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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The breast tumor kinase BRK is expressed in a high percentage of human breast tumors and breast tumor cell lines. We found that BRK is a nuclear tyrosine kinase that phosphorylates the RNA binding protein Sam68 (Src associated during mitosis, 68 kDa). BRK and Sam68 colocalize in Sam68/SLM nuclear bodies (SNBs) in human breast tumor cell lines. In functional studies, expression of BRK abolished the ability of Sam68 to bind RNA and act as a cellular Rev homologue. In addition to Sam68, we found that BRK also phosphorylates the Sam68-like mammalian proteins SLM-1 and SLM-2. We examined expression of Sam68, SLM-1, and SLM-2 in the mouse mammary gland using RNase protection assays. In the normal mouse mammary gland only expression of Sam68 was detected. We are currently examining expression of SLM-1 and SLM-2 in human breast tumor cell lines. While Sam68 is a substrate for Src family kinases during mitosis, BRK is the first identified tyrosine kinase that can phosphorylate Sam68 and regulate its activity within the nucleus, where it resides during most of the cell cycle. Since Sam68 has been implicated in cell cycle regulation, increased expression of BRK may alter the ability of Sam68 to regulate cell growth.

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Introduction

The Intracellular Breast Tumor Kinase BRK

The human tyrosine kinase BRK (**Br**east tumor **k**inase) is an intracellular kinase of the form SH3-SH2-YK, where SH3 is a polyproline binding motif, SH2 recognizes phosphorylated tyrosine in a sequence specific context, and YK is the tyrosine kinase catalytic domain. Unlike members of the SRC family, BRK has a very short unique amino terminus and appears to lack a consensus myristoylation motif. While BRK is most closely related to members of the Src family, it is highly diverged, with nearly equivalent homology to the proto-oncogenes p60-YRK (Q02977); p59-FYN (P27446); p90 v-YES [61504) and its cellular homologue c-YES; FRK/RAK (P42685); and c-Src itself, all with 44-45% protein identity. The BRK gene has only two intron boundaries conserved with the SRC family members (Lee et al., 1998; Mitchell et al., 1997) suggesting that BRK is part of a distinct family of nonreceptor tyrosine kinases.

A fragment of the Brk cDNA was initially cloned from cultured normal human melanocytes using reverse transcription PCR with degenerate primers corresponding to the conserved regions of tyrosine kinase catalytic domains and named PTK6 (protein tyrosine kinase 6) (ACCESSION NM_005975) (Lee et al., 1993). A portion of the catalytic domain coding sequence was subsequently cloned using reverse transcription PCR and RNA isolated from involved axillary nodes from a patient with metastatic breast cancer (Mitchell et al., 1994). The full length BRK cDNA was then isolated from the MCF-7 and T-47D breast cancer cell lines (Mitchell et al., 1994). BRK cDNA clones were also isolated from a normal human small intestinal cDNA library (Llor et al., 1999). Mouse BRK was isolated in a screen for proteins that regulate the differentiation of

intestinal epithelial cells and was named Sik (Src-related intestinal kinase) (Siyanoya et al., 1994; Vasioukhin et al., 1995).

BRK Expression in Breast Cancer

BRK has been detected in breast and colon tumors and in breast, colon and metastatic melanoma tumor cell lines (Barker et al., 1997; Easty et al., 1997; Llor et al., 1999; Mitchell et al., 1994). The sequence of BRK isolated from tumor cells and normal cells appears identical (Lee et al., 1998; Mitchell et al., 1997), suggesting that BRK which is overexpressed in tumor cells is the normal protein.

BRK protein was present at high levels in T-47D, ZR75-30, BT-474, BT-20, MDA-MB-453, and MDA-MB-361 breast tumor cell lines; moderate levels in SKBR-3, MDA-MB-231 breast tumor cell lines and the MCF-10A breast epithelial line from a patient with fibrocystic disease, and at low or zero levels in the PMC42, MDA-MB-157, MDA-MB-468, and Cal51 breast tumor cell lines (Barker et al., 1997). While BRK expression could not be detected in normal breast tissue (Llor et al., 1999), it is expressed in a high percentage of breast tumors (Barker et al., 1997). Of 41 primary breast tumor samples quantified by Western blotting relative to cytokeratin 18, BRK was overexpressed five fold or more in 27%, and overexpressed two fold or more in 61%, relative to normal breast tissue. One line expressed 43-fold higher levels of BRK protein (Barker et al., 1997).

The Nuclear RNA-binding protein Sam68 is a substrate of BRK

Sam68 (Src associated in mitosis, 68 kDa) is a KH domain containing RNA binding protein (Wong et al., 1992) that was first identified as a major target of Src during mitosis (Fumagalli et al., 1994; Taylor and Shalloway, 1994). The KH domain is an RNA-binding domain that was first described in hnRNPK. KH domain containing

proteins have been shown to play important roles in development, and these include the human FMR1 (fragile X mental retardation syndrome) gene (De Boulle et al., 1993), the mouse Qk1 (quaking) gene required for myelination (Ebersole et al., 1996), the C. elegans GLD-1 gene that is required for germ cell differentiation (Jones and Schedl, 1995), and the Drosophila Who/How gene required for muscle differentiation (Baehrecke, 1997). These proteins are members of a new subfamily of KH domain-containing RNA-binding proteins having single KH domains named the STAR (**S**ignal **T**ransducers and **A**ctivators of **R**NA) family (Vernet and Artzt, 1997).

Sam68 has been shown to preferentially bind RNA with UAAA motifs, and has been observed to localize in novel nuclear bodies called Sam68/SLM nuclear bodies (SNBs) in cancer cell lines (Chen et al., 1999). Previously we found that Sam68 coimmunoprecipitated with BRK from nuclear extracts prepared from human MCF-7 and HT29 breast and colon carcinoma cells (Derry et al., 2000). In breast cancer cell lines, BRK is present in SNBs where it associates with and can phosphorylate Sam68 (Derry et al., 2000)

Although the biological function of Sam68 is unknown, Sam68 can function as a cellular homologue of Rev by transporting unspliced HIV mRNA into the cytoplasm (Reddy et al., 1999). In our studies, we found that phosphorylation of Sam68 by BRK/Sik negatively regulates its RNA binding ability and its ability to function as a Rev cellular homologue (Derry et al., 2000). These data strengthen the possibility that the BRK/Sik signaling cascade can regulate the RNA-binding functions of the STAR proteins. The ability of Sam68 to act in RNA transport suggests a role in posttranscriptional regulation of gene expression, which may be negatively regulated by BRK/Sik within the nucleus. Thus, the phosphorylation of Sam68 by BRK/Sik within the

nucleus of breast cancer cells may have important physiological significance and may contribute to regulation of gene expression during development/progression of breast cancer.

Recently, two **S**am68-like-**m**ammalian proteins, SLM-1 and SLM-2 were identified (Di Fruscio et al., 1999). SLM-1 and SLM-2 have characteristic Sam68 SH2 and SH3 domain binding sites. In addition, both SLM-1 and SLM-2 colocalize with endogenous Sam68 in Sam68 nuclear bodies (SNBs) in cancer cell lines (Chen et al., 1999). The roles of Sam68 and related proteins in breast cancer have not been studied. However, since BRK is aberrantly expressed in BRK cancers, it is possible that inappropriate phosphorylation of BRK substrates such as Sam68 and the Sam68-like mammalian proteins SLM1 and SLM2 may contribute to the development of breast cancer.

BODY

The goal of our grant is to characterize substrates of BRK and to determine the biological significance of phosphorylation of these substrates in breast cancer. During our first year of funding we have determined that BRK efficiently phosphorylates both SLM-1 and SLM-2, but not the related STAR protein hnRNPK. We have determined the expression patterns of the three known BRK substrates Sam68, SLM-1 and SLM-2 in tissues of the mouse. Interestingly none of these substrates is expressed in normal breast tissue. Expression patterns of these proteins in breast cancer cells is under investigation.

Work proposed in the Idea grant *(taken from technical abstract of funded proposal)*

We hypothesize that induction of BRK in breast tumors leads to modification of STAR family proteins resulting in changes in gene expression that contribute to development of breast cancer. To test this, we will examine expression and

phosphorylation of STAR protein substrates of BRK in normal and cancerous breast cells (Aims 1 and 2). Then we will determine if altering BRK activity results in changes in RNA or protein expression, and if these changes require functional STAR proteins (Aim 3). The proposed experiments will enhance our understanding of BRK signaling and the STAR proteins in breast tumors. BRK and/or factors regulated by the BRK signaling pathway may provide therapeutic targets for the treatment of breast cancer.

Key Research Accomplishments

Using cotransfection, immunoprecipitation, and immunoblotting, we have determined that BRK efficiently phosphorylates both SLM-1 and SLM-2. Following transfections with myc-tagged Sam68 (myc-Sam68), myc-SLM1 and myc-Slm2, immunoprecipitations were performed using anti-myc antibodies. Immunoprecipitated proteins were incubated with GST-Sik and ^{32}P-ATP and the phosphorylation status of the proteins was determined following SDS-PAGE and autoradiography **(Figure 1A)**. The myc-tagged proteins are clearly phosphorylated following addition of GST-Sik. Results for duplicate immunoprecipitations are shown.

To determine the specificity of phosphorylation, HeLa cells were transfected with SikY-F and myc-Sam68, or myc-SLM1, myc-SLM2, myc-hnRNPK, or truncated myc-Sam68(68-347). SikY-F contains a substitution of the carboxy terminal negative regulatory tyrosine, leading to a more active protein. Cells were lysed and the proteins analyzed by immunoblotting with anti-Sik, anti-myc or anti-phosphotyrosine antibodies. Only tyrosine phosphorylation of myc-Sam68, myc-SLM1 and myc-SLM2 increased in these cotransfection experiments with SikY-F **(Figure 1B)**. The related protein hnRNPK as well as a truncated form of Sam68 were not phosphorylated indicating substrate specificity.

Currently the functions of the BRK substrates Sam68, SLM1, and SLM2 are poorly understood, and to date no comprehensive analysis of their expression during normal breast development has been performed. A major part of mammary gland development occurs after birth, beginning in puberty with ductal proliferation and completed during pregnancy with the appearance of differentiated secretary lobules. At puberty gonadal hormones promote the development of the mammary fatpad. Differentiation of a small number of alveolar cells occurs in estrus in virgin mice, while a rapid increase in the number and size of alveoli occurs during pregnancy resulting in terminal differentiation (Robinson et al., 1995).

In order to determine if STAR proteins play a role during normal mammary gland development, we examined their expression in the mouse mammary gland at different stages of differentiation. RNA expression was examined by RNase protection using total RNA from multiple different tissues and different stages of mammary gland development. This included mammary gland tissue isolated from virgin mice and female mice that had previously raised litters **(Figure 2)**. As expected mouse BRK(Sik) was expressed at highest levels in the gastrointestinal tract and skin. No expression was detected in normal mammary gland. Sam68 was ubiquitously expressed and detected in the mouse mammary gland. However, we were unable to detect SLM1 or SLM2 expression in the adult mouse mammary gland.

Reportable Outcomes

During the first year of funding we have completed a significant portion of Aim 1, "To characterize the expression of STAR family RNA-binding proteins in normal breast tissue and breast cancer cells." We are currently examining expression and phosphorylation of Sam68, SLM1 and SLM2 in breast cancer cell lines. A manuscript

describing the specific phosphorylation of SLM-1 and SLM-2 by BRK and the expression of these substrates is in preparation.

Wenjun Bie, Xin Ye, Jason Derry, Stephane Richard and Angela L. Tyner. SLM-1 and SLM-2 (T-STAR) are specific substrates of the BRK/Sik kinase. In preparation.

In related studies, a manuscript describing the tight linkage of BRK/Sik and the related kinase Srm and possible evolutionary consequences has been submitted.

Michael S. Serfas and Angela L. Tyner. Brk, Srm, Frk, and Src42A form a distinct family of intracellular Src-like tyrosine kinases. Submitted to *Oncology Research*.

In addition an abstract has been submitted for presentation at the 2002 ERA OF HOPE Department of Defense (DOD) Breast Cancer Research Program (BCRP) Meeting Meeting in Orlando, Florida.

Angela L. Tyner, Stephane Richard, Jason J. Derry, Xin Ye, and Wenjun Bie. THE BREAST TUMOR KINASE BRK IS A NUCLEAR TYROSINE KINASE THAT PHOSPHORYLATES AND REGULATES THE RNA-BINDING PROTEIN SAM68 AND THE SAM68-LIKE MAMMALIAN PROTEINS SLM-1 AND SLM-2

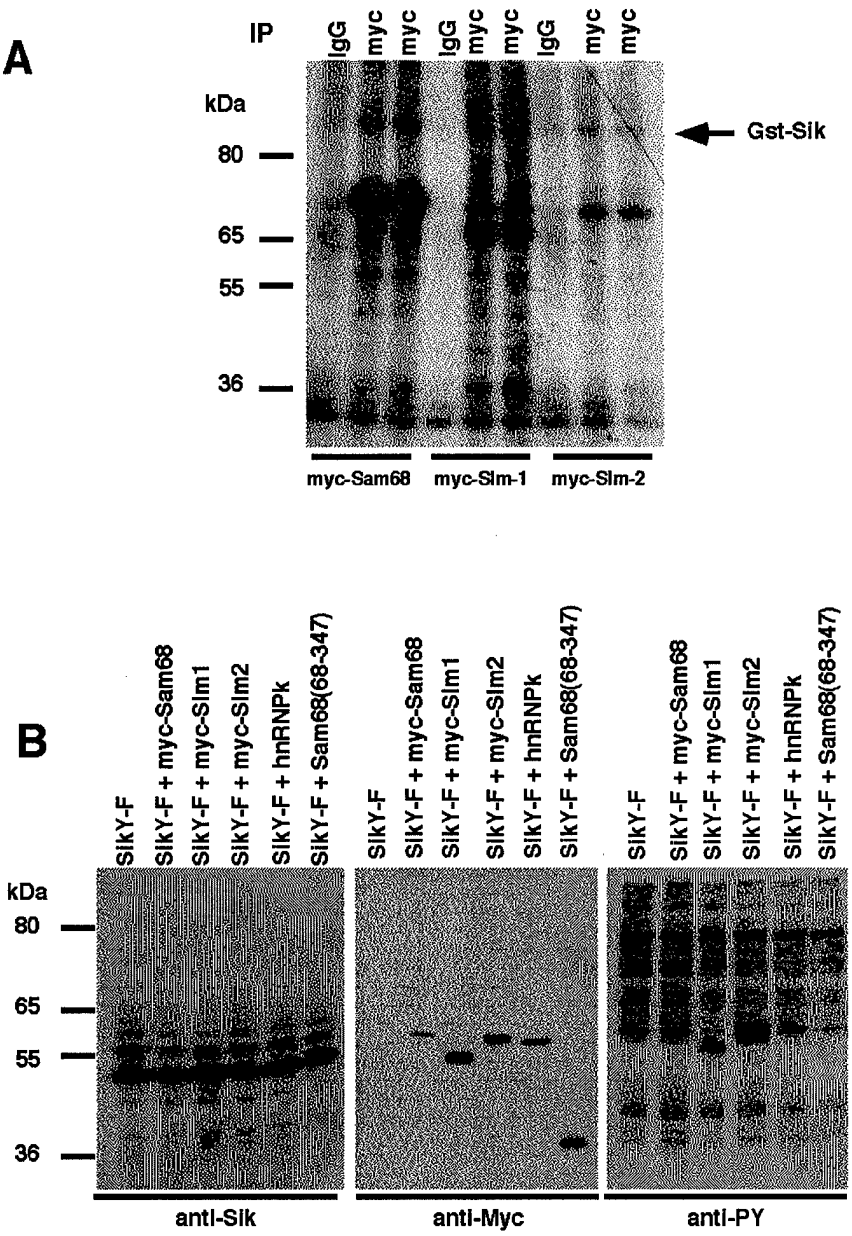

Figure 1. Specific Phosphorylation of the STAR Proteins SLM-1 and SLM-2 by the BRK/Sik kinase. A) Immunoprecipitated myc-tagged Sam68, SLM1, and SLM2 are phosphorylated by GST-Sik. B) Sik Y-F phosphorylates myc-Sam68, myc-SLM1, and myc-SLM2 but not the related protein hnRNPK. Tyrosine phosphorylation is demonstrated using anti-phosphotyrosine antibodies (anti-PY).

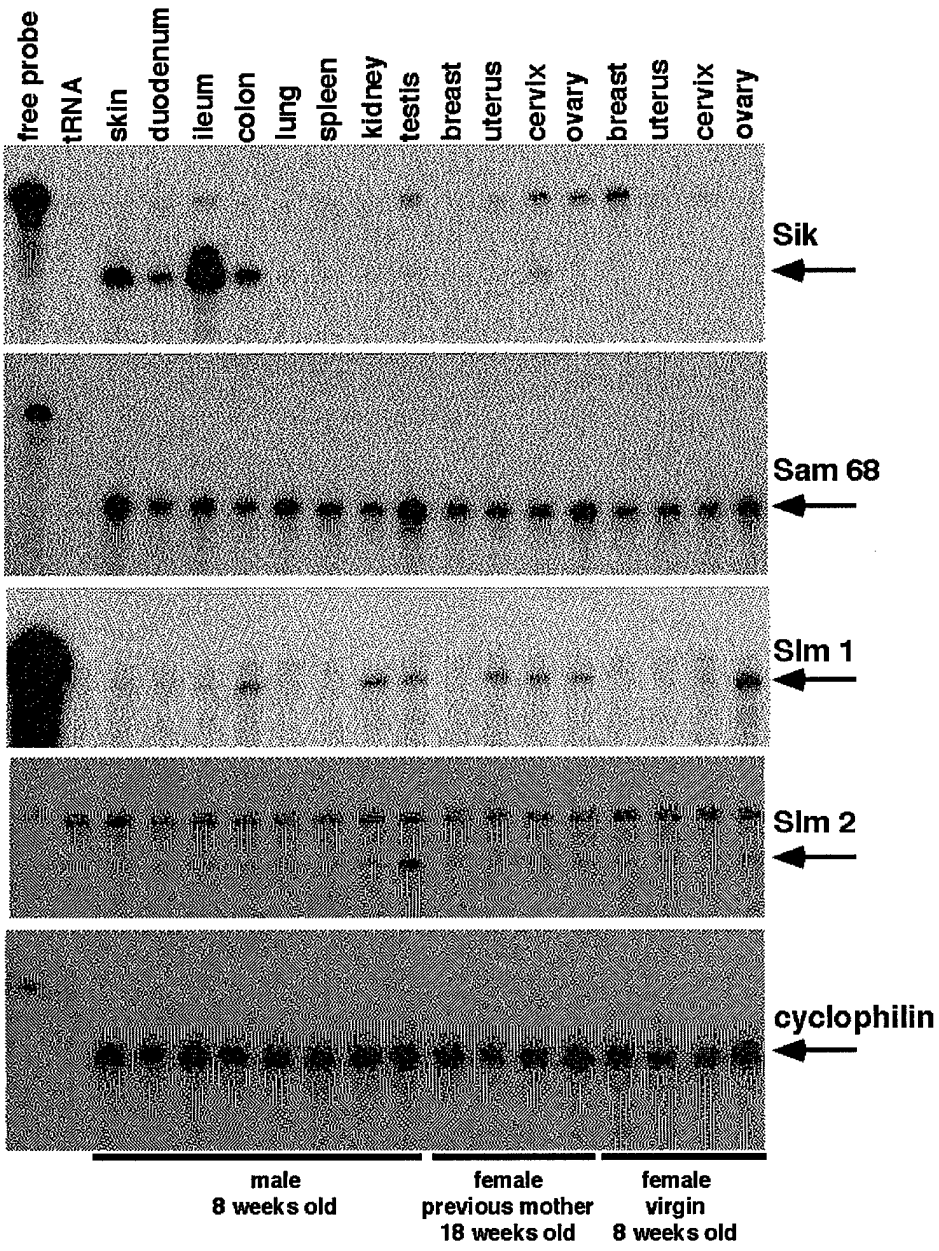

Figure 2. Expression Patterns of BRK/Sik Substrates in the Mouse as Detected Using RNAse protection Assays. RNAse protection assays were performed with total RNAs from a variety of tissues from the mouse (male mice and virgin female and breeder females as indicated) and ^{32}P-labled anti-sense probes specific for Sik (mouse BRK), Sam68, SLM-1, and SLM-2. Cyclophilin expression was examined as a control. Sam68 is ubiquitously expressed, while SLM-1 and SLM-2 have a much more restricted pattern of expression. SLM1 is expressed at low levels in skin and intestine, tissues in which Sik(BRK) is expressed. Although

BRK is induced in a high percentage of breast tumors, mouse BRK (Sik) and its substrates are not expressed in normal adult female breast tissue.

Conclusions

Our experiments have led to the identification new specific substrates of the breast tumor tyrosine kinase BRK, and will lead to a better understanding of signal transduction pathways regulated by BRK in breast cancer. Thus far Sam68 is the only BRK/Sik substrate expressed at significant levels in the normal mammary gland. We are currently examining expression of Sam68, SLM1, ad SLM2 in breast tumor cells. Aberrant expression and/or phosphorylation of these STAR proeins may contribute to the development of breast cancer. These proteins may provide new prognostic indicators for breast cancer and be potential targets for therapeutic intervention.

ABBREVIATIONS

BRK: **Br**east Tumor **K**inase

Sam68: **S**rc-**a**ssociated in **m**itosis, **68** kDa

Sik: **S**rc-related **i**ntestinal **k**inase (original name for mouse BRK)

SLM-1: **S**am68-**l**ike-**m**ammalian protein-1

SLM-2: **S**am68-**l**ike-**m**ammalian protein-2

SNBs: **S**am68/SLM **N**uclear **B**odies

STAR: **S**ignal **T**ransducers and **A**ctivators of **R**NA

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