AD_____

GRANT NUMBER DAMD17-96-1-6175

TITLE: A Novel Tyrosine Kinase Expressed in Breast Tumors

PRINCIPAL INVESTIGATOR: Angela L. Tyner, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
Chicago, Illinois 60612-7205

REPORT DATE: September 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commanding General
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1998	Annual (19 Aug 97 - 18 Aug 98)

4. TITLE AND SUBTITLE
A Novel Tyrosine Kinase Expressed in Breast Tumors

5. FUNDING NUMBERS
DAMD17-96-1-6175

6. AUTHOR(S)
Angela Tyner, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois
Chicago, IL 60612-7205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The breast tumor kinase BRK was isolated from a normal human small intestinal cDNA library that was screened with the cDNA encoding the mouse epithelial specific tyrosine kinase SIK. While BRK and SIK share only 80% sequence identity, Southern blot hybridizations confirmed that the two proteins are orthologues. *Sik* was mapped to mouse distal chromosome 2, which shows conservation of synteny with human chromosome 20q13.3 where the BRK gene is located. Chromosome 20q13 is frequently amplified in a number of tumor types, including breast and colon tumors. Increased BRK expression has been detected in a high proportion of human breast tumors. However, when SIK expression was examined at different stages of normal mammary gland differentiation in the mouse, no expression was detected. Overexpression of wildtype SIK in the normal murine mammary gland cell line NMuMG did not result in increased cell proliferation or foci formation. We are in the process of comparing the activities of SIK and BRK, and determining what growth factors may regulate this epithelial cell specific tyrosine kinase. SIK/BRK appears to play a role in signal transduction in the normal gastrointestinal tract, and its overexpression may be linked to the development of breast and colon tumors.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, tyrosine kinase, Sik, Brk	26
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

AT Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

AT For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

AT In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

AT In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

AT In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Angela L. Tyner 9/17/98

PI - Signature Date

TABLE OF CONTENTS

INTRODUCTION

SIK is a novel intracellular tyrosine kinase that was first identified in a screen for tyrosine kinases that regulate differentiation of intestinal epithelial cells (1). Although it is related to the Src-family of tyrosine kinases and contains both SH2 and SH3 domains, it has a very short unique amino terminus and is not myristoylated (2). Expression of SIK is restricted to epithelial cells and has been detected in the skin and all linings of the alimentary canal. Transcription of the *Sik* gene in these tissues is initiated in cells as they migrate away from the proliferative zone and begin the process of terminal differentiation. SIK expression is developmentally regulated and is first detected at mouse embryonic day 15.5 in the differentiating granular layer of the skin (2).

The role of SIK in regulating differentiation was examined in mouse keratinocytes (3). Addition of calcium to cultured mouse keratinocytes induces a terminal differentiation program and a cascade of tyrosine phosphorylation. SIK was activated within two minutes following calcium addition to keratinocytes. It was found to bind a rapidly phosphorylated 65 kDa GAP-associated protein (GAP-A.p65) through its SH2 domain. Overexpression of SIK in embryonic mouse keratinocyte cell line resulted in increased expression of the differentiation marker filaggrin during differentiation. This supported the hypothesis that SIK, the only known tyrosine kinase activated in keratinocytes within minutes following calcium addition, is involved in a signal transduction pathway that may promote differentiation.

We have found that the breast tumor kinase BRK is the human orthologue of the SIK tyrosine kinase. A portion of BRK catalytic domain was cloned using PCR and degenerate primers corresponding to the conserved regions of tyrosine kinase catalytic domains, and RNA isolated from involved axillary nodes from a patient with

metastatic breast cancer (4). The full length BRK cDNA was isolated from a library prepared from the T-47D breast cancer cell line. BRK transcripts were detected by Northern blot hybridization in the T-47D and MCF-7 breast tumor cell lines, but not in normal breast, liver, placenta, pancreas, or other tissues. By RNase protection, it was detected in one out of five grade III breast carcinoma RNAs, but not in two normal breast samples. By RT-PCR, BRK transcripts could be detected in three out of seven tumors but not in four normal breast RNA samples. In subsequent studies BRK was surveyed by Western blotting and found to be 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 (5). Of 41 primary breast tumor samples quantified by Western blotting relative to cytokeratin 18, BRK was overexpressed by 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.

BRK was also cloned from melanoma cells and named PTK-6 (6). BRK mRNA levels were undetectable in seven primary melanoma lines, two normal samples of melanocytes, and in biopsies from metastatic melanomas. However, BRK was present in 2/22 metastatic melanoma cell lines (7), and it is possible that it is expressed in 10% of primary melanoma and melanocyte cultures because the sample size thus far examined has been small (7).

Using human/hamster somatic cell hybrids PTK 6 (BRK) was mapped to human chromosome 20 (6). Fluorescence in situ hybridization was used to further localize the BRK gene to 20q13.3 (8). 20q13.3 was one of five regions found to be

amplified in homogeneously staining regions of chromatin of three primary breast carcinomas (9). In addition to being amplifed in breast tumors and breast tumor cell lines (10, 11), amplification at 20q13 has been detected in other epithelial tumors including gastric and gastro-esophogeal tumors (12), and colon tumors (13). Progress that we have made elucidating the relationship between BRK and SIK and the possible role that this kinase may play in epithelial cell cancers is discussed below.

BODY

Experimental Methods

Mapping of Mouse *Sik*

Mapping of *Eef1a2* was carried out using DNA from the Jackson Laboratory Interspecific Backcross BSS panel (14). This panel is made up of 94 N2 offspring derived from the cross (C57BL/6J X SPRET/Ei)F1 X SPRET/Ei. Over 3310 loci have been mapped in this cross. *Eef1a2* was found to map to within the most distal group of markers on Chr 2. A BAC library constructed from 129/Sv ES cell DNA (Research Genetics) was screened with primers corresponding to *Eef1a2*. Two independent clones were isolated, each of which had an insert of approximately 65-70 kb. Each of these clones was found by sequencing to contain the whole of the coding and 3'UTR sequence of the *Sik* gene in addition to *Eef1a2*. One clone also contained the 5'UTR sequence of *Sik*.

Sequences compared in Figure 1B include mouse: *Pltp*: U37226 (1051265), nucleotides (nt) 67-1548; *Eya2*: U81603, nt 166-1587; *Gnas*: Y00703 (51127), nt 20-1204; *Pck1*: AI037119, nt 1-546, AA562908, nt 470-539, AI021099, nt 1-59, AA080172, nt 18-478, AA286042, nt 458-569, AA106463, nt 9-537, AA110781, nt 118-515; *Lama5*: U37501 (2599231), nt 5906-10820; *Eef1a2*: L26479 (1220409), nt

134-1525; *Col9a3*: X91012 (975686), nt 1-333, AA027742 (1493761), nt 1-104; *Sik*: U16805, nt 286-1641; human: PLTP: L26232 (468325), nt 88-1569; EYA2: Y10261 (1834488), nt 196-1617; GNAS: X04408 (31914), nt 69-1253; PCK1: nt 122-1990 of L05144 (189944) excluding 589-609 (for which mouse EST sequence was unavailable); LAMA5: 1-4930 nt of GenBank locus AB011105 (NCBI accession 3043589); EEF1A2: 84-1475 nt of X70940 (38455); COL9A3: 1564-2012 nt of L41162 (1196420); BRK: X78549 814-2169.

Ribonuclease protection assays

Expression of *Sik* was analyzed by ribonuclease protection assay, as described (15) using [^{32}P] α-CTP labeled antisense RNA probes. A pBlueScript SK II+ plasmid containing a 205 bp fragment encoding a portion of the *Sik* catalytic domain (1), was linearized at an Xba I site in the polylinker, and in vitro transcription was performed using T7 polymerase (Promega, Madison, WI). A 198 bp StuI-ApaI fragment of the BRK cDNA was subcloned pBlueScript SK II - . This plasmid was linearized with Hind III, and in vitro transcription was performed with T7 polymerase. Linearized templates used for in vitro transcription were purified on 5% acrylamide gels. As controls for RNA levels and integrity, RNase protections were also performed with antisense probes for mouse cyclophilin (pTRI-cyclophilin-Mouse ; Ambion, Austin, TX).

Tissues were dissected from ICR mice and homogenized in guanidine thiocyanate solution with 2-mercaptoethanol (16). Total RNA was isolated by CsCl gradient centrifugation. Twenty µg of each total RNA sample, or an equal amount of baker's yeast tRNA (Boehringer Mannheim, Indianapolis, IN) was precipitated with ethanol and resuspended in 30 ul of hybridization buffer containing 2 X 10^5 cpm of probe. Samples were hybridized 12-18 hr at 55°C, then treated for 30 min at 37°C

with 350 ul of ribonuclease digestion buffer (10 mM TrisCl, pH 7.5, 5 mM EDTA, 300 mM NaCl) containing 0.23 units RNase A and 25 units RNase T_1 per sample (Boehringer Mannheim) or 300 ul of ribonuclease digestion buffer (10 mM Tris·Cl, pH 7.5, 5 mM EDTA, 200 mM NaOAc) containing 5 units RNase ONE (Promega) per sample. The samples were subjected to proteinase K digestion, phenol:chloroform extraction, ethanol precipitation, and were then analyzed on a denaturing acrylamide gel.

Western blot analyses

Thirty μg of protein per lane were subjected to electrophoresis through a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes using a tank blotting unit (Bio-Rad, Melville, NY). Filters were blocked for 1 hour in 5% nonfat dry milk in TBS-T buffer containing 10 nm Tris-HCl, pH 7.5, 500 mM NaCl and 0.1% Tween 20, and then incubated for one hour with BRK (C-17) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or they were blocked with TBS-T buffer containing 100 mM NaCl and ß-actin antibody (Sigma, positive control). The higher NaCl substantially reduced background obtained with the commercially available BRK polyclonal antibody. Subsequently, the membranes were stained with appropriate horseradish peroxidase [HRP] conjugated secondary antibodies and antibody detection was accomplished using the SuperSignal ULTRA chemiluminiscence substrate (Pierce).

Infection of NMuMG Cells with SIK expression Constructs

As described previously, retroviral expression constructs in the vector pLXSN containing wildtype SIK or SIK in which the conserved lysine in the catalytic domain has been mutated to methionine (kinase-dead SIK) have been generated. Infection of the normal murine mammary gland cell line NMuMG was done as previously

described (17). The BOSC 23 packaging cell line was transiently transfected with plasmids using the $CaPO_4$-precipitate procedure. Within 48 hours after transfection the supernatant from these cells was used for infection of the NMuMG cell line. In experiments with control plasmid MFG-lacZ, encoding β-galactosidase (18), between 30 and 60% of the cells were infected. At 48 hours after infection the cells were split 1:4 into selective media containing 250 μg/ml of active G418 (Gibco BRL) and were refed every 2 days. Upon reaching the confluence the cells were split 1:4 once again. After 2 weeks in selective media there were no dead or dying cells in the culture.

Immunoprecipitations and in vitro kinase assays

For immunoprecipitations, cells were washed twice with PBS, overlaid with ice cold LB buffer [50 mM HEPES pH 7.4, 150 mM NaCl, 1% NP 40, 2mM Na_3VO_4, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 10 mg/ml aprotinine, pepstatine A and leupeptine, 1 mM PMSF], or LB/SDS buffer [LB plus 1% sodium deoxycholate and 0.1% SDS], incubated on ice for 30 min and then harvested. Protein concentrations were measured using the BCA protein assay (Pierce). One mg of total cellular protein was used for immunoprecipitations with the anti-SIK antibodies. Protein extracts were precleared by incubation with 40 μl of 50% slurry of protein A-sepharose (Pharmacia Biotech Inc., Piscataway, NJ) for 1 hour at 4°C. Proteins were incubated with 1μg of antibody for 1 hour at 4°C. 40 μl of a 50% slurry of protein A-sepharose was added and incubation was continued for 1 h. The sepharose beads were washed 5 times with LB or LB/SDS, resuspended in 1 x

SDS sample buffer [62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol] and boiled for 5 min.

For in vitro kinase assays, the sepharose beads were washed 5 times in LB/SDS, followed by one wash in kinase buffer [50 mM HEPES pH7.4, 50 mM NaCl, 10 mM $MnCl_2$, 0.05% Triton X100]. They were then resuspended in 20 µl of the same buffer containing 20 µCi of [γ-^{32}P] ATP. After careful mixing, the reaction mixture was incubated at 30°C for 15 min. An equal volume of 2 x SDS sample buffer, containing 30 mM EDTA and 1mM of cold ATP, were added and the reaction mix was boiled for 5 min. The phosphorylated proteins were detected following SDS-PAGE electrophoresis, incubation in 1 M KOH at 55°C for 2 hours and exposure to X-ray film.

Indirect immunohistochemistry with tyramide amplification

Paraffin-embedded biopsy samples sectioned at 5-8 micrometers thick were deparaffinized, hydrated, and preincubated in block buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% TWEEN 20, 1:50 normal goat serum) for 40 minutes. Sections were then incubated with 0.2 ug/ml BRK antibody (Santa Cruz) in block buffer overnight at 4 C, washed, and incubated with 1:250 biotinylated goat anti-rabbit antibody (Vector) in block buffer for 30-60 min. After washing, the TSA-indirect kit (DuPont/NEN) was used according to manufacturer's instructions. Briefly, sections were treated with strepavidin-horseradish peroxidase, reacted for 5 minutes with biotinyl tyramide reagent, visualized with 1:500 FITC-Avidin DCS (Vector), and mounted with Vectashield mounting medium (Vector).

For control sections, 4 ng per ng of BRK antibody, of the peptide from which the BRK antibody was raised (Santa Cruz) were added to block buffer, 10-15 minutes before use.

Results

Mapping of *Sik* to a region of the mouse genome that shares conservation of synteny with human chromosome 20q13.3

Like SIK, BRK is also a 451 amino acid protein with common sequences found in SRC family kinases, such as SH2 and SH3 domains and a putative regulatory tyrosine at the carboxy terminus. In contrast to members of the SRC family, SIK and BRK lack sites for myristoylation at the amino terminus. SIK and BRK also have the sequence HRDLAARN in their catalytic domains, in contrast to the sequence HRDLRAAN shared by members of the SRC family. While the functional domains of BRK and SIK are conserved, SIK and BRK share only 80% amino acid identity, and 83% nucleotide identity.

Comparison of the nucleotide sequences encoding *Sik* and BRK indicates that there are no apparent insertions or deletions and the sequence differences largely reflect changes in GC content of the DNA code. Of 231 differences within the coding sequence, 159 are positions where G or C is present in the human sequence, while the corresponding mouse nucleotide is A or T. Many of the alterations in the protein sequence appear to be driven by mutations of CpG dinucleotides. Of 86 CpG dinucleotides in the human, only 18 are conserved with the mouse sequence, which has 24 in total. Throughout the known promoter and mRNA sequence, the observed/expected (o/e) CpG ratio of *Sik* averages 0.23 and does not exceed 0.3 within a 500-bp window, while BRK averages an o/e ratio of 0.63, peaking at 0.8 in a 5' CpG island but not falling below 0.4. The average level for mammalian genomic

DNA is 0.26 (19). The loss of CpG content in *Sik* is evenly distributed throughout the promoter and coding sequence, with BRK always having at least double the *Sik* o/e CpG ratio.

We have found that the mouse *Sik* gene is linked to the gene *Eef1a2* that encodes translation elongation factor alpha. A BAC library constructed from 129/Sv ES cell DNA was screened with primers corresponding to *Eef1a2* (20). Two independent clones were isolated, each of which had an insert of approximately 65-70 kb. Each of these clones was found by sequencing to contain the whole of the coding and 3'UTR sequence of the *Sik* gene in addition to *Eef1a2*. One clone also contained all 5'UTR sequence of *Sik*. The *Sik* gene must therefore map to the distal end of mouse chromosome 2, within 60 kb of *Eef1a2*, in a region of conserved synteny with human chromosome 20q13 (Figure 1). Since BRK has been mapped to human chromosome 20q13.3 (8), this provides further evidence that these two genes are orthologous. In wasted mice the *Eef1a2* gene is deleted (20), while the *Sik* gene is intact and appropriately expressed.

The coding sequences of BRK and the linked genes EEF1a2 and LAMA5 contain 82-89% G+C in the third codon position, which places them well above the threshold of the H3 isochore (75%), the G+C-richest fraction of DNA which forms 3% of the human genome and contains 28% of human genes (21). H3 isochores, which are usually over 300 kB in length, are most concentrated in T (telomeric, thermally resistant, or H3+) bands, and occur more sparsely in T' (H3*) bands; one of the human genome's 28 H3+ bands maps to the telomere of chromosome 20 (22, 23). Although the overall values for G+C% and 5-methylcytosine in human and mouse are nearly identical (19), the mouse genome lacks the very GC-rich H3 isochore (24, 25). Accordingly, the mouse *Sik, Eef1a2,* and *Lama5* genes contain 14-19% less G+C in

the third codon position (Figure 1b). Interestingly, the tightly linked *Col9a3* gene also has 18% less G+C, although its third codon G+C is much lower. It has been estimated that 20% of the CpG islands present in the human have been lost in mouse orthologues, primarily in tissue-specific genes (26). The case of BRK and *Sik* suggests that this can occur by effects on the isochore level which increase CpG loss uniformly on a megabase scale. Thus, the mapping of these genes to regions of conserved synteny in human and mouse chromosomes near the telomere allows the physical extent and nature of T band changes between the species to be examined directly.

***Sik* expression is not induced during normal mammary gland differentiation**

Since significant levels of BRK mRNA have been detected in human breast tumor cell lines and in primary breast tumors, we hypothesized that BRK/*Sik* may play a role in normal differentiation of the mammary gland. We examined *Sik* expression in the mammary gland. Mammary gland differentiation is regulated hormonally and requires pregnancy for establishment of terminal differentiation (for review see (27). During involution following weaning, a number of morphological and biochemical changes also occur. We examined *Sik* expression in the mammary glands of virgin, pregnant, and lactating mice and at different times following weaning (involution). At no time did we detect significant expression in the normal mammary gland. In contrast, *Sik* expression was easily detected in the skin and small intestine (Figure 2). No significant *Sik* expression was detected in the mammary gland at different stages using in situ hybridization and immunohistochemistry (data not shown), ruling out the possibility that *Sik* expression was induced in a small subset of cells in the mammary gland.

BRK expression in tumor cell lines

Levels of BRK protein were examined by Western blotting using protein extracts from a breast tumor cell line that expresses high levels of BRK and four human colon carcinoma tumor cell lines, including SW480, HT29, T84, and Caco-2. Of these, Caco-2 cells are the least tumorigenic (28). These cells differentiate in the absence of inducers, after reaching confluence, and as they are maintained in culture, they polarize, form microvilli, and express increasing levels of brush border enzymes, such as sucrase isomaltase (29). BRK expression was detected in the colon carcinoma cell lines. The SW480 and HT29 cells express high levels of BRK protein, which are equivalent to or exceeding that found in the breast tumor cell line MCF7 (Figure 3).

Expression of SIK in NMuMG cells

As seen in Figure 4, we have generated NMuMG (normal murine mammary gland) cell lines expressing the SIK tyrosine kinase. NMuMG cells do not express SIK. This is consistent with our results examining the expression of SIK at different stages of breast development in vivo (Figure 2). So far, we have been unable to detect any alterations in growth properties of cells overexpressing wildtype SIK. In addition, we have not been able to co-immunoprecipitate the EGF receptor using anti-SIK antibodies and protein extracts from NMuMG cells overexpressing SIK, or from HT-29 cells overexpressing SIK. We have not detected increased SIK activity following EGF addition to these cell lines.

In preliminary experiments, it appears that SIK may be influenced by hepatocyte growth factor. When 200 scattering units/ml of HGF was added to SIK expressing NMuMG cell lines for 0.5, 2, and 5 minutes, increased SIK tyrosine phosphorylation was detected (Figure 4A). In the presence of no added HGF, SIK is

not significantly tyrosine phosphorylated. After 0.5 minutes, a significant increase in SIK tyrosine phosphorylation was detected, while there are no changes in SIK protein levels (Figure 4B). In the coming year we will try to determine if SIK activity increases following HGF addition. We also perform studies to determine if SIK associates with the HGF receptor Met.

Localization of BRK in Breast Cancer Cells

As reported last year, we have used cell fractionation and immunohistochemistry to localize SIK that is overexpressed in NMuMG cells. Sik was found in membrane, cytosolic and nuclear fractions in NMuMG cells. In contrast SIK expression is almost exclusively nuclear in primary mouse keratinocyte cultures. We have examined BRK expression in twelve archival human breast tumor samples using immunohistochemistry with tyramide amplification. Strong BRK expression was detected in five tumors, weak Brk expression was detected in three tumors, and no Brk expression was detected in four of the tumor samples. There was no consistent pattern of Brk protein localization in these tumors. In some cases BRK was cytoplamic, and in others in was apparent in granules. In one differentiated ductal carcinoma BRK protein was present in the basal cell membranes. As a control, the primary BRK antibody was preincubated with control peptide, which strongly reduced staining in adjacent sections. Further studies will be required to understand the significance of variable BRK localization in these tumors.

CONCLUSIONS

From its initial cloning from breast tumor tissue, BRK has been suspected to be a proto-oncogene. BRK is a highly diverged 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. BRK is most closely homologous (45%) to SRK1 [P42686], a SRC-like tyrosine kinase from Spongilla lacustris, and it shares 45% homology and 6 of 7 introns with DSRC41, a SRC-like gene from Drosophila. Nonetheless, BRK 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 cDNA encoding the intracellular breast tumor tyrosine kinase BRK was initially cloned from metastatic breast tumor mRNA, and its expression was only detected in human breast tumors and in breast tumor cell lines (4). We isolated BRK encoding cDNA clones from a normal human small intestine cDNA library, using a mouse *Sik* probe. The genes encoding *Sik* and BRK do not appear to be tightly conserved. In contrast to mouse and human SRC, which share a high degree of sequence identity (99%), mouse SIK and human BRK share only 80% sequence identity. Because of this relatively low level of homology, we confirmed that *Sik* and BRK are orthologues of one another by performing a series of Southern blot experiments. Previously, we found that radiolabeled probes specific for BRK and *Sik* recognized an identical simple set of bands in both mouse and human genomic DNA, indicating that no genes with closer homology existed in either genome. In addition, we have now mapped the *Sik* gene to the distal portion of mouse chromosome 2,

which shows conservation of synteny with human chromosome 20q13.3 where BRK is located.

We were unable to detect *Sik* expression at any stage of normal mammary gland development in the mouse. BRK expression in breast tumors and breast tumor cell lines, but not in normal breast tissue, suggested a role for BRK expression in carcinomas. Others have found BRK expressed at appreciable levels in approximately two-thirds of the breast tumors that were examined (5). Overexpression of BRK in the HB4a human mammary cell line mitogenically sensitizes these cells to EGF (30). In addition, overexpression of BRK in these cells resulted in increased growth in soft agar, indicating that BRK overexpression can contribute to a transformed phenotype (30).

Mapping of *Sik* to the telomeric region of chromosome 2 explains the high level of divergence of the *Sik* gene to some extent, as the mouse genome does not have the high G+C rich H3 regions found in human telomeres (21). In addition, it has been estimated that 20% of the CpG islands present in the human have been lost in mouse orthologues, primarily in tissue-specific genes (26).

Some activities of SIK appear to differ than those of BRK which were reported by another group (30). In contrast to BRK, SIK overexpression in the NMuMG cell line does not result in increased proliferation and/or foci formation. In addition, we have been unable to demonstrate that SIK plays any positive role in EGF signaling. It is possible that the divergent mouse sequence encodes a protein lacking features of the human orthologue. Alternatively, the BRK cDNA that was cloned from tumor cell RNA, may contain a mutation that alters the encoded protein's activities. We are the first to demonstrate that BRK is expressed in the normal epithelial linings of the intestinal tract and skin. In the coming year with will compare the sequence of BRK

expressed in normal intestine and skin, with BRK expressed in a variety of tumor cell lines to determine if mutations of BRK are present in tumors. We will start by performing Cleavase Fragment length Polymorphism (CFLP) analysis (31, 32), followed by sequencing if alterations are detected.

In all of the cellular studies that we have done so far, we have used wildtype SIK expression constructs. We will now repeat some of these studies with BRK expression constructs to determine if BRK expression in mouse breast cells results in increased sensitivity to EGF and foci formation. These experiments will be important to perform prior to carrying out the proposed transgenic mouse experiments. It is possible that targeted expression of BRK to the mammary gland will result in cellular transformation and tumor formation, while targeted expression of SIK will not.

We detected BRK expression in 8/12 archival breast tumor samples. Although BRK maps to a region of human chromosome 20 that is frequently amplified in a variety of tumor types, it still must be determined if increased BRK expression in tumors ultimately contributes to transformation. Like SIK, we have found that BRK is present throughout the normal human gastrointestinal tract and skin, and we localized BRK expression to differentiating epithelial cells in the colon, where the highest levels of protein and mRNA were found in epithelial cells in the middle and upper colonic crypts. These data support the hypothesis that wildtype BRK may play a role in a signal transduction pathway associated with differentiation. It will be important to determine if BRK overexpression plays a role in the development of breast and colon cancers.

FIGURE LEGENDS

Figure 1. (A) The map position of *Sik* relative to other markers in the Jackson Laboratory BSS Interspecific Backcross. Only those genes whose human homologues have been mapped are shown. The raw typing data for all the markers is available on the WWW at http://www.jax.org/resources/documents/cmdata. Distances between markers are given to the left of the Chromosome as centiMorgans +/- standard error. (B) Isochore G+C differences in distal mouse chromosome 2 and human chromosome 20q. Known sequence for the syntenic genes above are analyzed for total G+C content, third codon position G+C content, and observed/expected CpG ratio for human, mouse, and the degree to which the human exceeds mouse content (Δ). Percent sequence identity (with <0.6% gaps) is also indicated. Note that the GC-rich distal genes, including Brk/Sik, vary in a manner consistent with loss of the most GC-rich isochores in the mouse, but additionally in correlation with map position. Asterisk indicates that mouse Pck1 sequence was assembled from mouse EST fragments and is less reliable. The segments examined are restricted to coding sequence and usually include all coding sequence, but segments of Lama5 and Col9a3 compared use only 3' sequence because 5' sequence is unknown.

Figure 2. *Sik* is not expressed in normal mouse breast tissue. RNase protection assays were performed with total RNA isolated from virgin, pregnant, lactating, and involuting breast tissue, as well as small intestine and skin. Cyclophilin expression was examined in the same samples as a control.

Figure 3. BRK expression in colon carcinoma cell lines. Western blotting was performed with total protein extracts from MCF7, SW480, HT29, T84, and Caco-2 cells. SW480 and HT-29 cell extracts contain highest levels of BRK, while Caco-2 cells contain too low a level of BRK to be visible in the 10 minute exposure at left. In the 5 hour exposure at right, BRK protein levels can be seen to increase during early differentiation of Caco-2 cells. In the long exposure, the T84 cell line can be seen to have a weak upper band corresponding to the upper band in Caco-2 cells (not shown). The membrane was stripped and probed with anti-β-actin antibody as a control (2 minute exposure).

Figure 4. Increased SIK tyrosine phosphorylation following HGF addition to NMuMG cell lines. Proteins in total cell lysates (TCL), or protein immunoprecipitated with the anti-SIK antibody from cells infected with the pLXSN vector alone, or vector driving expression of wildtype Sik, were probed with anti-phosphotyrosine antibody (A) or anti-SIK antibody (B).

REFERENCES

1. E. Y. Siyanova, M. S. Serfas, I. A. Mazo, A. L. Tyner, *Oncogene* **9**, 2053-7 (1994).

2. V. Vasioukhin, et al., *Oncogene* **10**, 349-57 (1995).

3. V. Vasioukhin, A. L. Tyner, *Proc Natl Acad Sci U S A* **94**, 14477-82 (1997).

4. P. J. Mitchell, et al., *Oncogene* **9**, 2383-2390 (1994).

5. K. T. Barker, L. E. Jackson, M. R. Crompton, *Oncogene* **15**, 799-805 (1997).

6. S. T. Lee, K. M. Strunk, R. A. Spritz, *Oncogene* **8**, 3403-10 (1993).

7. D. J. Easty, et al., *Int J Cancer* **71**, 1061-5 (1997).

8. S. H. Park, K. H. Lee, H. Kim, S. T. Lee, *Cytogenet Cell Genet* **77**, 271-2 (1997).

9. M. Muleris, A. Almeida, M. Gerbault-Seureau, B. Malfoy, B. Dutrillaux, *Genes Chromosomes Cancer* **14**, 155-63 (1995).

10. A. Kallioniemi, et al., *Proc Natl Acad Sci U S A* **91**, 2156-60 (1994).

11. F. Courjal, C. Theillet, *Cancer Res* **57**, 4368-77 (1997).

12. W. El-Rifai, et al., *Cancer Res* **58**, 34-7 (1998).

13. J. Schlegel, et al., *Cancer Res* **55**, 6002-5 (1995).

14. L. B. Rowe, et al., *Mamm Genome* **5**, 253-74 (1994).

15. M. S. Serfas, A. L. Tyner, *Am. J. Physiol.* **265**, G506-G513 (1993).

16. J. M. Chirgwin, A. L. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294-5299 (1979).

17. W. S. Pear, G. P. Nolan, M. L. Scott, D. Baltimore, *Proc. Natl. Acad. Sci. USA* **90**, 8392-8396 (1993).

18. G. Dranoff, et al., *Proc. Natl. Acad. Sci. U S A* **90**, 3539-3543 (1993).

19. K. Jabbari, S. Caccio, J. P. Pais de Barros, J. Desgres, G. Bernardi, *Gene* **205**, 109-18 (1997).

20. D. M. Chambers, J. Peters, C. M. Abbott, *Proc Natl Acad Sci U S A* **95**, 4463-8 (1998).

21. D. Mouchiroud, et al., *Gene* **100**, 181-7 (1991).

22. S. Saccone, S. Caccio, J. Kusuda, L. Andreozzi, G. Bernardi, *Gene* **174**, 85-94 (1996).

23. S. Saccone, A. De Sario, G. Della Valle, G. Bernardi, *Proc Natl Acad Sci U S A* **89**, 4913-7 (1992).

24. G. Bernardi, *Annu Rev Genet* **23**, 637-61 (1989).

25. G. Bernardi, *Gene* **135**, 57-66 (1993).

26. F. Antequera, A. Bird, *Proc Natl Acad Sci U S A* **90**, 11995-9 (1993).

27. L. Hennighausen, G. W. Robinson, *Genes Dev* **12**, 449-55 (1998).

28. J. E. de Vries, et al., *Br J Cancer* **71**, 271-7 (1995).

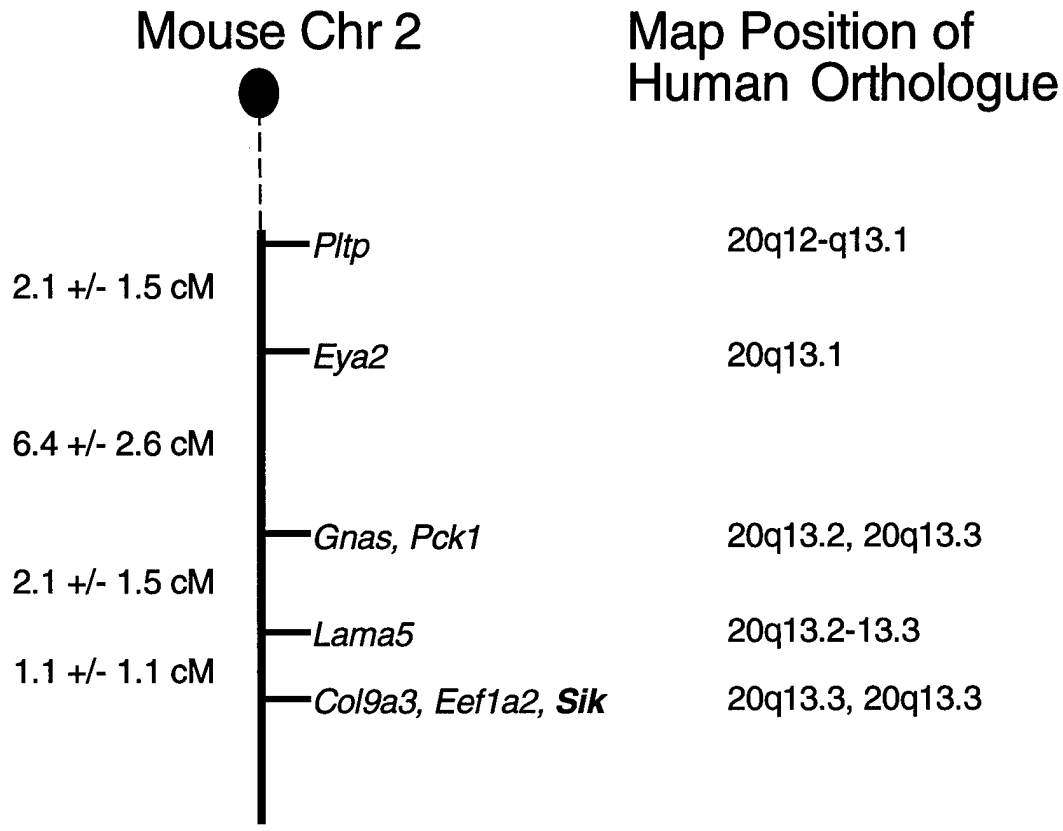
29. M. Pinto, et al., *Biol. Cell* **47**, 323-330 (1983).

30. T. Kamalati, et al., *J. Biol. Chem.* **271**, 30956-30963 (1996).

31. M. A. D. Brow, et al., *Focus* **18**, 2-5 (1996).

32. V. Lyamichev, M. A. D. Brow, J. E. Dahlberg, *Science* **260**, 778-783 (1993).

Figure 1



A. *Sik* maps to the distal arm of mouse chromosome 2

Gene	Cons	CDS G+C% H	M	Δ	3rd Codon G+C% H	M	Δ	CpG Obs/Exp H	M	Δ
Pltp	83	59.9	57.1	+2.8	77.3	72.5	+4.8	0.43	0.54	-0.11
Eya2	85	55.6	56.3	-0.7	67.7	69.4	-1.7	0.51	0.53	-0.02
Gnas	95	55.0	55.8	-0.8	73.4	75.7	-2.3	0.64	0.63	+0.01
Pck1	84*	55.9	56.1	-0.2	71.4	62.0	+9.6	0.42	0.47	-0.05
Lama5	76	67.1	59.7	+7.4	82.4	68.2	+14.2	0.53	0.38	+0.15
Eef1a2	88	62.1	56.6	+5.5	89.0	72.0	+17.0	0.81	0.61	+0.20
Col9a3	81	69.3	62.6	+6.7	61.1	42.9	+18.2	0.48	0.18	+0.30
Sik	83	64.0	55.3	+8.7	88.5	69.9	+18.6	0.63	0.23	+0.40

B. Differences in CpG content of Genes Linked to *Sik* and BRK

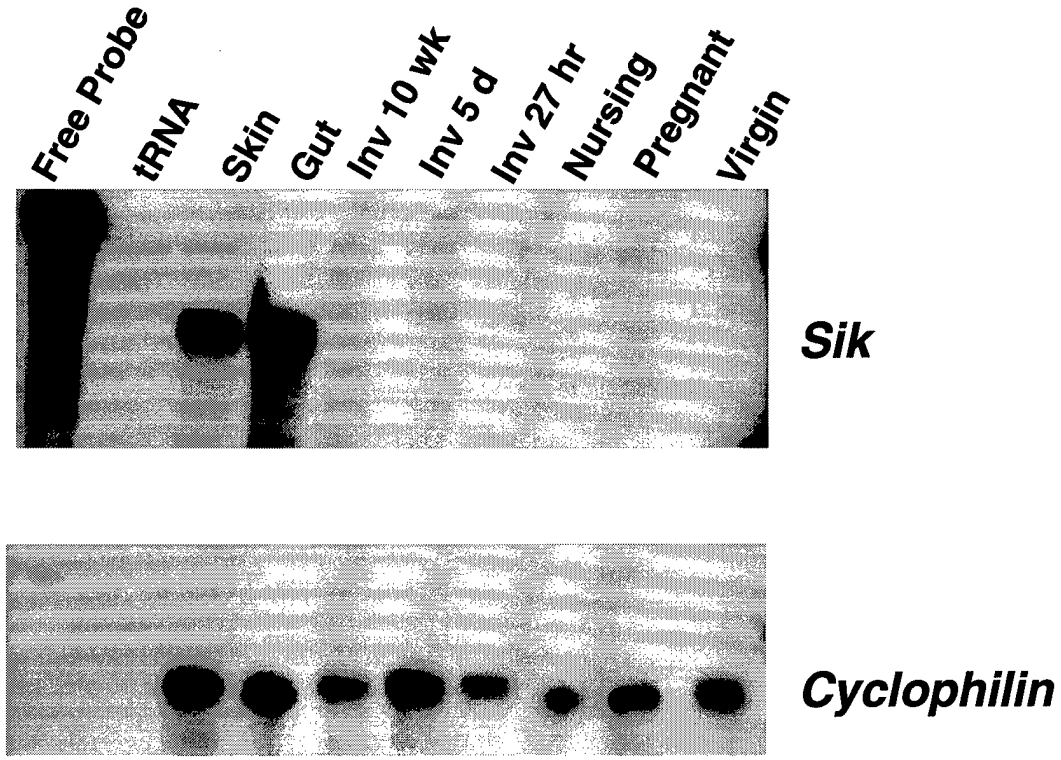


Figure 2. *Sik* is not expressed in normal mouse breast tissue

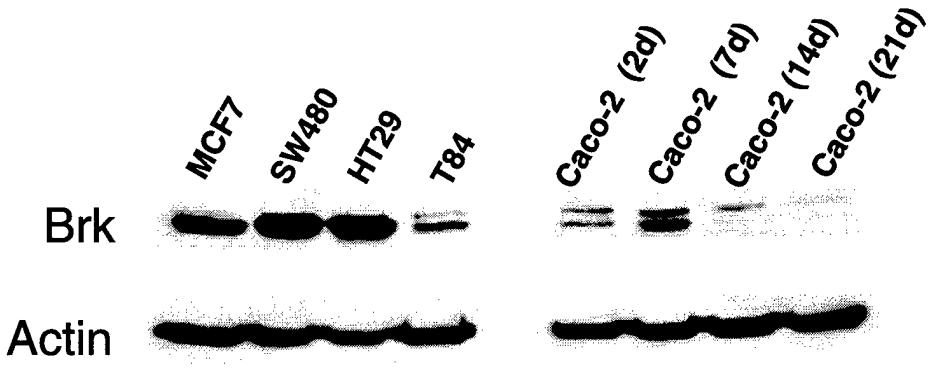
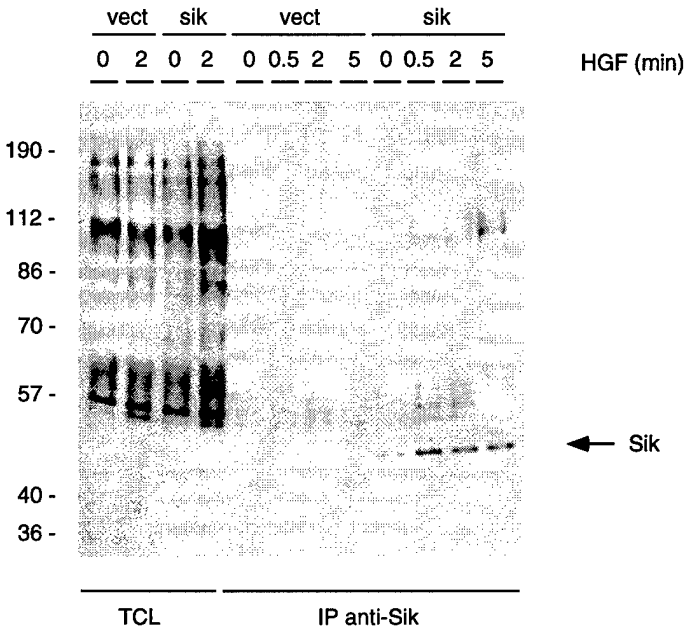
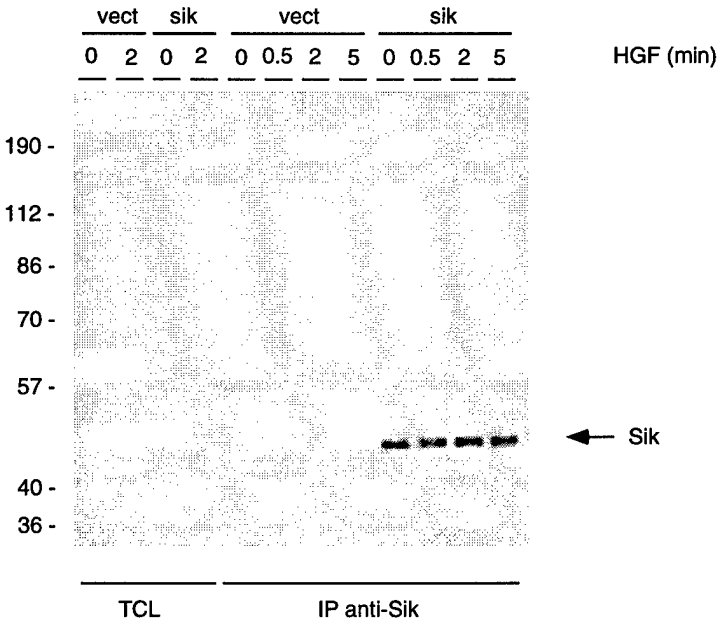


Figure 3. BRK is expressed in breast and colon tumor cell lines

Figure 4



A. Tyrosine phosphorylation of Sik increases after treatment with HGF
Immunoblot with anti-phosphotyrosine antibodies following HGF treatment of NMuMGcells containing empty vector or the wildtype Sik expression construct. TCL: Total Cell Lysate; IP anti-Sik: Proteins immunoprecipitated with the anti-SIK antibody.



B. Sik protein levels do not change after treatment with HGF
Immunoblot with anti-Sik antibodies following HGF treatment of NMuMG cells containing empty vector or the wildtype Sik expression construct.