell line BT20, as well as the invasive breast tumor cell line MDA-231, for Sam68 phosphorylation using the α-440 antibodies. MDA-231 and BT20 have been previously reported to express moderate and high levels of BRK expression, respectively (31). Cell extracts were prepared, separated by SDS-PAGE, and the phosphorylation of Sam68 Tyr440 was visualized by immunoblotting. Comparable phosphorylation of Sam68 was detected in breast cancer cells MDA-231, MDA-468, and BT-20 but not in HeLa cells derived from human epithelial cervical carcinoma (Fig. 4A, top panel). Each cell lysate contained equivalent levels of Sam68, as detected with anti-Sam68 antibodies. Anti-BRK antibodies confirmed the presence of BRK in the breast tumor cell lines and weakly expressed in HeLa cells (Fig. 4A, middle panel). To determine whether all tyrosines in the NLS of Sam68 are phosphorylated, cell lysates from MDA-MB-468 breast cancer cells were immunoprecipitated with control IgG or with the anti-Sam68 antibody. The immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with anti-435pY, anti-440pY, and anti-443pY antibodies. Immunoprecipitated Sam68 was easily detected by all phosphoantibodies (Fig. 4B). These data concur with the overexpression experiments in HeLa cells that Sam68 is indeed tyrosine phosphorylated by BRK on tyrosines 435, 440, and 443.
We next stained human breast tumor tissue arrays (Ambion) with anti-BRK, anti-440pY, and anti-Sam68 antibodies (TABLE ONE). The anti-BRK staining detected the presence of BRK and the anti-440pY detected phosphorylated Sam68 at Tyr440. The anti-Sam68 antibodies stained all the cells and tissues as expected for a ubiquitously expressed protein (supplemental Fig. S2). We focused our results on the infiltrating ductal carcinomas (IDCs) because the array contained 175 different samples of this particular tumor type. 20/175 (11.4%) IDCs stained positively with anti-440pY antibodies, whereas 29/175 (16.6%) IDCs stained positive for BRK expression and 8 of the BRK-positive IDCs contained phosphorylated Tyr440. The predicted coincidence of BRK and phospho-Sam68 in the represented sample set is 3.3/175 (1.9%) and our observed overlap of 8/175 (4.6%) is therefore statistically relevant. Thus, the expression of BRK may be, in part, responsible for Sam68 phosphorylation in IDCs.

**BRK Is Required for the Tyrosine Phosphorylation of Sam68 in Response to EGF Treatment**—Because BRK has been shown to potentiate the mitogenic signals of EGF (33), we investigated first whether endogenous Sam68 was phosphorylated in response to EGF treatment. We stimulated serum-starved MDA-231 cells with EGF (100 ng/ml) and observed a time-dependent activation of Sam68 phosphorylation (Fig. 5A). The level of Sam68 Tyr440 phosphorylation peaked at ~30 min stimulation compared with ~15 min for EGF receptor (pp180) phosphorylation. These data indicate the phosphorylation of Sam68 is enhanced in EGF signaling in MDA-231 cells. We further showed that

| Breast histology               | Total spots on array | Positively stained spots |
|--------------------------------|----------------------|--------------------------|
|                                | Sam68    | Phospho-Sam68 | BRK    |
| Infiltrating ductal carcinoma  | 175      | 100          | 11.4   | 16.6   |
| Lobular carcinoma              | 8        | 100          | 25     | 37.5   |
| Medullary carcinoma            | 3        | 100          | 0      | 33.3   |
| Metastatic breast adenocarcinoma| 3        | 100          | 0      | 0      |

**TABLE ONE**

Expression pattern of SNBs, phosphorylated Sam68, and BRK in high density breast tissue MicroArrays (Ambion Inc.)

Predicted coincidence of BRK and phospho-Sam68 is 1.9%, we observed a statistically significant coincidence of 4.6% (8/175). Statistical analyses were performed using SSPS software.

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| Medullary carcinoma            | 3        | 100          | 0      | 33.3   |
| Metastatic breast adenocarcinoma| 3        | 100          | 0      | 0      |
Sam68 phosphorylated on Tyr^440 relocalized to perinuclear structures in response to EGF treatment (Fig. 5B). To determine whether or not BRK played a role in EGF-induced phosphorylation of Sam68, we depleted BRK from MDA-231 cells by treating cells with BRK or control siRNA (Fig. 6A). We achieved ~60% knockdown of BRK expression in MDA-231 using the BRK-specific siRNA. Treatment of these cells with EGF diminished the EGF-induced phosphorylation of Sam68 by ~50% (Fig. 6B). These data demonstrate that Sam68 is a physiological substrate of the EGF receptor signaling pathway that requires BRK in the MDA-231 breast cancer cell line.

**Sam68 Suppresses BRK-induced Cell Proliferation in Primary Astrocytes**—The expression of BRK is known to be up-regulated in human breast tumors suggesting that BRK induces cell cycle progression (31). To investigate the interplay between BRK and Sam68 in cell proliferation, we generated adenoviruses expressing each protein. In addition, the adenoviruses also express GFP under a separate promoter and GFP serves as a marker of infection. Primary rat astrocytes are devoid of BRK and express low levels of Sam68 (42) and are suitable primary cells to examine the role of Sam68 and BRK in cell proliferation. Astrocytes were infected for 24 h with control (AdGFP), AdSam68, AdBRK, or AdSam68/AdBRK adenoviruses and cell proliferation was examined by BrdUrd incorporation. The BrdUrd incorporation was visualized by immunofluorescence staining (Fig. 7A) and expressed as the mean ± S.D. (Fig. 7B, n > 400 cells). Approximately 95% of the astrocytes were transduced with the adenoviruses (Fig. 7A). Fewer astrocytes transduced with AdSam68-incorporated BrdUrd (~10%) compared with the control AdGFP (~30%) (Fig. 7B). In contrast, the infection of astrocytes with AdBRK increased the number of cells incorporating BrdUrd from ~30% with AdGFP to ~60%. However, co-infection of AdSam68 and AdBRK also resulted in a reduction of BrdUrd incorporation from ~60 to ~25% (Fig. 7B). To further define what stage of the cell cycle accounts for the observed defects, we carried out flow cytometry analysis using propidium iodide and BrdUrd labeling (Fig. 7C). In control infected cultures, ~19.4% of the cells were in S phase, as compared with ~25.2% for cells infected with AdBRK and ~13.4% in the case of AdSam68 (Fig. 7C). However, co-infection with AdSam68 and AdBRK resulted in few cells in S phase of the cell cycle, as only 9.3% of cells were observed in the S phase. These findings demonstrate that the Sam68 cell cycle inhibition overcomes the BRK-induced cell cycle progression.

**DISCUSSION**

In the present study we report the generation of new phospho-specific antibodies against three phosphorylated tyrosines in the NLS of
STAR protein Sam68. We provide evidence that BRK phosphorylates Sam68 on all three tyrosines in the NLS and demonstrate that tyrosine 440 is essential in the nuclear localization of Sam68. Furthermore, we observe that phosphorylation of Sam68 by BRK induces multiple SNBs and also suppresses BRK-induced cell proliferation. We show that EGF stimulation of breast cancer cell line MDA-231 enhanced the tyrosine phosphorylation of Sam68 that was inhibited by BRK siRNA treatment. Finally, we reveal the prevalence of phosphorylated Sam68 in breast cancer cells and infiltrating ductal carcinomas. Our findings position Sam68 downstream of BRK in breast cancer cells.

The NLS of Sam68 contains an RXHPY(Q/G)R motif located at its distal end. This motif, originally identified and mapped in QKI-5, is also conserved in other QKI homologs as well as mammalian Sam68, SLM-1, SLM-2, and non-STaR protein Drosophila HNF-4 homologs, all of which are nuclear (43). The RXHPY(Q/G)R motif carries a conserved tyrosine (Tyr440) in Sam68. Indeed tyrosine 440 was critical for the nuclear localization of Sam68 as substituting this tyrosine with phenylalanine blocked nuclear entry Tyr440 (Fig. 3). Consistent with tyrosine phosphorylation altering the localization of Sam68 we noted that the induction of phosphorylation Tyr440 in response to EGF treatment led to relocation of Sam68. The localization of Sam68 is known to be regulated by virus infection (44, 45) and arginine methylation (46), but this is the first report showing that tyrosine phosphorylation regulates its cellular localization.

We first reported the localization of Sam68 within dynamic nuclear structures that we called SNBs (35). These SNBs were observed in immortalized and transformed cells, and the prevalence of SNBs correlated with the tumorigenicity of some cancer cell lines (35). SNBs are preferentially positioned in proximity to the nucleoli and seemed to be the site of RNA metabolism as they were also found to contain heterogeneous nuclear ribonucleoprotein K (47), scaffold attachment factor-B (SAF-B)/HAP (48), and splicing factor YT521 (15). Also, we previously identified BRK in SNBs in the HT29 colon adenocarcinoma cell line (3). In its unphosphorylated state, endogenous and transfected Sam68 show a general diffuse nuclear localization and usually three distinct SNBs (35). Upon BRK phosphorylation, Sam68 relocates to multiple SNBs (Fig. 2). The BRK-induced effect was specific to SNBs, as the localization of Cajal bodies and PML bodies were unaltered with BRK overexpression (data not shown). Interestingly, SNBs were barely found in Src 3T3 cells (35), a cell type that contains tyrosine-phosphorylated Sam68 (1, 2), suggesting that the relocating effect is BRK-specific.

The tumor suppressor activities of Sam68 have been previously reported (20). Taylor et al. (21) used an inducible expression system to show that overexpression of Sam68 induces a G1 cell cycle arrest, an effect that was independent of the RNA binding properties of Sam68. Furthermore, random homozygous knock-out of Sam68 in NIH3T3 cells has been associated with neoplastic transformation of mouse fibroblasts and tumorigenesis (20). We confirmed by adenoviral delivery into primary rat astrocytes that Sam68 and BRK have opposing effects on cell cycle progression (Fig. 7). Sam68 inhibited cell cycle progression, whereas BRK promoted cell proliferation of primary rat astrocytes. The intracellular localization of BRK is nuclear in normal prostate epithelial cells and benign prostate hyperplasia, and cytoplasmic in high grade prostate intraepithelial neoplasia (34). Our data support the hypothesis that Sam68 may function as a tumor suppressor, a characteristic shared by other STaR proteins such as SLM-2 (49), GLD-1 (50), and Quaking (51).

The ability of BRK to induce cell proliferation is not surprising. BRK is overexpressed in ~60% of breast carcinomas, but not in normal mammary tissue (24). Knockdown of BRK in the breast carcinoma cell line T47D resulted in significant suppression of cell proliferation (32). Moreover, BRK has been shown to associate with and sensitize EGFR to proliferative responses and also to potentiate anchorage-independent growth of mammary epithelial cells (33). This BRK-dependent mitogenic sensitization may be achieved through its enhancement of erbB3 phosphorylation and subsequent activation of phosphatidylinositol 3-kinase/Akt pathway (29). These reports show that BRK confers a proliferative advantage on cells. In the present study, we show elevated expression of phosphorylated Sam68 in MDA-231, MDA-468, and BT20 breast cancer cell lines (Figs. 4–6). These cell lines have been shown to overexpress the EGFR receptor (52). Moreover, we show for the first time that Sam68 is tyrosine phosphorylated downstream of the EGFR receptor with slower kinetics than the EGFR receptor implying that there is an intermediate protein. Because BRK siRNA treatment attenuated the Sam68 phosphorylation of Tyr440, this implies that BRK is the tyrosine kinase that mediates this phosphorylation. Therefore, the tyrosine phosphorylation of Sam68 in breast cancer cell lines may be part of a mechanism of inactivating its anti-proliferative functions, by altering its RNA binding properties and stimulating its adaptor functions (3, 7).

In conclusion, this study demonstrates that Sam68 is phosphorylated by BRK on tyrosine residues in the NLS in breast cancer cell lines and primary tumors. We also show that tyrosine 440 is a key modulator of Sam68 localization by BRK and EGF treatment. Our study also identifies phosho-Sam68 in BRK-positive breast cancer cell lines and tissues and reveals that Sam68 attenuates the ability of BRK to stimulate cell proliferation. These findings suggest that overexpression of Sam68 or blocking Sam68 Tyr440 phosphorylation may be valuable therapeutic targets for suppression of BRK-induced tumor progression.

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REFERENCES

1. Fumagalli, S., Totty, N. F., Hsuan, J. I., and Courtneidge, S. A. (1994) Nature 368, 871–874
2. Taylor, S. J., and Shalloway, D. (1994) Nature 368, 867–871
3. Derry, J. I., Richard, S., Valderrama Carvajal, H., Ye, X., Vasioukhin, V., Cochrane, A. W., Chen, T., and Tyner, A. L. (2000) Mol. Cell. Biol. 20, 6114–6126
4. Lang, V., Mege, D., Semichon, M., Gary-Gouy, H., and Bismuth, G. A. (1997) Eur. J. Immunol. 27, 3360–3367
5. Lukong, K. E., and Richard, S. (2003) Biochim. Biophys. Acta 1653, 73–86
6. Di Fruscio, M., Chen, T., Bonyadi, S., Lasko, P., and Richard, S. (1998) J. Biol. Chem. 273, 30122–30130
7. Richard, S., Yu, D., Blumer, K. J., Hausladen, D., Olszewy, M. W., Connelly, P. A., and Shaw, A. S. (1995) Mol. Cell. Biol. 15, 186–197
8. Bedford, M. T., Frankel, A., Yaffe, M. B., Clarke, S., Leder, P., and Richard, S. (2000) J. Biol. Chem. 275, 16030–16036
9. Wong, G., Muller, O., Clark, R., Conroy, L., Moran, M. F., Polakis, P., and McCormick, F. (1992) Cell 69, 551–558
10. Chen, T., Cóte, J., Carvajal, H. V., and Richard, S. (2001) J. Biol. Chem. 276, 30803–30811
11. Lin, Q., Taylor, S. J., and Shalloway, D. (1997) J. Biol. Chem. 272, 27274–27280
12. Iih, M., Haga, I., Li, Q. H., and Fujisawa, J. (2002) Nucleic Acids Res. 30, 5452–5464
13. Wang, L. L., Richard, S., and Shaw, A. S. (1995) J. Biol. Chem. 270, 2010–2013
14. Taylor, S. J., Anafi, M., Pawson, T., and Shalloway, D. (1995) J. Biol. Chem. 270, 10120–10124
15. Hartmann, A. M., Nayler, O., Schwaiger, F. W., Obermeier, A., and Stamm, S. (1999) Mol. Biol. Cell 10, 3909–3926
16. Reddy, T. R., Xu, W. D., and Wong-Staal, F. (2000) Oncogene 19, 4071–4074
17. Coyle, J. H., Guzik, B. W., Bor, Y. C., Jin, L., Eisner-Smerage, L., Taylor, S. J., Rekosh, D., and Hammarskjöld, M. L. (2003) Mol. Cell. Biol. 23, 92–103
18. Matter, N., Herrlich, P., and Konig, H. (2002) Nature 420, 691–695
19. Babic, I., Jakymiw, A., and Fujita, D. J. (2004) Oncogene 23, 3781–3789
20. Liu, K., Li, L., Nisson, P. E., Gruber, C., Jesse, J., and Cohen, S. N. (2000) J. Biol. Chem. 275, 40195–40201
21. Taylor, S. J., Renwick, R. J., and Shalloway, D. (2004) BMC Cell Biol. 5, 1–12
22. Barlat, I., Maurier, F., Duchesne, M., Guitard, E., Tocone, B., and Schweighofer, F. (1997) J. Biol. Chem. 272, 3129–3132
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23. Li, Q. H., Haga, I., Shimizu, T., Itoh, M., Kurosaki, T., and Fujisawa, J. (2002) *FEBS Lett.* **525**, 145–150
24. Serfas, M. S., and Tyner, A. L. (2003) *Oncol. Res.* **13**, 409–419
25. Haegeman, A., Heap, D., Bie, W., Derry, J. J., Richard, S., and Tyner, A. L. (2004) *J. Biol. Chem.* **279**, 54398–54404
26. Mitchell, P. J., Sara, E. A., and Crompton, M. R. (2004) *Oncogene* **19**, 4273–4282
27. Zhang, P., Ostrander, J. H., Faivre, E. J., Olsen, A., Fitzsimmons, D., and Lange, C. A. (2005) *J. Biol. Chem.* **280**, 1982–1991
28. Harvey, A. J., and Crompton, M. R. (2003) *Oncogene* **22**, 5006–5010
29. Kamalati, T., Jolin, H. E., Fry, M. J., and Crompton, M. R. (1997) *J. Biol. Chem.* **271**, 30956–30963
30. Harvey, A. J., and Crompton, M. R. (2003) *Oncogene* **22**, 4212–4220
31. Chen, T., Boisvert, F. M., Bazett-Jones, D. P., and Richard, S. (1999) *Mol. Biol. Cell* **10**, 3015–3033
32. Ishidate, T., Yoshihara, S., Kawasaki, Y., Roy, B. C., Toyoshima, K., and Akiyama, T. (1997) *FEBS Lett.* **409**, 237–241
33. Massie, B., Couture, F., Lamoureux, L., Mosser, D. D., Guilbault, C., Jolicoeur, P., Belanger, F., and Langelier, Y. (1998) *J. Virol.* **72**, 2289–2296
34. Fessart, D., Simaan, M., and Laporte, S. A. (2005) *Mol. Endocrinol.* **19**, 491–503
35. Pilotte, J., Larocque, D., and Richard, S. (2001) *Genes Dev.* **15**, 845–858
36. McCarrey, K. D., and de Vellis, J. (1980) *J. Cell Biol.* **85**, 890–902
37. Massie, B., Couture, F., Lamoureux, L., Mosser, D. D., Guilbault, C., Jolicoeur, P., Belanger, F., and Langelier, Y. (1998) *J. Virol.* **72**, 2289–2296
38. Serfas, M. S., and Tyner, A. L. (2003) *Oncol. Res.* **13**, 409–419
39. Haegeman, A., Heap, D., Bie, W., Derry, J. J., Richard, S., and Tyner, A. L. (2004) *J. Biol. Chem.* **279**, 54398–54404
40. Mitchell, P. J., Sara, E. A., and Crompton, M. R. (2004) *Oncogene* **19**, 4273–4282
41. Zhang, P., Ostrander, J. H., Faivre, E. J., Olsen, A., Fitzsimmons, D., and Lange, C. A. (2005) *J. Biol. Chem.* **280**, 1982–1991
42. Kool, J., van Zaane, W., and Terleth, C. (2001) *Cell Growth & Differ.* **12**, 535–541
43. Jones, A. R., and Schedl, T. (1995) *Genes Dev.* **9**, 1491–1504
44. Galarnneau, A., and Richard, S. (2005) *Nat. Struct. Mol. Biol.* **12**, 691–698
45. Lev, D. C., Kim, L. S., Melnikova, V., Ruiz, M., Ananthaswamy, H. N., and Price, J. E. (2004) *J. Cancer* **91**, 795–802