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13. ABSTRACT *(Maximum 200 Words)*

Efforts to identify genes that regulate intestinal epithelial cell differentiation led to the isolation of a novel epithelial-specific tyrosine kinase named Sik, for Src-related intestinal kinase. We identified the human homologue of Sik and determined that it is the breast tumor kinase BRK, which had been isolated from a metastatic breast tumor. Sik/BRK is expressed in differentiating epithelial cells of the skin and gastrointestinal tract. While BRK is induced in a high percentage of breast tumors examined, we could not detect Sik/BRK expression at any stage of normal mammary gland development. In breast cancer cell lines, BRK is in novel nuclear structures called Sam68/SLM nuclear bodies (SNBs), where it associates with the RNA-binding protein Sam68. Phosphorylation of Sam68 by Sik/BRK inhibits Sam68 RNA-binding and the ability of Sam68 to act as an HIV1 Rev cellular homologue in nuclear RNA export. Sam68 has been shown to play a positive role in promoting mitosis, and is a member of a growing family of RNA-binding proteins called STAR (Signal Transducers and Activators of RNA). Sik/BRK may regulate gene expression associated with breast cancer development by modifying the activities of the Sam68 and related STAR proteins.

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Table of Contents

INTRODUCTION

Sik is an intracellular tyrosine kinase that was first identified in a screen for tyrosine kinases in intestinal epithelial cells (Siyanova et al., 1994). Although it is related to the Src family and contains SH2 and SH3 domains, it has a very short unique amino terminus and is not myristoylated (Vasioukhin et al., 1995). Sik expression is restricted to differentiating epithelial cells and it is found in the skin and all linings of the alimentary canal. Addition of calcium to cultured primary mouse keratinocytes induces cell differentiation and rapid activation of Sik (Vasioukhin and Tyner, 1997). Overexpression of Sik in an embryonic mouse keratinocyte cell line resulted in increased expression of the differentiation marker filaggrin during calcium-induced differentiation, suggesting that Sik is involved in a signal transduction pathway that may promote differentiation (Vasioukhin and Tyner, 1997).

The human orthologue of Sik is called BRK (breast tumor kinase) (Llor et al., 1999; Mitchell et al., 1994). BRK expression has been detected in breast tumors (Barker et al., 1997), colon tumors (Llor et al., 1999), and melanomas (Easty et al., 1997; Lee et al., 1993). Like Sik, BRK is expressed in skin and gastrointestinal epithelial cells that are undergoing terminal differentiation. While BRK appears to play a role in signal transduction in normal epithelial linings, its overexpression appears to be linked to the development of a variety of epithelial tumors. The seemingly paradoxical roles of Sik/BRK during differentiation and tumorigenesis are poorly understood.

During the funding period, we determined that Sik/BRK is not expressed during normal mammary gland differentiation, although it is expressed at high levels in up to 2/3 of breast tumors examined. We found that is a nuclear tyrosine kinase that phosphorylates Sam68. Sam68 (Src-associated in mitosis, 68 kDa) is an RNA binding protein that was identified as a major target of Src during mitosis when the nuclear membrane breaks down (Fumagalli et al., 1994; Taylor and Shalloway, 1994). Sam68 contains a STAR (Signal Transduction and Activation of RNA) domain of approximately 200 amino acids that is also referred to as the GSG (GRP33/Sam68/GLD1) domain (reviewed in (Vernet and Artzt, 1997). A conserved KH (hnRNP**K** **H**omology) RNA binding domain is located within the STAR domain. KH domains play crucial roles in proteins encoded by several developmentally important genes, including 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). The Sam68 KH domain is important for its nuclear localization and self-association (Chen et al., 1997).

Sam68 can bind and be phosphorylated by Src and Src family members (Richard et al., 1995) and the Tec family member Itk (Bunnell et al., 1996) following nuclear breakdown during mitosis. It has also been found to be phosphorylated on serine and threonine residues by Cdc2/cyclin B complexes (Resnick et al., 1997). Sam68 preferentially binds RNA with UAAA motifs (Lin et al., 1997; Resnick et al., 1997), but tyrosine phosphorylation by Fyn impairs its RNA binding ability (Wang et al., 1995). Sam68 also associates with phospholipase Cγ, and the adaptor proteins Grb2 (Richard et al., 1995) and Nck (Lawe et al., 1997). Sam68 appears to play a role posttranscriptional control of gene expression, as it was recently shown to be a functional homologue of the HIV-1 Rev protein that is involved in nuclear export of RNA containing the Rev response element (Reddy et al., 1999).

Sik/BRK is the only identified tyrosine kinase that colocalizes with Sam68 within the nucleus. We found that Sik/BRK is active within the nucleus and that it can phosphorylate Sam68 in vivo. Phosphorylation of Sam68 within the nucleus inhibits its RNA-binding functions and may have important physiological significance and may contribute to the posttranscriptional control of gene expression in breast cancers.

BODY

NOTE: References (Derry et al., 2000; Llor et al., 1999; Serfas and Tyner, 1998) cite support from the DOD grant and contain described data. These papers are included in the appendix.

Task 1: To determine the patterns of Sik (mouse BRK) expression during normal mammary gland development in the mouse using in situ hybridization. To examine Sik RNA expression at different stages using Northern blot analyses, RNase protection, or quantitative PCR.

Sik expression is not induced during normal mammary gland differentiation (Llor et al., 1999)

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 (Hennighausen and Robinson, 1998). 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) using RNase protection assays. 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. No significant *Sik* expression was detected in the mammary gland at different stages using in situ hybridization and immunohistochemisry, ruling out the possibility that *Sik* expression was induced in a small subset of cells in the mammary gland (Llor et al., 1999).

We also examined Sik expression in the NMuMG (normal murine mammary gland) cell line. We found that NMuMG cells do not express Sik. This is consistent with our results in vivo, where we found that Sik is not expressed at any stage of normal breast development (Llor et al., 1999).

Task 2: To examine BRK expression in human breast tumors to examine expression in histologically distinct types of breast tumors.

BRK expression in tumor cell lines (Llor et al., 1999)

Using Western blotting, we examined BRK expression in cells lines including the normal murine mammary gland cell line NMuMG, the human breast tumor cell line MCF-7, and the human Hela cervical carcinoma cell line. While no BRK expression could be detected in the NMuMG cells, high levels of BRK were present in epithelial tumor cell lines, including MCF-7 and Hela. Using immunohistochemistry and confocal microscopy, we examined the intracellular localization of BRK protein in these cell lines. In the estrogen sensitive MCF-7 cell a significant amount of BRK protein is present in nuclear structures.

BRK expression in human tumors

We examined BRK expression in a collection of archival human breast tumors using immunohistochemistry. The three most strongly staining tumors are shown in Figure 1. In each of the tumors, BRK displays a different pattern of localization, but in all of them it appears cytoplasmic within the cell. While the exclusion of signal from nuclei in the views shown is not always striking, direct comparison of the fluorescent and phase contrast images for the sections makes it clear that nuclei are not strongly stained (not shown).

The surprising range of possible expression patterns - granular, basolateral membrane, focal adhesions, uniformly cytoplasmic, and nuclear - even among cells with the same overall level of

expression, strongly suggest that BRK, which does not contain any apparent nuclear localization motif whether monopartite or bipartite, must be subject to interactions with other proteins which contain the appropriate sorting signals. One such protein may be Sam68, which is described in more detail below.

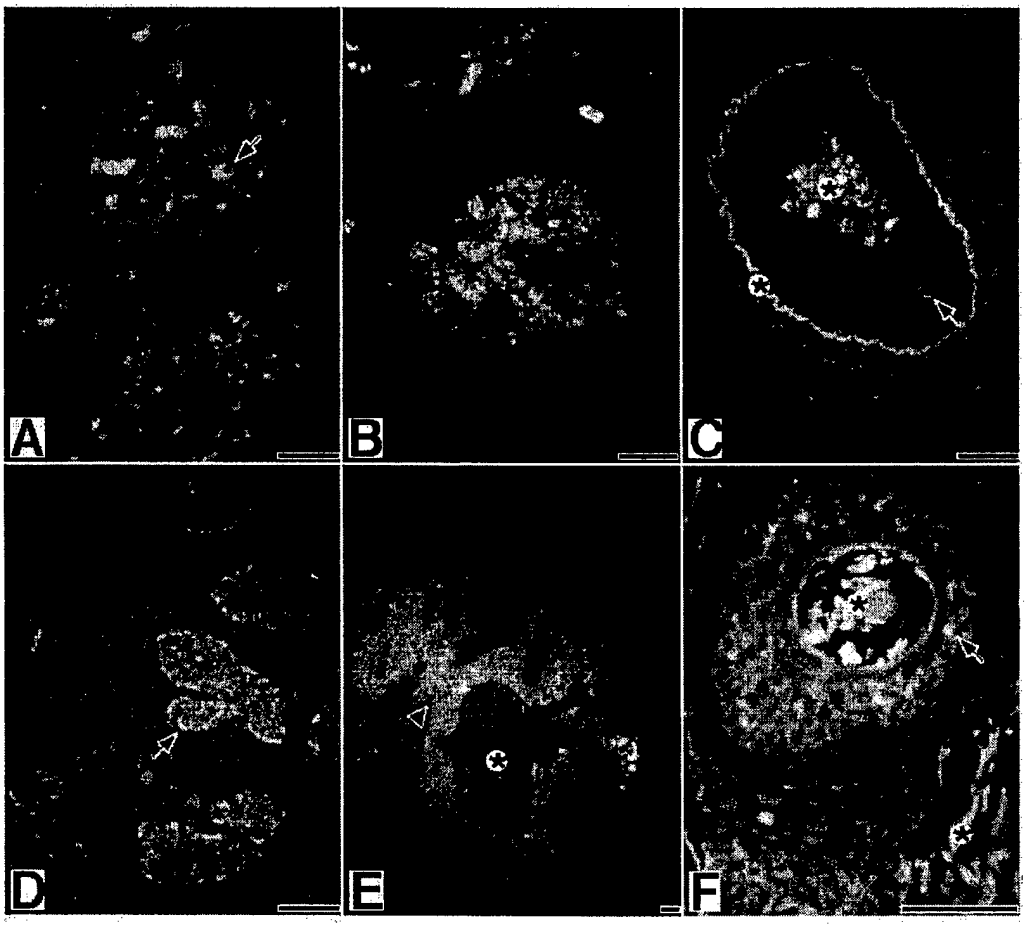

Figure 1. Variable patterns of BRK expression in three human breast tumors. Although BRK is detected in the nuclei of breast tumors cells in culture, its expression was primarily cytoplasmic or membrane associated in tumor cells. (A-B) BRK is present throughout the cytoplasm of archival tumor BC1047, but it varies at a cellular level within the tumor, showing substantial variegation within all parts of the tumor mass on the sections examined. (C) Tissue from another region of tumor BC1047 sample, which contains a consistent epithelial monolayer despite the mass of necrotic tissue, presumably overgrown tumor cells, which occupies its lumen, appears to express a much lower level of BRK, which is tightly localized to cytoplasmic granules. (D) Tumor BC613 maintains a more duct-like pattern of growth and appears to maintain BRK at or near the basolateral membrane. (E) Tumor BC440 seems to stain more homogenously with BRK throughout all of the cellular cytoplasm. (F) A rare duct, perhaps of a sweat or sebaceous gland, in a sample taken from a benign cyst, expresses a cytoplasmic/membrane pattern of BRK with occasional regions of stronger staining.

Task 3: To generate an antibody specific for BRK, so BRK protein expression can be studied.

In order to generate an antibody that would recognize BRK, we ordered a BRK peptide corresponding to 17 carboxy terminal amino acids of the BRK protein and this peptide was injected into

two rabbits to produce a polyclonal anti-sera. The peptide and antisera were prepared by Research Genetics, Huntsville, Alabama. We affinity purified BRK anti-bodies from the sera received from Research Genetics. In control western blots the BRK polyclonal anti-sera was not highly specific or reactive. As we were testing the polyclonal BRK antisera that we generated, an anti-BRK antibody also became commercially available from Santa Cruz Biotechnology, Inc. The commercially available antibody had much greater specificity than the antibody that we prepared and were in the process of characterizing. For expression studies, we have been using the commercially available Santa Cruz BRK antibody (sc-1188).

Task 4: To examine the interactions between the type I (EGFR family) receptor tyrosine kinases and BRK(Sik) using human breast tissue, tumors, and cell lines.

We were not 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 did not detect increased Sik activity following EGF addition to these cell lines. These results differ from those obtained by Mark Crompton's group (Kamalati et al., 1996).

Task 5: To clone mutant and wildtype Sik (BRK) constructs and to derived stable cell lines expressing these constructs

We successfully generated wildtype and mutant Sik expression constructs to be used for examining Sik function in various cell lines. The full length *Sik* cDNA cloned into the pBluescript II SK vector was used in the construction (Vasioukhin et al., 1995). First, the *Sik* cDNA was subcloned into the pAlter vector (Promega, Madison, WI) using BamHI and KpnI sites. Then, the 3'-nontranslated region was deleted by partial digestion with SstI and self-ligation. Next, the *Sik* coding sequence was cloned into the EcoRI site of the pLXSN retroviral expression vector (Miller et al., 1993).

The oligonucleotide-mediated Altered Sites in vitro Mutagenesis System (Promega Corp., Madison, WI) was used for preparation of the mutant kinase deficient Sik cDNA. The complete Sik cDNA was cloned into the pAlter vector using BamHI and SalI sites. The oligonucleotide used for catalytic domain mutagenesis 5'-GTGGCTGTGATGGTGATCTCT-3' has a substitution of A for T which changes the lysine at position 219 of wild type Sik to methionine. A second oligo, 5'-CACCAGGTTTGAGAACC-3', which has a substitution of A for T which changes the tyrosine at position 447 to phenylalanine was used for mutation of the putative regulatory carboxy tyrosine. The entire sequence of the mutant cDNAs was confirmed by sequencing with Sequenase (USB, Cleveland, OH) using dideoxynucleotide chain termination method (Sanger et al., 1977).

Transfection of the normal murine mammary gland cell line NMuMG was done as previously described (Pear et al., 1993). Briefly, 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 beta-galactosidase (Dranoff et al., 1993), 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 ug/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.

Task 6: To determine the biological activity of the BRK(Sik) kinase in cell lines. We will determine if BRK(Sik) expression results in altered growth characteristics, cellular transformation or induction of differentiation.

Ectopic expression of Sik in the NMuMG normal murine mammary gland cell line and in Rat1A cells

We generated NMuMG (normal murine mammary gland) cell lines expressing the wildtype, putative activated (Y-F), and kinase defective (K-M) Sik tyrosine kinase. NMuMG cells do not express Sik. We were unable to detect any alterations in growth properties of cells overexpressing wildtype or dominant negative Sik protein.

Sik and BRK do not have an amino-terminal glycine residue and are not myristoylated. Lack of myristoylation probably contributes to the nuclear localization of these proteins, since they are not anchored at the membrane. Several proteins such as Src and Akt require myristoylation for inducing cellular transformation. Mutation of NH2-terminal glycine of c-Src prevents both myristoylation and morphological transformation (Kamps et al., 1985). Akt is a serine/threonine kinase that acts downstream of phosphatidylinositol 3-kinase and plays a role in cell survival. The viral v-Akt is myristoylated and a potent oncogene, while the cellular form of Akt is not myristoylated and not oncogenic (Ahmed et al., 1993). When directed to membranes by the addition of a Src myristoylation sequence, Akt becomes constitutively active and oncogenic.

We generated a series of myristoylated Sik expression constructs in the vector pLXSN to determine targeting Sik to the membrane would influence cell growth. Sequences corresponding to the c-Src-myristoylation signal were ligated to the 5' ends of our Sik cDNA expression constructs. These constructs were introduced into Rat1A cells that are more susceptible to transformation than epithelial cells. Wildtype Sik expressing cells also did not form colonies, when compared with cells transfected with the empty expression vector. However, cells expressing the myristoylated Sik construct formed colonies in soft agar, while the myristoylated Sik construct with the inactivating K-M mutation did not form colonies. Wildtype and the myristoylated forms of Sik were expressed at equivalent levels. A figure of colonies is shown in Figure 2. These data suggest that Sik activity may be dependent upon its intracellular localization.

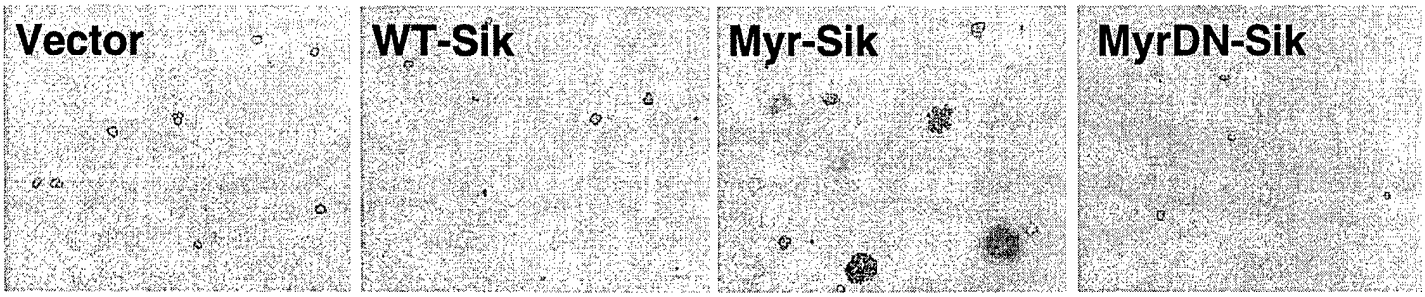

Figure 2. Addition of the Src myristoylation signal to the Sik cDNA results in kinase dependent anchorage independent growth. RAT1A cells were transfected with wildtype Sik (WT-Sik), myristoylated Sik (Myr-Sik) and myristoylated kinase defective dominant negative Sik (K-M) (MyrDN-Sik). Only cells containing the active myristoylated Sik formed colonies in soft agar. These data indicate that intracellular localization of Sik is important for its biological activities.

BRK is present in the nucleus and phosphorylates the RNA-binding protein Sam68 (Derry et al., 2000)

We found that BRK is in nuclear structures in tumor cell lines. The RNA-binding protein Sam68 (Src-associated in mitosis, 68 kDa) was also shown to be in novel nuclear structures called Sam68/SLM

Nuclear Bodies (SNBs) that are predominant in tumor cell lines. Sam68 is an RNA binding protein that was initially identified as a major target of Src during mitosis (Fumagalli et al., 1994; Taylor and Shalloway, 1994). It contains a KH RNA-binding domain that was first described in hnRNP K. Sam68 belongs to a new subfamily of KH domain-containing RNA-binding proteins having single KH domains called the STAR (**S**ignal **T**ransducers and **A**ctivators of **R**NA) family (Vernet and Artzt, 1997). Several STAR proteins play important roles in development including 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). Sam68 has been shown to preferentially bind RNA with UAAA motifs (Lin et al., 1997). Although the function of Sam68 is unknown, Sam68 has been shown to be required for cell cycle progression (Barlat et al., 1997), and can function in the transport of HIV mRNA (Reddy et al., 1999).

Using immunohistochemistry we determined that BRK colocalizes with Sam68 in the novel Sam68/SLM nuclear bodies (Derry et al., 2000). Using coimmunoprecipitation and immunoblotting, we have shown that BRK is associated with Sam68 within the nuclei of these cells in vivo. BRK is unique because it is present in the nucleus where Sam68 resides during most of the cell cycle, indicating that BRK may be an important physiological regulator of this RNA-binding protein. Using cotransfection, immunohistochemistry, and confocal microscopy, we determined that BRK is active and phosphorylates Sam68 in vivo (Derry et al., 2000).

The SH2 and SH3 domains of Sik/BRK are involved in interactions with Sam68 (Derry et al., 2000)

To determine which part of Sik interacts with Sam68, different domains of Sik were expressed in bacteria as GST-fusion proteins and used in GST-pull down assays. Lysates from NMuMG cells transfected with wildtype Sik, Sik Y-F, or Sik K-M and GFP-Sam68 expression constructs were incubated with GST alone, GST-Sik SH2+SH3, GST-Sik SH2 and GST-Sik SH3 bound to beads. Bound proteins were detected by immunoblotting with anti-Sam68 AD1 antibody. GFP-Sam68 and the endogenous Sam68 were observed to associate with the Sik SH3 and Sik SH2+SH3 domain fusion proteins. Association of GFP-Sam68 with the Sik SH2 domain was only observed when it was coexpressed with wildtype Sik or Sik Y-F, but not kinase defective Sik K-M.

Phosphorylation of Sam68 by BRK inhibits its RNA binding ability, and its ability to substitute for the HIV-1 Rev protein in RNA transport (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). We found that phosphorylation of Sam68 by BRK 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 signaling cascade regulates the RNA functions of Sam68. The ability of Sam68 to act in RNA transport suggests a role in posttranscriptional regulation of gene expression, which can be negatively regulated by BRK within the nucleus.

Task 7: Years 3-4: To generate MMTV- Sik (mutant and wildtype) kinase transgenic mice.
Task 8: Year 4: To determine if directing the expression of inappropriate levels or patterns of Sik in transgenic mice will result in altered breast development and/or increased tumorigenesis.

Generation of Sik Transgenic Mice

Although we originally proposed to use the MMTV promoter to generate transgenic mice, we decided to use the keratin 14 promoter to drive expression of wildtype Sik in transgenic mice. Keratin 14 has been reported to be expressed in the mammary gland (Kao et al., 1995; Stingl et al., 1998; Wetzels et

al., 1989). In carrying out work in tasks 1 and 2, we did not detect Sik in the normal mammary gland. K14 is also expressed in the basal cells of the skin prior to the induction of Sik as cells terminally differentiate (Vasioukhin et al., 1995; Vassar et al., 1989). By using the K14 promoter we could compare the effects of ectopic expression of Sik in two tissues at the same time, skin and breast. The hypothesis that we tested was that ectopic expression of Sik may induce abnormal differentiation, changes in cell turnover, and tumor formation.

The transgenic expression construct consisted of the full-length Sik cDNA inserted into the BamHI site of pK14-HGH (a gift of Linda Degenstein and Elaine Fuchs). The resulting construct was a 6.7 kB EcoRI-HindIII fragment within the cloning site of the pGem 3Z plasmid vector (Promega), which contains approximately 2100 bp of the cytokeratin 14 promoter/enhancer region, followed by approximately 2100 bp of Sik cDNA sequence, followed by 2150 of HGH genomic sequence including three introns and a polyadenylation sequence, followed by 490 bp of cytokeratin 14 3' noncoding region and polyadenylation site. In linearized and purified form, this vector was injected into mouse embryos (Hogan et al., 1994) by Roberta Franks, who runs the UIC Cancer Center mouse core facility. Eighty-six offspring produced and were screened by Southern blot hybridization of tail biopsy DNA samples. Eleven founder transgenic animals were identified. Of these founders, four lines were chosen for further study. No significant morphological changes or changes in proliferation, differentiation or tumorigenesis were observed in the skin and breast tissue in the Sik wildtype transgenic mice and their offspring. It is possible that we directed Sik overexpression to cells that do not express the appropriate substrates or adaptor proteins to mediate a biological response.

ADDITIONAL STUDIES PERFORMED THAT ARE NOT INCLUDED IN ORIGINAL STATEMENT OF WORK THAT CITED SUPPORT OF THE DOD GRANT

Mapping of *Sik* to a region of the mouse genome that shares conservation of synteny with human chromosome 20q13.3 (Llor et al., 1999)

While the functional domains of human BRK and mouse Sik are conserved, Sik and BRK share only 80% sequence identity. We compared mouse Sik and human BRK at many levels. These proteins are expressed in the same patterns and behave similarly in biochemical assays. We 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* (Chambers et al., 1998). 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. Since BRK has been mapped to human chromosome 20q13.3 (Park et al., 1997), this provides further evidence that these two genes are orthologous (Llor et al., 1999). In wasted mice the *Eef1a2* gene is deleted (Chambers et al., 1998), while the *Sik* gene is intact and appropriately expressed (C. M. Abbott, personal communication).

Ryk is expressed in a differentiation-specific manner in epithelial tissues and is strongly induced in decidualizing uterine stroma (Serfas and Tyner, 1998)

Michael Serfas, a graduate student supported by the DOD grant, also examined expression of the tyrosine kinase Ryk and the resulting publication acknowledged his DOD support. Ryk is a ubiquitously expressed tyrosine kinase-like receptor of unknown activity and associations. We examined ryk expression in adult mouse epithelial tissues and during embryonic development at the histological level.

Ryk RNA is present at greatly increased levels in cells at particular stages of epithelial differentiation: the basal layer of skin and tongue epithelia, the intervillous layer and some crypt bases of the intestine, and the lower matrix region of the hair follicle. Although ryk RNA is expressed at similar levels in a variety of tissues from embryonic day 10.5 to 18.5, specific induction of ryk RNA can be seen by in situ hybridization in the basal layer of skin and hair follicle at day 15.5 to 16.5, and protein staining localizes to the hair follicle by immunohistochemistry. At day 4.5 and 6.5, little if any ryk is present in the blastocyst, but it is transiently induced at a high level in mature decidual cells of the uterine stroma. We review a number of independent isolations of ryk, including fruit fly and nematode members of the ryk family. Because ryk is induced in epithelial cells seeking a final place in a differentiated tissue, or during remodeling of the endometrium, and a homologous gene, derailed, is known to regulate muscle and nerve target seeking in Drosophila, we hypothesize that ryk may also be involved in cellular recognition of appropriate context.

KEY RESEARCH ACCOMPLISHMENTS

- Sik expression is not induced during normal mammary gland differentiation (Llor et al., 1999).
- Sik is not expressed in the normal murine mammary gland cell line (Derry et al., 2000).
- Sik/BRK can be found in the cytoplasm, at the membrane and in the nucleus of tumor cells.
- Ectopic expression of myristoylated Sik that targets the protein to the membrane results in anchorage independent growth.
- Sik/BRK colocalizes with the RNA binding protein Sam68 in Sam68/SLM nuclear bodies (SNBs) in MCF7 cells (Derry et al., 2000).
- The SH2 and SH3 domains of Sik/BRK are involved in binding Sam68 (Derry et al., 2000).
- Phosphorylation of Sam68 by Sik/BRK inhibits its RNA binding ability, and its ability to substitute for the HIV-1 Rev protein in RNA transport (Derry et al., 2000).
- *Sik* maps to a region of the mouse genome that shares conservation of synteny with human chromosome 20q13.3 where BRK is located (Llor et al., 1999).

REPORTABLE OUTCOMES

Derry, J., S. Richard, H. Valderrama Carvaja, X. Ye, A. W. Cochrane, V. Vasioukhin, T. Chen, and **A. L. Tyner**. 2000. Sik/BRK phosphorylates Sam68 in the nucleus and negatively regulates its RNA binding ability. Molecular and Cellular Biology **20** 6114-6126.

Tyner, A.L., Derry, J. Richard S., Chen Taiping, and X. Ye. 2000. The Src-related Intestinal Kinase Sik is present in the nucleus and phosphorylates the RNA-binding protein Sam68. Gastroenterology **118** (4): 2915.

Llor, X.,Serfas, M.S., Bie, W., Vasioukhin, V., Polonskaia, M., Derry, J., Abbott, C.M., and **A. L. Tyner**. 1999. BRK/SIK expression in the gastrointestinal tract and in colon tumors. Clinical Cancer Research **5**, 1767-77.

Serfas, M.S. and **A. L. Tyner**. 1998. Ryk is expressed in a differentiation-specific manner in epithelial tissues and is strongly induced in decidualizing uterine stroma. Oncogene **17**, 3435-3444.

DOD Era of Hope Meeting Abstracts/Presentations

Tyner A. L, Ye X., Richard S., Chen T. .and J. Derry. 2000. The breast tumor kinase SIK/BRK is present in the nucleus and phosphorylates the RNA-binding protein Sam68.

Tyner A .L., Vasioukhin, V., Serfas M .S., and X. Llor. 1997. Characterization of the novel breast tumor kinase BRK.

Degrees Received

Graduate Student	Michael S. Serfas (received his Ph.D. in 1999)
Graduate Student	Jason Derry (will receive Ph.D. in 2001)

CONCLUSIONS

BRK is expressed in breast tumors and breast tumor cell lines (Barker et al., 1997; Kamalati et al., 1996). Since we had detected BRK expression in differentiating cells of the normal epithelial linings of the gastrointestinal tract and skin, we hypothesized that BRK would also be expressed at some stage of normal beast development. This did not prove to be the case. We could not detect expression of the mouse orthologue of BRK (Sik) at any stage of normal breast development in the mouse, or in the nontransformed breast epithelial cell line NMuMG (Llor et al., 1999).

We determined that Sik/BRK may be present in the nucleus. Nuclear tyrosine kinases have the unique ability to coordinate signal transduction events in the cytoplasm with specific changes in the nucleus. Only a small number of nuclear tyrosine kinases have been identified, including Abl, Rak, Fes, Fer, and Wee1 (reviewed in (Pendergast, 1996). The Sik/BRK kinase lacks a clear nuclear localization and has no DNA binding domain (Vasioukhin et al., 1995). Nevertheless, Sik/BRK is present in the nucleus where it associates with and can phosphorylate the RNA binding protein Sam68 (Derry et al., 2000). BRK/Sik also lacks a myristoylation signal for membrane targeting but it was also found at the membrane in some cells. BRK/Sik association with other proteins appears to regulate its intracellular activities.

Both Sik and its human orthologue BRK are present in the nuclei of mouse and human cells respectively (Derry et al., 2000). NMuMG cells were isolated from the mammary glands of Namru mice and have epithelial growth characteristics and do not form malignant lesions when introduced into nude mice (Hynes et al., 1985). Ectopically expressed Sik is localized diffusely within the nuclei of immortalized NMuMG cells, while BRK protein appears in specific structures in the MCF7 breast adenocarcinoma cell line. These data complement earlier studies by Chen and Richard (Chen et al., 1999), who found that SNBs (Sam68 nuclear bodies) were predominant in transformed cells. SNBs are novel unique dynamic structures that disassemble when transcription is inhibited with actinomycin D (Chen et al., 1999). When GFP-Sam68 and wildtype Sik are introduced into MCF-7 or HT-29 cells, they localize to the SNBs, which become tyrosine phosphorylated (Derry et al., 2000). The consequences of Sam68 tyrosine phosphorylation by Sik within the nucleus and in SNBs need to be further explored.

We have shown that Sik can bind Sam68 through both its SH3 and SH2 domains. The binding affinities of specific SH2 domains are influenced by sequence context. For example, Src family members prefer the sequence pTyr-Glu-Glu-Ile, while the SH2 domains of p85 and PLC-gamma select the general

motif pTyr-hydrophobic-X-hydrophobic (Songyang et al., 1993; Songyang et al., 1994). Using a technique employing degenerate phosphopeptide libraries to predict the specificity of individual SH2 domains, it was determined that the Sik SH2 domain may bind to phosphorylated proteins with p-YEEY, YEDY, YDEY and YDDY motifs (Z. Songyang and L. C. Cantley, personal communication). Interestingly, Sam68 contains the sequence YEDY in its carboxy terminus and this is a putative binding site for the Sik SH2 domain. This sequence may also be the target of Sik, as we have shown that Sam68 lacking the carboxy terminus is not phosphorylated by Sik (Derry et al., 2000).

RNA binding proteins may regulate gene expression by a number of mechanisms (reviewed in (Siomi and Dreyfuss, 1997). They may alter RNA structure to regulate interaction with trans-acting factors, or provide localization or targeting signals. Although its cellular function is unknown, Sam68 has been shown to be able to functionally substitute for the HIV-1 Rev protein, which plays an essential role in the nuclear export of unspliced and partially spliced viral transcripts and export of the HIV genome (Reddy et al., 1999). This implicates Sam68 in the posttranscriptional regulation of gene expression. We found that Sik kinase activity can negatively regulate the ability of Sam68 to function as a cellular homologue of Rev. Our data strengthen the possibility that signaling cascades can regulate the RNA function of Sam68 and related STAR proteins. The ability of Sam68 to act in RNA transport suggests a role in posttranscriptional regulation of gene expression, which can be negatively regulated by Sik within the nucleus. Sam68 may also serve as an adaptor for Sik, bringing it into proximity of other, as of yet unidentified, substrates.

Sam68 is the first substrate identified for the Sik/BRK kinase (Derry et al., 2000). Sik/BRK expression is initiated in differentiating cells of the skin and gastrointestinal tract (Vasioukhin et al., 1995), and its activity is induced in primary keratinocytes that are stimulated to differentiate (Vasioukhin and Tyner, 1997). It is possible that Sik phosphorylation of Sam68 within the nucleus regulates an aspect of RNA transport and posttranscriptional gene regulation associated with specific events occurring during normal epithelial cell differentiation. Overexpression or inappropriate localization of Sik/BRK in breast cancer may result in altered tyrosine kinase activity and phosphorylation of Sam68. It will be important to determine the functional role of nuclear Sam 68 tyrosine phosphorylation in breast cancer in future studies.

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Sik (BRK) Phosphorylates Sam68 in the Nucleus and Negatively Regulates Its RNA Binding Ability

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Sik (mouse Src-related intestinal kinase) and its orthologue BRK (human breast tumor kinase) are intracellular tyrosine kinases that are distantly related to the Src family and have a similar structure, but they lack the myristoylation signal. Here we demonstrate that Sik and BRK associate with the RNA binding protein Sam68 (Src associated during mitosis, 68 kDa). We found that Sik interacts with Sam68 through its SH3 and SH2 domains and that the proline-rich P3 region of Sam68 is required for Sik and BRK SH3 binding. In the transformed HT29 adenocarcinoma cell cell line, endogenous BRK and Sam68 colocalize in Sam68-SLM nuclear bodies (SNBs), while transfected Sik and Sam68 are localized diffusely in the nucleoplasm of nontransformed NMuMG mammary epithelial cells. Transfected Sik phosphorylates Sam68 in SNBs in HT29 cells and in the nucleoplasm of NMuMG cells. In functional studies, expression of Sik abolished the ability of Sam68 to bind RNA and act as a cellular Rev homologue. While Sam68 is a substrate for Src family kinases during mitosis, Sik/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.

The Src-related intestinal kinase Sik is an intracellular tyrosine kinase that we identified in a screen for tyrosine kinases in intestinal epithelial cells (34). Although it is related to the Src family and contains SH2 and SH3 domains, it has a very short unique amino terminus and is not myristoylated (41). Sik expression is restricted to differentiating epithelial cells, and it is found in the skin and all linings of the alimentary canal. Addition of calcium to cultured primary mouse keratinocytes induces cell differentiation and rapid activation of Sik (42). Overexpression of Sik in an embryonic mouse keratinocyte cell line resulted in increased expression of the differentiation marker filaggrin during calcium-induced differentiation, suggesting that Sik is involved in a signal transduction pathway that may promote differentiation (42).

The human orthologue of Sik is called BRK (breast tumor kinase) (24, 25). Increased BRK expression has been detected in colon tumors (24), breast tumors (2, 25), and melanomas (11, 22). Neither Sik nor BRK expression has been detected in normal mammary tissue, but both proteins are expressed in normal epithelial cells that are undergoing terminal differentiation in the gastrointestinal tract (24, 41). While BRK appears to play a role in signal transduction in normal epithelial linings, its overexpression appears to be linked to the development of a variety of epithelial tumors. The seemingly paradoxical roles of Sik and BRK during differentiation and tumorigenesis are poorly understood.

To date, no substrates of Sik and BRK have been identified. Here we report that Sam68 (Src associated in mitosis; 68 kDa) is a substrate of Sik that can be phosphorylated by Sik within the nucleus. Sam68 is an RNA binding protein (47) that was first identified as a major target of Src during mitosis (14, 39). Thus far, Sam68 has been shown to be a substrate of Src family kinases (14, 29, 39, 46), ZAP70 (20), and the insulin receptor (31). Although Sam68 resides in the nucleus during most of the cell cycle, none of these tyrosine kinases colocalize with Sam68 within the nucleus. Sam68 has also been shown to be a substrate of Cdc2 during mitosis (28). Sam68 has been proposed to function as a multifunctional adapter protein for Src kinases (29, 38), and it can associate with phospholipase Cγ1, the p85 subunit of phosphatidylinositol-3-kinase (31), and the adapter proteins Grb2 (29), Nck (21), and Grap (40).

Sam68 has been shown to preferentially bind RNA with UAAA motifs (23). The RNA binding activity of Sam68 is negatively regulated by Src kinases (45), and Sam68 may function as a protein that links signaling cascades by Src kinases to RNA metabolism. The type of RNA binding motif present in Sam68 is called the hnRNP K homology (KH) domain (15, 33). Sam68 is part of a subfamily of KH domain-containing proteins, because it contains an extended KH domain embedded in a larger domain called the GSG (GRP33-Sam68-GLD1) domain (10, 19). This protein module is also referred to as the STAR (signal transduction and activation of RNA) domain (43). The GSG domain of Sam68 has been shown to be required for RNA binding (5, 23), RNA-dependent oligomerization (5), and protein localization (4). Sam68 has been observed to localize in novel nuclear bodies called Sam68-SLM nuclear bodies (SNBs) in cancer cell lines (4). Although the function of Sam68 is unknown, Sam68 has been shown to be required for cell cycle progression (3) and can function as a cellular homologue of Rev by transporting unspliced human immunodeficiency virus (HIV) RNA into the cytoplasm (27).

Here we report that both Sik and BRK colocalize with Sam68 within the nucleus. We show that Sik is active within the

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nucleus and that it can phosphorylate Sam68 in vivo. In addition, we demonstrate that phosphorylation of Sam68 by Sik negatively regulates its RNA binding ability and its ability to function as a Rev cellular homologue. Phosphorylation of Sam68 within the nucleus may have important physiological significance and may contribute to the posttranscriptional control of gene expression during the differentiation of epithelial linings.

MATERIALS AND METHODS

Expression constructs. For the preparation of the mutant Sik cDNAs, we used the oligonucleotide-mediated Altered sites in vitro mutagenesis system (Promega). The Sik cDNA was cloned into the pAlter plasmid, and the oligonucleotide with the sequence 5′-CACCAGGTTTGAGAACC-3′, with a substitution of A for T resulting in substitution of the tyrosine at position 447 with phenylalanine, was used to generate the Sik Y-F construct. This type of mutation has been shown to lead to constitutive activation of the Src family of tyrosine kinases (8). Preparation of the kinase defective Sik expression construct Sik K-M was previously described (42). Wild-type Sik, Sik Y-F, and Sik K-M coding sequences were cloned into the vector pcDNA3. The GST-Sik constructs were as previously described (42).

The Fyn expression construct and Myc-Sam68 (68–347) (also called P1234) were previously described (29), as was Myc-Sam68 (5). The coding region of mouse hnRNPK was amplified using the expressed sequence tags AA544863 and AA183839 and the oligonucleotides with the sequences 5′-CAGGAATTCACT AGTCTTAGAAAA-3′ and 5′-AATGAATTCCGAACAGCCAGAAGA-3′, and it was digested with *Eco*RI and subcloned in frame in Myc-Bluescript (29). The DNA fragments encoding the Sam68 proline motifs P0, P1P2, P3, P4, and P5 were amplified by PCR using Myc-Sam68 as a DNA template (5). The DNA was digested with *Bam*HI and *Eco*RI and subcloned in the respective sites of pGEX-KG (16). The sequences of the oligonucleotides are as follows: P0, 5′-CGT GGA TCC AAG GAC CCG TCA GGT-3′ and 5′-GCG GAA TTC TCA AGC GCC TCC TCT GGG CCC AC-3′; P1P2, 5′-CGG GGA TCC CCC GCC ACC CAG CCG CCG-3′ and 5′-GCG GAA TTC TCA CGG CTG TGG CTG ACG GGG GC-3′; P3, 5′-AAC GGA TCC CCT GAA CCC TCT CGT GGT-3′ and 5′-GCG GAA TTC TCA AGC TCC TCT AGG TGG TCC AAC-3′; P4, 5′-CGT GGA TCC CCA GTG AGA GCT CCA TCA CC-3′ and 5′-GCG GAA TTC TCA CCC AGC TGT CCG AGC TCT TG-3′. For construction of GST-Sam68 (331–443), Myc-Sam68 was digested with *Xho*I and the DNA fragment corresponding to amino acids 331 to 443 was subcloned in frame into Myc-BS. The resulting plasmid was digested with *Bam*HI and *Kpn*I, and the fragment was subcloned into the *Bam*HI-*Hin*dIII sites of pGEX-KG. The *Kpn*I and *Hin*dIII sites were made blunt. For construction of GST-Sam68 (354-393), Myc-Sam68 was used as a template for the following primers: forward, CCCGGATCCATT CAGAGAATACCTTTG, and reverse, ATAGAATTCTTACTCCCCTTGACT CTGGC. The DNA fragment was digested with *Bam*HI and *Eco*RI and subcloned into the corresponding sites in pGEX-KG. Myc-pcDNA Sam68 was constructed by digesting Myc-BS Sam68f (5) with *Eco*RI and subcloning the fragment in frame in Myc-pcDNA (6). Myc-pcDNA Sam68Δ C was constructed by digesting Myc-pcDNA Sam68 with *Xho*I and religating. This deletes amino acids 348 to 443 of Sam68, and the translation terminates in the vector.

Peptides P0, P3, and P4 used in competition assays were synthesized by the W. M. Keck Biotechnology Resource Center, New Haven, Conn., and their sequences are as follows: P0, biotin-RLTPSRPSPLPHRPRGGGGGPRGG; P3, biotin-GVSVRGRGAAPPPPPVPRGRGVGP; P4, biotin-TRGATVTRGVPP PPTVRGAPTPR.

Cell lines. Cell lines were obtained from the American Type Culture Collection. NMuMG cells were generally transfected using the LipofectAMINE Reagent (Gibco/BRL). HeLa cells were maintained in Dulbecco modified Eagle medium (DMEM) with 1.0 mM sodium pyruvate and 10% bovine calf serum (HyClone, Logan, Utah) and were transfected with the vaccinia virus T7 expression system and lysed as previously described (29). COS7 cells were maintained in DMEM supplemented with 10% bovine calf serum and were transfected using the DEAE-dextran method.

Subcellular fractionation. Cells were washed two times in 1× phosphate-buffered saline (PBS) and one time in hypotonic lysis buffer (HLB; 20 mM Tris-HCl [pH 7.5], 1 mM $MnCl_2$, 2 mM EGTA) for 5 min on ice. Cells were then treated with 1.5 ml of HLB (with 20 μg of leupeptin/ml and 1 mM phenylmethylsulfonyl fluoride [PMSF]) and shaken for 20 min on ice. Cells were scraped and homogenized in a Dounce homogenizer (50 to 60 strokes) and spun for 10 min at 2,300 rpm, 4°C. The supernatant from this spin was kept as cytosolic and membrane fractions. The pellet was washed in 1 ml of HLB, spun 4 min at 5,000 rpm at 4°C, and resuspended in 1 ml of Dignum buffer (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MnCl_2$, 0.1 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol [DTT], 0.5 mM PMSF, 2 μg of leupeptin/ml, 2 μg of aprotinin/ml, 1 mM $NaVO_4$). After shaking for 15 min at 4°C, samples were spun at 14,000 rpm for 10 min at 4°C. The supernatant was kept as the nuclear protein fraction.

Antibodies, immunoprecipitations, and immunoblotting. Anti-Sik polyclonal antibodies N20 (sc-915) and C17 (sc-916) were purchased from Santa Cruz Biotechnology. Immunoblot analyses were performed with a combination of the two mouse Sik antibodies, N20 and C17, at a 1:5,000 dilution for increased sensitivity. BRK was detected with the Santa Cruz Biotechnology BRK antibody (C-17, sc-1188) or the Sik N20 antibody. The monoclonal antibody anti-Myc 9E10 (Santa Cruz Biotechnology) and antiphosphotyrosine antibodies 4G10 (1:10,000) and PY-20 (1:2,000) (both from Santa Cruz Biotechnology) and RC20-HRPO (1:5,000) (Transduction Laboratories) were also used. The anti-AD1 rabbit polyclonal antibody specific for Sam68 was generated using a peptide from amino acids 330 to 348 of mouse Sam68 (4). For immunoblotting, the designated primary antibody was followed by either goat anti-rabbit antibody, goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) (ICN), HRP-conjugated donkey anti-rabbit antibody, or HRP-conjugated protein A (Transduction Laboratories), and chemiluminescence was used for protein detection.

Immunoprecipitations were performed as previously described (42). Anti-BRK antibodies or anti-Sam68 AD-1 and 50 μl of protein G-Sepharose (Amersham or Pharmacia Biotech) were incubated with 1 to 2 mg of cell lysate for 3 to 16 h at 4°C. As controls, lysates were incubated with Sepharose beads and rabbit serum, rabbit immunoglobulin G (IgG), or alone.

Sik-GST fusion protein in vitro binding assays. Glutathione *S*-transferase–Sik (GST-Sik) and GST-Sam68 fusion proteins were prepared as described previously (29, 42). Cell lysates were precleared by incubating with GST-saturated glutathione beads for 30 min. Precipitations were performed by incubating lysates with GST, GST-SikSH2/3, Sik SH2, or Sik SH3 for 45 min at 4°C, followed by incubation with glutathione-Sepharose beads (Amersham) for 30 min. Precipitates were eluted with sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with anti-Sam68 antibody.

Immunofluorescence. Cells were grown on chamber slides (Falcon) and fixed in methanol at −20°C for 5 min or Carnoy's fixative for 5 min at room temperature. Cells transfected with green fluorescent protein (GFP) constructs were fixed in 4% paraformaldehyde for 5 min at room temperature and permeabilized in 50% methanol–50% acetone for 15 min at −20°C. Slides were blocked in 2% goat serum or 3% bovine serum albumin in TNT buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.05% Tween 20) for 30 to 40 min. Slides were then incubated with anti-BRK or anti-Sam68 AD-1 antibodies (1:250) overnight at 4°C, washed, incubated with biotinylated goat anti-rabbit antibody (Vector Laboratories) (1:250) for 1 h at room temperature, washed, blocked 30 min with blocking reagent (from DuPont NEN), and incubated with streptavidin-HRP (DuPont NEN) (1:100). After washing, tyramide amplification was performed using the TSA-Indirect Kit (DuPont NEN) according to manufacturer's directions. Reactions were visualized with rhodamine-avidin (Vector Laboratories) (1:500), and slides were mounted with Vectashield mounting medium (Vector Laboratories). Controls for specificity of signal were performed by preincubating BRK antibodies with the immunogenic BRK peptide (1:4) for 30 min at room temperature or by incubating sections with normal rabbit sera alone.

For double antibody labeling in HT29 and MCF-7 cells, cells were stained as above with rabbit anti-BRK antibody and visualized with rhodamine, followed by incubation with anti-Sam68 (Transduction Laboratories) (1:50) for 1 h at room temperature and anti-mouse IgG fluorescein isothiocyanate (FITC) conjugate (Sigma) (1:64), and they were analyzed by confocal microscopy.

For double antibody labeling of cells transfected with GFP-Sam68 and Sik constructs, cells were stained overnight with antiphosphotyrosine antibody conjugated to HRP (RC20-HRPO; Transduction Laboratories) at a 1:2,000 dilution at 4°C and were incubated with biotinyl tyramide and rhodamine-avidin (1:500). Next, slides were treated with 1.0% H_2O_2 for 15 min to inactivate HRP, followed by incubation with the second antibody, anti-Sik (C-17) at a 1:250 dilution, followed by HRP-conjugated goat anti-rabbit antibody, biotinyl tyramide, and then streptavidin Alexa 350 conjugate (Molecular Probes) (1:500) for visualization. Controls were performed by substituting rabbit serum, rabbit IgG, or blocking buffer alone for the first and second antibodies. Omission of Sik antibody in the second antibody incubation, followed by tyramide amplification, resulted in no Alexa 350 signal in these double staining experiments. Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) (Boehringer Mannheim) for 3 min and washed before mounting.

poly(U) binding assays. Following transfections with Sam68Δ1–67 (45) and Sik Y-F or Fyn, HeLa cells were lysed on ice in 1% Triton X-100, 25 mM Tris (pH 7.4), 150 mM NaCl, 25 mM NaF, and 100 μM sodium orthovanadate. Lysates were centrifuged to remove insoluble material, and one-fourth of the total cell lysate was added to 20 μl of agarose-poly(U) beads (Pharmacia Biotech Inc.) or agarose beads as a control for 30 min, 4°C. Beads were washed twice with lysis buffer and eluted in Laemmli sample buffer. For assessment of total protein expression, 2.5% of the cell lysate was blotted.

REV assays. COS7 cells were transfected with a total of 3.5 μg of DNA supplemented with empty pcDNA3.1. Each transfection contained 0.125 μg of Rev response element (RRE) chloramphenicol acetyltransferase (CAT) reporter plasmid pDM128 (17), with 1.5 μg of Rev expression vector B1-SVH6Rev (7, 37), 1.5 μg of Rev mutant B1-SVH6RevM10, 1.5 μg of pcDNA-Sam68ΔC, or 1.5 μg of pcDNA-Sam68. Increasing amounts of expression vectors for Sik K-M and Sik Y-F (0.05, 0.8, and 1.6 μg) were added with pcDNA-Sam68. The β-galactosidase expression vector, pCH110 (0.125 μg; Pharmacia-Amersham Inc.) was included in all transfections for measuring the efficiency of transfection. Forty-

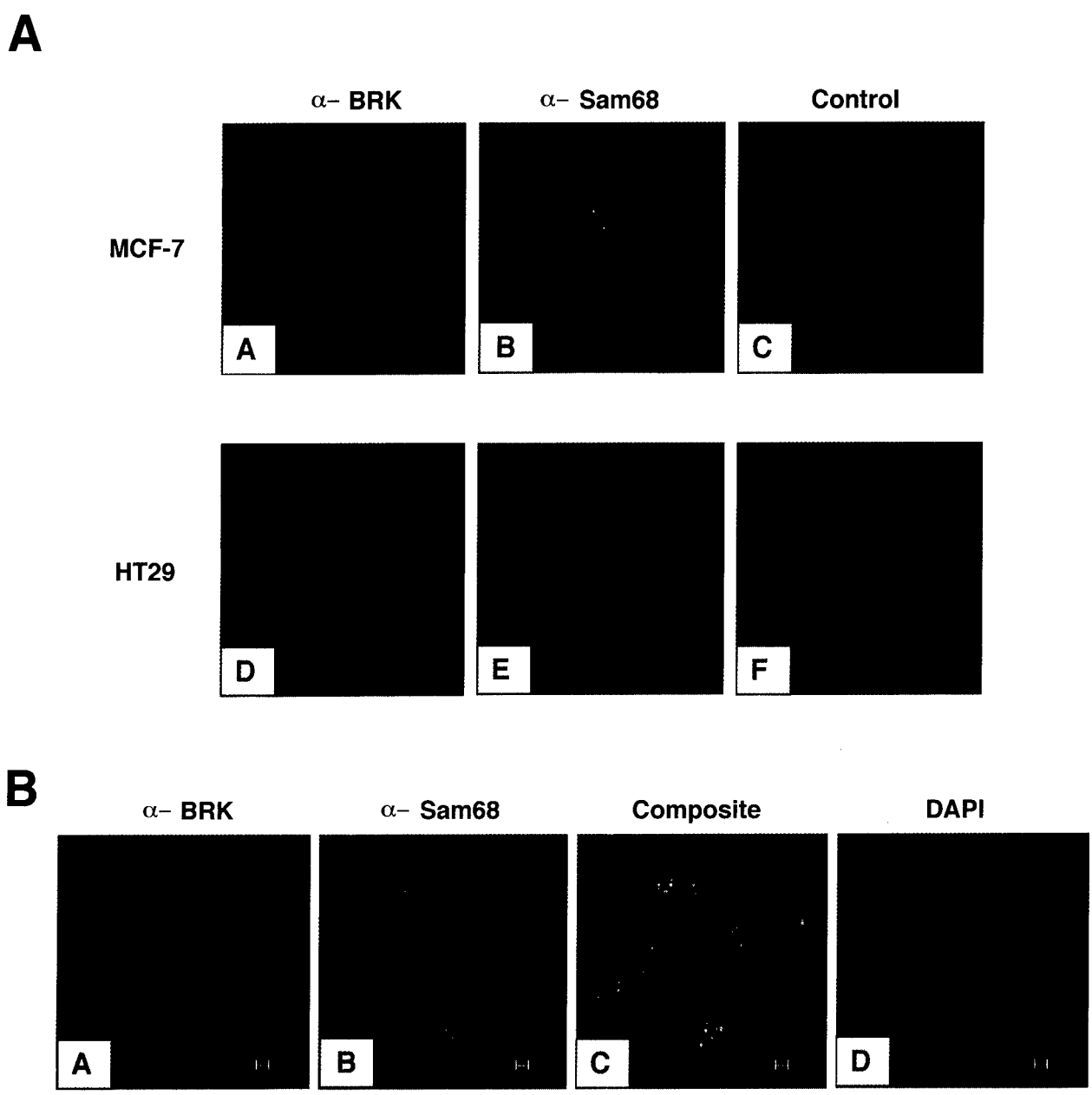


FIG. 1. BRK and Sam68 localize in nuclear structures in breast and colon tumor cell lines. (A) BRK and Sam68 are found in nuclear structures in human tumor cell lines. Immunofluorescence was used to examine the localization of endogenous BRK and Sam68 in the MCF-7 (A, B) and HT29 (C, D) tumor cell lines. Cells were fixed and stained with antibodies against BRK (A, D) or Sam68 (B, E), BRK antibody preincubated with immunogenic peptide (C), or control rabbit sera (F). (B) BRK localizes to SNBs in HT29 cells. Cells were fixed and stained with antibodies against BRK followed by staining with antibodies against Sam68 and were analyzed by confocal microscopy. BRK was visualized with rhodamine (A), and Sam68 staining was visualized with FITC (B). A composite (C) shows colocalization of BRK and Sam68 as yellow spots in the nuclei (C). Nuclei were stained with DAPI (D). Bars, 5 μm.

eight hours after transfection, the cells were collected and resuspended in 150 μl of 0.25 M Tris-HCl, pH 7.8. The cell extracts were prepared by three freeze-thaw cycles, followed by a brief centrifugation to remove cell debris. CAT and β-galactosidase assays were performed as previously described (30). CAT activity was normalized to the β-galactosidase activity and did not exceed twofold.

RESULTS

BRK associates with Sam68 in human tumor cell lines. BRK is expressed in breast and colon tumors and tumor cell lines (2, 24). To better understand the role of BRK, we performed indirect immunofluorescence microscopy to visualize its cellular localization. We found that endogenous BRK localized into distinct nuclear dots in the MCF-7 and HT29 breast and colon tumor cell lines (Fig. 1A, panels A and D). The presence of BRK in nuclear dots was not due to the elevated expression of BRK in these cells because Sik overexpression in normal murine mammary gland cells was localized diffusely in the nucleus without the presence of nuclear dots (see Fig. 5 below). The RNA binding protein Sam68 was also observed in similar structures in these cells (Fig. 1A, panels B and E). When control nonimmune serum was used (Fig. 1A, panel F), or when the primary antibody was preincubated with

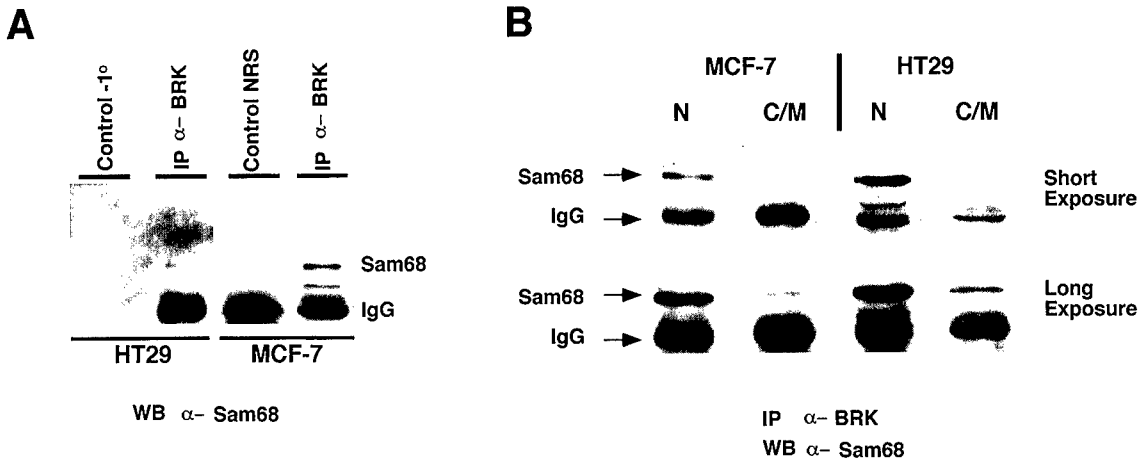


FIG. 2. BRK and Sam68 associate within the nuclei of HT29 and MCF-7 cells. (A) Sam68 coimmunoprecipitates with BRK from lysates of the HT29 colon and MCF-7 breast carcinoma cell lines. Cells were lysed, and 1 mg of total-cell lysate was incubated with anti-BRK antibodies, normal rabbit serum (NRS), or Sepharose beads alone as a control for nonspecific binding to beads. The immunoprecipitate was resolved by SDS-PAGE followed by immunoblotting with anti-Sam68 AD1 polyclonal antibodies. (B) Nuclear (N) and cytosolic and membrane (C/M) fractions from MCF-7 and HT29 cells were immunoprecipitated with anti-BRK antibodies, followed by immunoblotting with anti-Sam68 antibodies. A short exposure (10 s) shows interaction in the nuclear fraction only. A longer exposure (45 s) shows much weaker interaction in the cytosolic and membrane fraction.

the immunogenic peptide (Fig. 1A, panel C; BRK antibody plus BRK peptide, long exposure), no dots were observed, confirming the specificity of the signal.

Previously, Sam68 was reported to localize to novel nuclear structures termed SNBs that are novel and distinct from coiled bodies, gems, PML nuclear bodies, the perinucleolar compartment, and SC-35 speckles (4). SNBs contain nucleic acid that is most likely RNA, but their function remains unknown (4). We examined whether BRK colocalized with Sam68 in SNBs. HT29 cells were fixed and stained with antibodies against Sam68 (4) and BRK (BRK C-17), followed by secondary antibodies conjugated to FITC and rhodamine, respectively. The fluorescent signals were imaged using confocal microscopy. It was observed that most of the BRK nuclear dots colocalized with Sam68 SNBs (Fig. 1B). These findings demonstrate that BRK is a nuclear kinase that appears to be a component of SNBs in the HT29 colon cancer cell line.

The colocalization of BRK and Sam68 in SNBs suggested that these proteins might associate. To examine whether endogenous BRK associates with Sam68 in HT29 and MCF-7 cells, we performed coimmunoprecipitation experiments. Anti-BRK immunoprecipitates from HT29 and MCF-7 cells contained a band corresponding to Sam68 that was not detected when normal rabbit serum was used as a control or when the primary antibody was omitted (Fig. 2A). A significant increase in the amount of Sam68 that coprecipitated with BRK was detected when nuclear protein fractions were used for the immunoprecipitations (Fig. 2B). These findings suggest that BRK is a nuclear tyrosine kinase that associates with Sam68 in SNBs in cancer cell lines.

Sik phosphorylates Sam68 in vivo. Colocalization of BRK with Sam68 suggested that Sam68 may be a substrate for the Sik and BRK tyrosine kinase within the nucleus. To determine if Sam68 is a substrate of Sik, the normal murine mammary gland cell line NMuMG (18) was transiently transfected with wild-type, putative activated (Y-F), and kinase-defective (K-M) Sik. The putative activated form of Sik contains a Tyr-to-Phe substitution of the potential regulatory tyrosine at position 447 of Sik, while kinase-defective Sik contains a substitution of a conserved Lys at position 219 within the kinase catalytic domain with Met (42). Previously, we showed that Sik K-M has no kinase activity and acts as a dominant negative protein, but no enzymatic regulatory role has been demonstrated for the carboxy-terminal tyrosine of Sik (42). The different Sik expression constructs were cotransfected with a GFP-Sam68 fusion construct (4), and tyrosine phosphorylation of Sam68 was examined by immunoblotting with anti-phosphotyrosine antibodies. The NMuMG cell line does not express endogenous Sik (24) (Fig. 3A, middle panel, Sam68 + Vector). Tyrosine-phosphorylated GFP-Sam68 was detected in total-cell lysates from NMuMG cells cotransfected with wild-type Sik or Sik Y-F and the GFP-Sam68 expression construct, but not in cells cotransfected with vector alone or the kinase-defective Sik K-M construct (Fig. 3A, right panel). Higher levels of phosphorylated GFP-Sam68 and endogenous Sam68 were detected in cells expressing Sik Y-F than cells expressing wild-type Sik (Fig. 3A, right panel). The elevated tyrosine phosphorylation of Sam68 in Sik Y-F-transfected cells was not a result of elevated expression of Sik Y-F or GFP-Sam68 (Fig. 3A, left and middle panels). The association between Sik and Sam68 was further investigated in NMuMG cells transfected with GFP-Sam68 and Sik. Anti-Sam68 immunoprecipitates contained a coimmunoprecipitated phosphotyrosine protein with a molecular mass of 50 kDa that migrates in the expected position for autophosphorylated Sik (Fig. 3B, left panel). Moreover, Sik coimmunoprecipitated with both GFP-Sam68 and endogenous Sam68, because Sik could be detected in Sam68 immunoprecipitates from cells transfected with only wild-type Sik (Fig. 3B, right panel). Greater levels of Sik Y-F coimmunoprecipitated with Sam68 than wild-type Sik or Sik K-M. This may be explained by the increased ability of Sik Y-F to phosphorylate and then bind phosphorylated Sam68 through its SH2 domain (see Fig. 6A). No band corresponding to Sik was present in Sam68 precipitates from cells transfected with GFP-Sam68 and pcDNA3 (Vector) or in immunoprecipitations with IgG.

The ability of the different Sik constructs to phosphorylate Sam68 in HeLa cells transfected with wild-type Sik, Sik Y-F, and Sik K-M and Myc-tagged Sam68 was further examined using the vaccinia virus T7 expression system. Transfected HeLa cells were lysed, and the proteins were analyzed by immunoblotting with anti-Sik, anti-Myc, and anti-phospho-

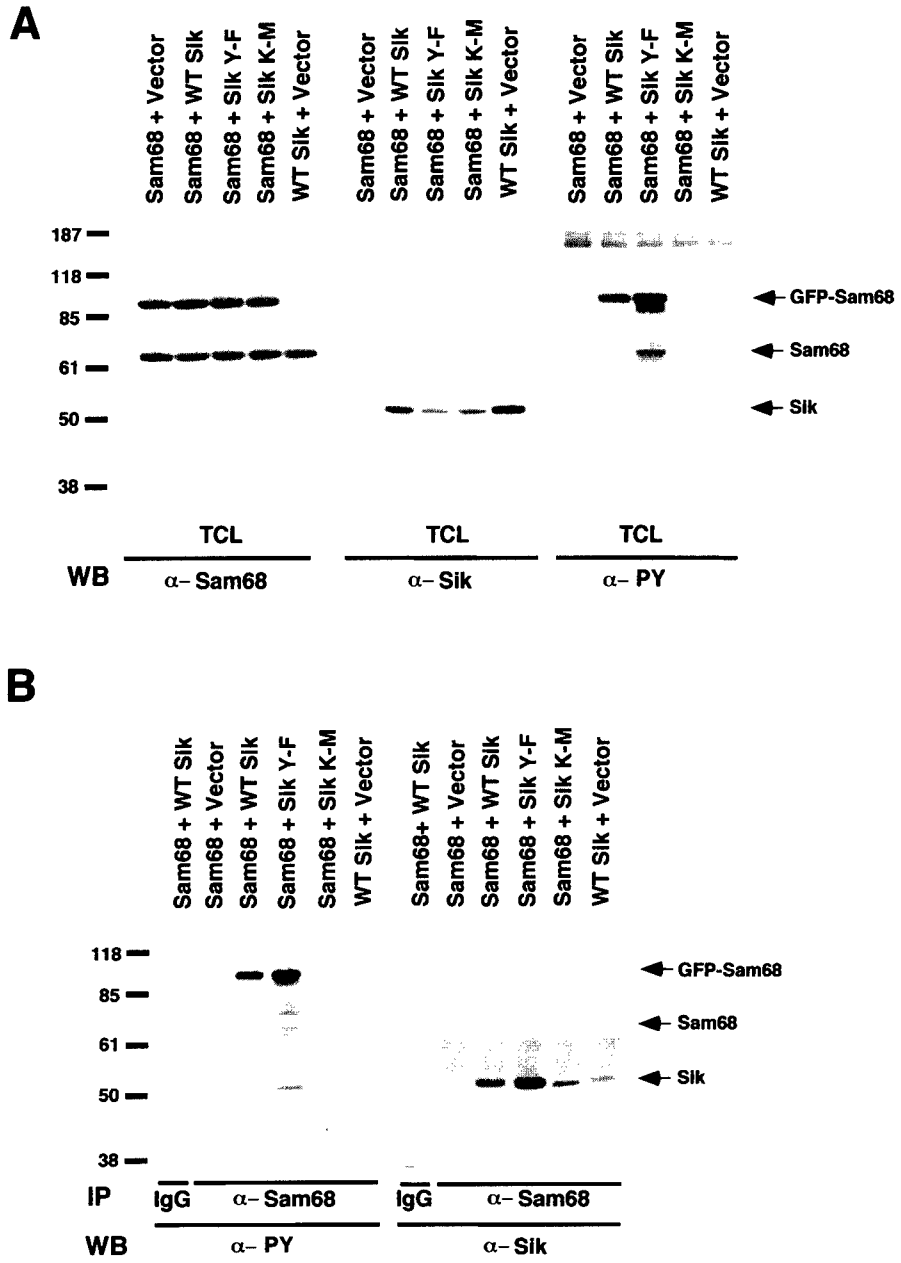


FIG. 3. Sik phosphorylates Sam68 in vivo. (A) Sam68 is tyrosine phosphorylated in NMuMG cells expressing wild-type Sik and Sik Y-F. NMuMG cells were cotransfected with GFP-Sam68 and Vector alone, wild-type (WT) Sik, Sik Y-F, or kinase-defective Sik K-M. Total-cell lysates were divided equally and immunoblotted with antibodies against Sam68, Sik, and phosphotyrosine. Tyrosine-phosphorylated Sam68 was detected only in lysates containing wild-type Sik or Sik Y-F. Although lower levels of Sik Y-F were expressed than wild-type Sik, the highest levels of tyrosine-phosphorylated Sam68 were detected in cells cotransfected with Sik Y-F, suggesting that tyrosine 447 of Sik negatively regulates its activity. (B) Sik associates with Sam68. Immunoprecipitations were performed with Sam68 antibody or IgG as a control and with lysates from the transfected cells in panel A. Immunoblotting was performed with antiphosphotyrosine or anti-Sik antibodies. Sik was observed to coprecipitate with Sam68 from lysates of cells transfected with Sik expression constructs. Sik antibody binding was detected using HRP-conjugated protein A. Tyrosine-phosphorylated Sik could also be detected in the Sam68 immunoprecipitates (left panel).

tyrosine antibodies. Several proteins, including one comigrating with Sam68, appeared heavily phosphorylated by the Sik Y-F construct (Fig. 4A, right panel). These data provide additional evidence that Sik is negatively regulated by phosphorylation of the carboxy-terminal tyrosine at position 447 and that substitution of Sik Y447F activates the kinase. The anti-Sik and anti-Myc immunoblots show equivalent expression of the proteins (Fig. 4A, left and middle panels).

The ability of Sik Y-F to phosphorylate other nuclear KH domain proteins, such as hnRNPK, was examined. A truncated form of Sam68, Sam68 (68–347), which contains amino acids 68 to 347 and lacks part of the amino terminus and the tyrosine-rich carboxy terminus, was also investigated as a substrate for Sik. HeLa cells were transfected with Sik Y-F alone or were cotransfected with Sik Y-F and Myc-Sam68, Myc-hnRNPK, or Myc-Sam68 (68–347). The cells were lysed and immunoprecipitated with control IgG or anti-Myc antibodies. The bound proteins were analyzed by immunoblotting with

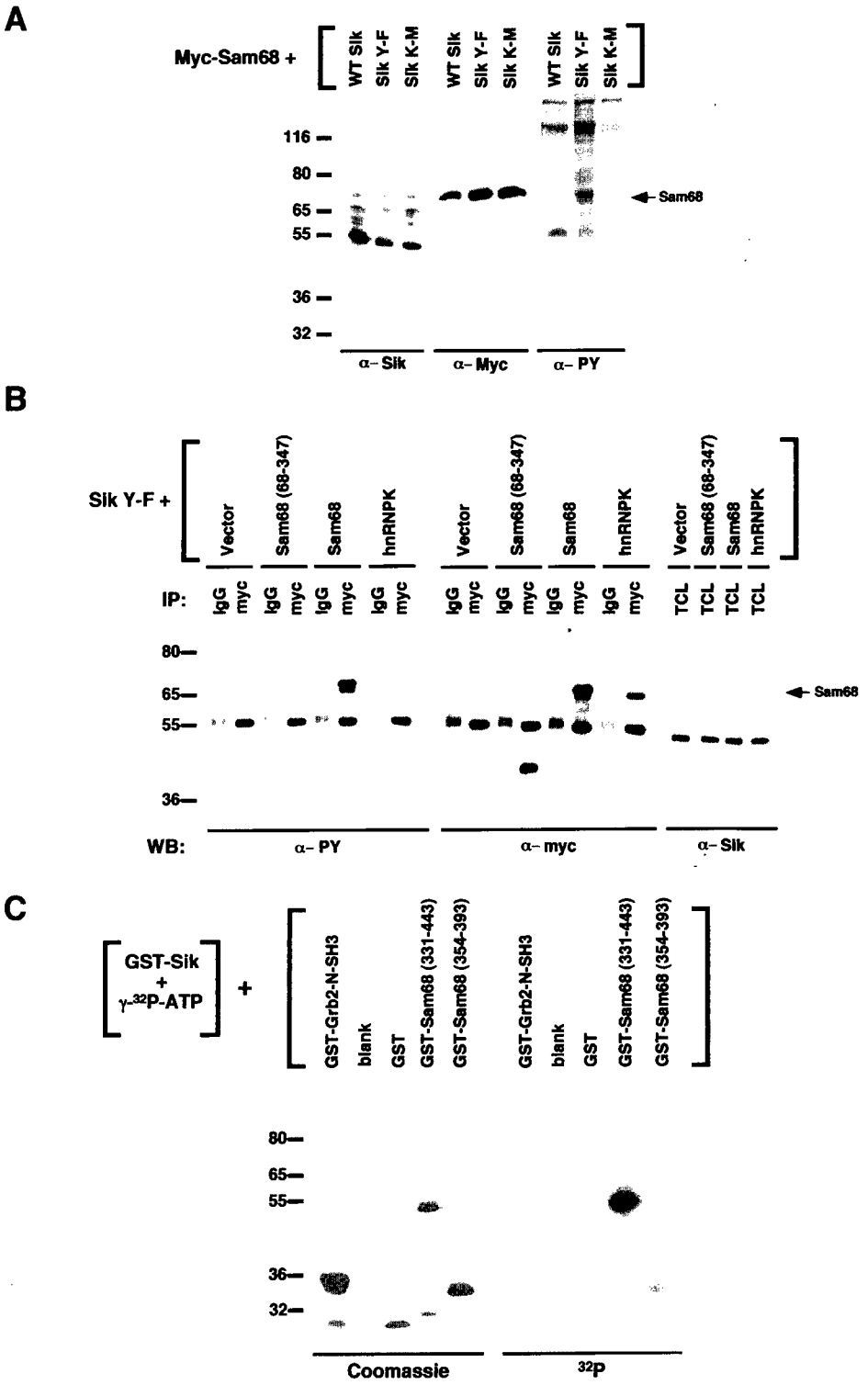


FIG. 4. Sik Y-F has increased kinase activity and specifically phosphorylates Sam68. (A) The carboxy-terminal tyrosine of Sik functions as a negative regulatory site. HeLa cells were cotransfected with Myc-tagged Sam68 and wild-type (WT) Sik, Sik Y-F, or Sik K-M (kinase defective). Cells were lysed, the proteins were separated by SDS-PAGE, and immunoblotting with anti-Sik, anti-Myc, and antiphosphotyrosine antibodies was performed. Increased phosphorylation of Myc-Sam68 was visible in total-cell lysates from Sik Y-F-transfected cells (right panel). (B) HeLa cells were transfected with Sik Y-F or were cotransfected with Sik Y-F and Myc-Sam68, Myc-hnRNPK, or truncated Myc-Sam68 (68-347). The cells were lysed and immunoprecipitated with IgG (control) or anti-Myc antibodies. The bound proteins were analyzed by immunoblotting with antiphosphotyrosine antibodies (left panel). The same membrane was subsequently immunoblotted with anti-Myc antibodies (middle panel). Total-cell extracts (TCL) were immunoblotted with anti-Sik antibodies (right panel). The band at ~55 kDa in lanes 1 to 16 represents the heavy chain of the immunoprecipitating antibodies. (C) Sik phosphorylates the carboxy terminus of Sam68. In vitro kinase assays were performed using full-length GST-Sik, [γ-^{32}P]ATP, and 2 μg of the following substrates: GST-Grb2-N-SH3 (negative control), GST alone, GST-Sam68 (331–443), or GST-Sam68 (354–393). The proteins were separated by SDS-PAGE and stained with Coomassie blue (left). The gel was dried, and the phosphorylated proteins were visualized by autoradiography (right).

antiphosphotyrosine, anti-Myc, and anti-Sik antibodies (Fig. 4B). A phosphotyrosine-containing protein with a molecular mass of 68 kDa (lane 6) was observed in anti-Myc immunoprecipitates from extracts transfected with wild-type Myc-tagged Sam68, but not with Myc-hnRNPK or Myc-Sam68 (68–347) (Fig. 4B, left panel). The membrane was reimmunoblotted with anti-Myc antibodies to confirm the equivalent expression of Myc-Sam68, Myc-hnRNPK, or Myc-Sam68 (68–347) (Fig. 4B, middle panel). Total-cell extracts were also verified for the equivalent expression of Sik Y-F by immunoblotting with anti-Sik antibodies (Fig. 4B, right panel). These data suggest that the C terminus of Sam68 is the target for the Sik tyrosine kinase.

To confirm that the carboxy terminus of Sam68 is directly phosphorylated by Sik, we incubated full-length GST-Sik with GST-Sam68 (331-443) and GST-Sam68 (354-393), two Sam68 carboxy terminus fusion proteins containing amino acids 331 to 443 and 354 to 393, respectively, in the presence of [γ-^{32}P] ATP. These two carboxy-terminal fragments of Sam68 were efficiently phosphorylated by Sik, whereas a control GST protein containing the amino-terminal SH3 domain of Grb2 and GST alone was not phosphorylated (Fig. 4C, right panel). The amounts of proteins used were determined to be equivalent as visualized by Coomassie blue staining (left panel). These data suggest that Sik directly and specifically phosphorylates the C terminus of Sam68.

Colocalization of Sik, Sam68, and phosphotyrosine in the nuclei of transfected cells. The localization of Sik and Sam68 was investigated in the NMuMG cell line. NMuMG cells were transfected with both wild-type Sik and GFP-Sam68 and were analyzed by confocal microscopy. The pattern of wild-type Sik expression was visualized by avidin-Alexa 350 (blue) (Fig. 5A, panel C) and was detected in the nucleus and at the membrane. Expression of the Sik Y-F and Sik K-M expression constructs was also detected in the nuclei and at the membranes of transfected cells (data not shown). GFP-Sam68, wild-type Sik, and phosphotyrosine colocalized in the nucleoplasm of the cells (Fig. 5A, panel D). Nuclear bodies were more commonly seen in cancer cell lines, but were not generally observed in the nontransformed NMuMG cell line (Fig. 5A and B). In experiments with control IgG, no specific fluorescent signal was detected.

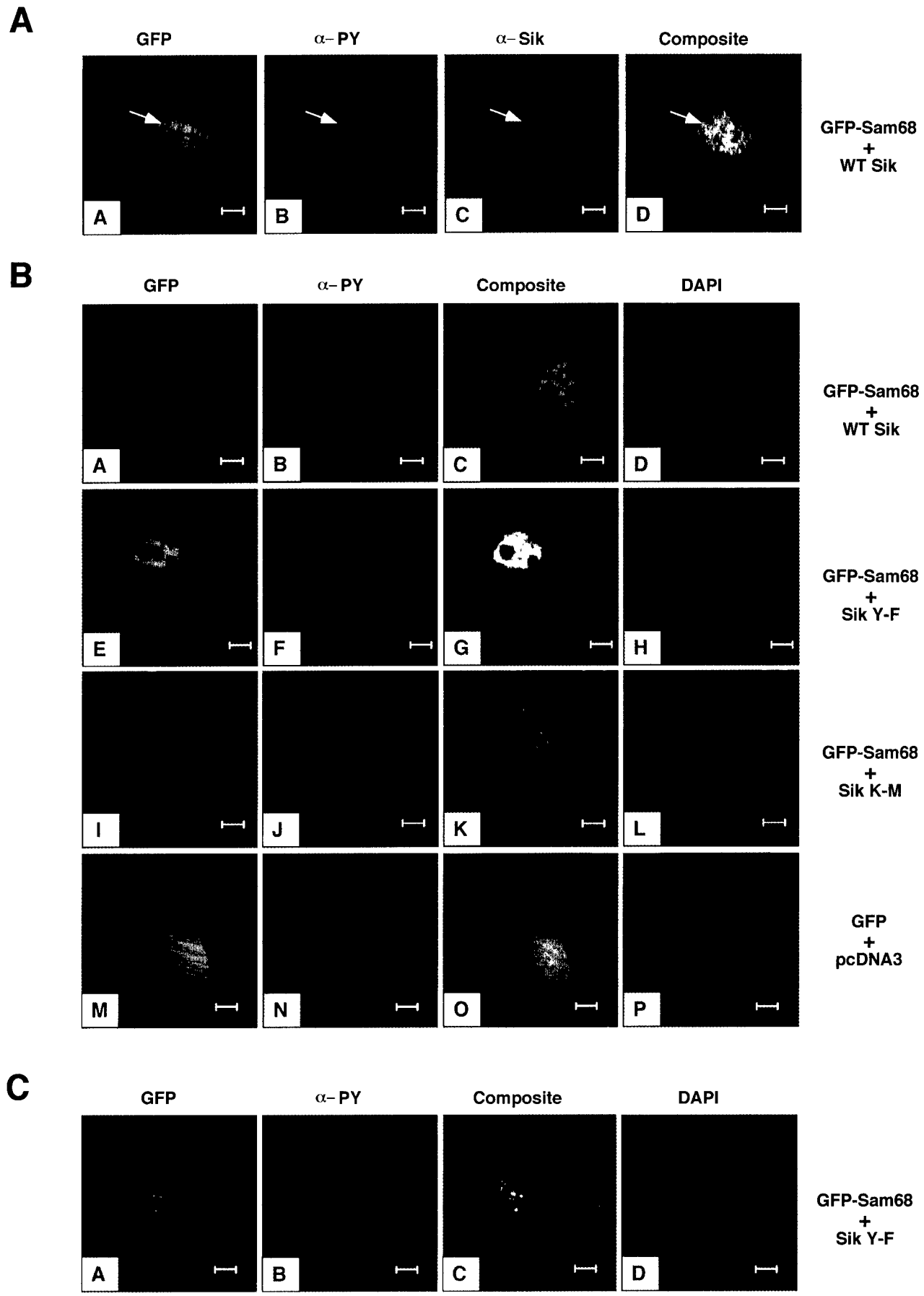
The localization and tyrosine phosphorylation of Sam68 in the presence of wild-type Sik, Sik Y-F, and Sik K-M were examined (Fig. 5B). Phosphotyrosine was readily detected only in the nuclei of cells transfected with active Sik and Sam68, suggesting that Sik is active within the nuclei and phosphorylates Sam68. Phosphotyrosine was detected in the nuclei of cells transfected with wild-type Sik and Sik Y-F (Fig. 5B, panels B and F), but not in kinase-defective Sik K-M-transfected cells (Fig. 5B, panel J). The majority of Sik-phosphorylated protein colocalized with Sam68 (Fig. 5B, panels C and G). We also observed that the intensity of the antiphosphotyrosine staining was greatest in cells transfected with Sik Y-F and Sam68. Cotransfection of the GFP expression vector pEGFP-C1 and the empty Sik expression vector pcDNA3 resulted in diffuse GFP fluorescence throughout the cell (Fig. 5B, panel M) and no detectable antiphosphotyrosine staining (Fig. 5B, panel N). These data provide further support that Sam68 is a substrate for Sik in vivo.

Since BRK and Sam68 were observed to colocalize in SNBs (Fig. 1), the presence of phosphotyrosine in these SNBs following transfection of the Sik expression constructs was examined. HT29 cells were transfected with GFP-Sam68 and wild-type Sik, Sik Y-F, or kinase-defective Sik K-M. The transfected cells were fixed, stained with antiphosphotyrosine antibodies, and analyzed by confocal microscopy. Tyrosine-phosphorylated protein colocalized with Sam68 in SNBs in these transformed cells transfected with wild-type Sik and Sik Y-F, but not with Sik K-M. The Sik Y-F transfection is shown in Fig. 5C. These data demonstrate that Sik most likely targets Sam68 in SNBs in cancer cell lines.

Sik-Sam68 interaction is mediated by both the SH3 and SH2 domains. To determine which part of Sik interacts with Sam68, different domains of Sik were expressed in bacteria as GST-fusion proteins and used in GST pull down assays. Lysates from NMuMG cells transfected with wild-type Sik, Sik Y-F, or Sik K-M and GFP-Sam68 expression constructs were incubated with GST alone, GST-Sik SH2+SH3, GST-Sik SH2, and GST-Sik SH3 bound to beads. Bound proteins were detected by immunoblotting with anti-Sam68 AD1 antibody (Fig. 6A). GFP-Sam68 and the endogenous Sam68 were observed to associate with the Sik SH3 and Sik SH2+SH3 domain fusion proteins (Fig. 6A). Association of GFP-Sam68 with the Sik SH2 domain was observed only when it was coexpressed with wild-type Sik or Sik Y-F, but not kinase-defective Sik K-M (Fig. 6A). We also observed association of endogenous phosphorylated Sam68 protein following longer exposures of the immunoblots. These data demonstrate that Sik associates with Sam68 through both its SH3 and SH2 domains.

Sam68 contains at least five proline motifs and interacts with the SH3 domains of several proteins, including Src, Fyn, and PLCγ-1 (14, 29, 39, 46). To determine which proline motif within Sam68 mediates interaction with the Sik SH3 domain, we used a GST pull down approach, with lysates from HeLa cells that express human BRK and GST fusion proteins representing the five proline (P0, P1P2, P3, and P4) motifs in Sam68. We found that GST-P3 was the main polypeptide that interacted with BRK (Fig. 6B). To further confirm the specific interaction of Sam68 P3 with the Sik SH3 domain, we tested the ability of peptides representing the proline-rich sequences P0, P3, and P4 to compete with binding of Sam68 with the GST-Sik SH3 domain. The Sam68 P3 peptide competed with the binding between Sam68 and the Sik SH3 domain. No significant competition was observed with the P0 and P4 peptides. Thus, the Sik SH3 domain appears to interact with one

FIG. 5. Wild-type Sik and Sik Y-F phosphorylate nuclear proteins that colocalize with GFP-Sam68 within the nucleus. (A) Localization of GFP-tagged Sam68, phosphotyrosine, and wild-type (WT) Sik in transfected NMuMG cells. Antiphosphotyrosine antibody was visualized with rhodamine (red), while anti-Sik antibody binding was visualized with avidin-Alexa 350 (blue). Wild-type Sik is present in the nucleus and at the membrane (panel C). (B) NMuMG cells were transfected with GFP-Sam68 and wild-type Sik (A to D), GFP-Sam68 and Sik Y-F (E to H), GFP-Sam68 and kinase-defective Sik K-M (I to L), or the GFP expression vector pEGFP-C1 and pcDNA3 (M to P). Cells were fixed 24 h after transfection, and tyrosine-phosphorylated proteins were localized using anti-phosphotyrosine antibodies (B, F, J, N). DAPI was used to stain the nuclei (D, H, L, P). In NMuMG cells, Sam68 displays diffuse, nuclear localization visible by green fluorescence (A, E, I). Cells cotransfected with GFP-Sam68 and wild-type Sik or Sik Y-F also stain strongly with the anti-phosphotyrosine antibody visualized using rhodamine (B, F), while no phosphotyrosine was detected in cells expressing kinase-defective Sik K-M (J). Panels C, G, K, and O are composites demonstrating colocalization that appears yellow. GFP alone is expressed throughout the cell (M) and is negative for anti-phosphotyrosine staining (N). (C) Increased phosphotyrosine in SNBs in HT29 cells following introduction of the activated Sik Y-F construct into HT29 cells. HT29 cells were transfected with GFP-Sam68 (A, C) and Sik Y-F, and tyrosine-phosphorylated proteins were localized using antiphosphotyrosine antibodies (B, C). Colocalization of Sam68 and the increased phosphotyrosine signal in SNBs are shown in panel C. No phosphotyrosine signal was detected in control cells transfected with kinase-defective Sik K-M (not shown). DAPI was used to stain the nuclei (D). Bars represent 5 μm.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Sik Y-F

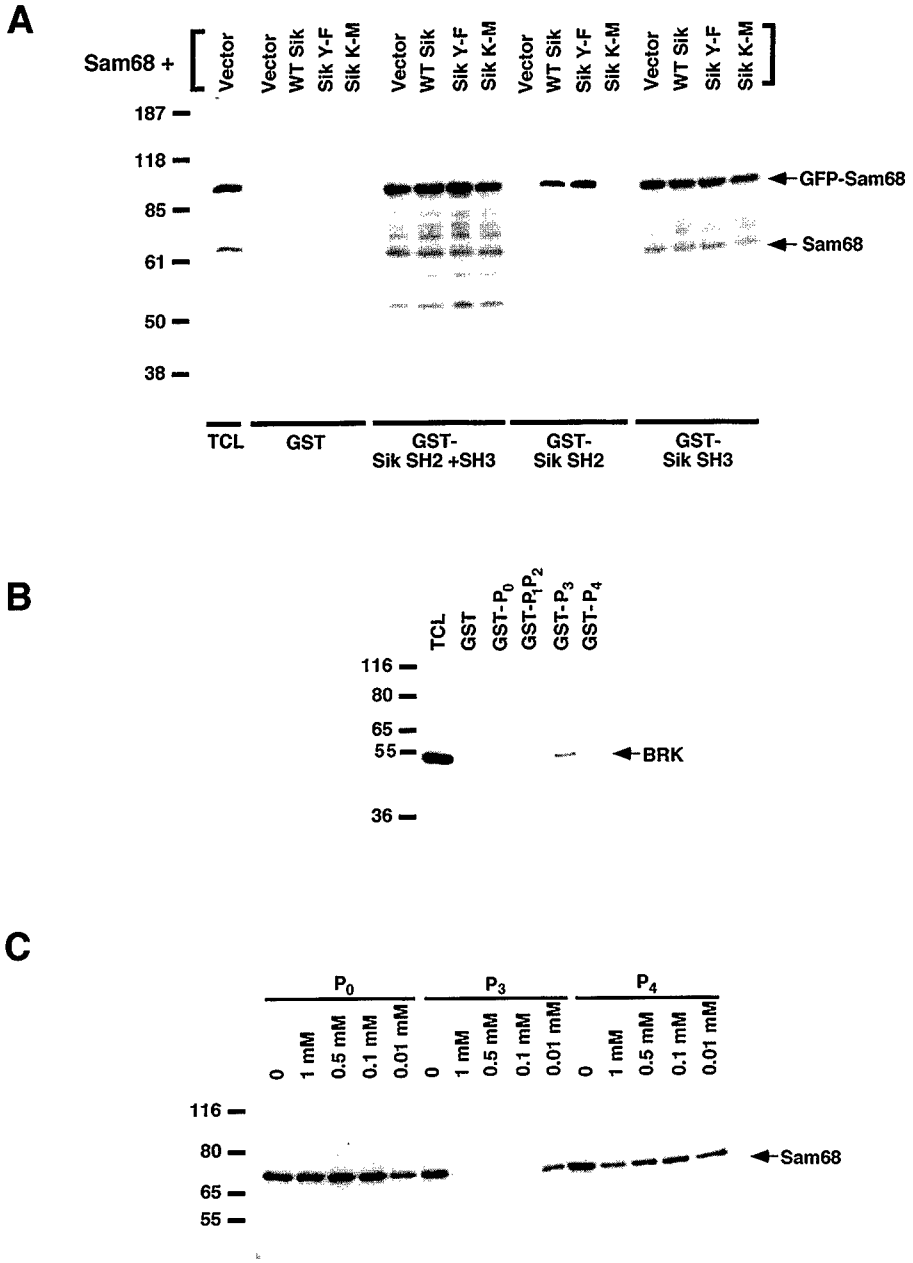

FIG. 6. The Sik SH2 and SH3 domains bind Sam68. (A) The ability of Sam68 to bind the SH2 and SH3 domains of Sik was tested. NMuMG cells were transfected with GFP-Sam68 and the expression vector pcDNA3 (Vector) or the wild-type Sik, activated Sik Y-F, or kinase-defective Sik K-M expression constructs. Cell lysates were divided equally and incubated with GST, GST-Sik SH2+SH3, GST-Sik SH2, and GST-SH3 covalently coupled to beads. Bound proteins as well as an aliquot of total-cell lysate from GFP-Sam68-transfected cells were separated by SDS-PAGE and immunoblotted with Sam68 AD1 polyclonal antibody. Positions of GFP-Sam68 and the endogenous Sam68 protein are indicated with arrows. GFP-Sam68 and endogenous Sam68 protein bound to the GST-Sik, SH2+SH3, and GST-Sik SH3 fusion proteins in all of the cell lysates. GFP-Sam68 binding to the GST-Sik SH2 domain was detected only in cells transfected with wild-type Sik or Sik Y-F, suggesting that phosphorylation by Sik is required for Sik SH2 binding. (B) Sam68 proline motif 3 (P3) associates with the SH3 domain of Sik. HeLa cell lysates were incubated with GST, GST-Sam68 P0, GST-Sam68 P1P2, GST-Sam68 P3, or GST Sam68 P4 covalently coupled to beads. The beads were washed, and the bound BRK was observed by immunoblotting. An aliquot of the HeLa cell lysate was used to represent total-cell lysate. BRK binding was only detected with the GST-Sam68 P3 fusion protein. (C) Beads containing covalently coupled GST-Sik SH3 protein were preincubated for 15 min at room temperature with the indicated concentration of Sam68 proline-rich peptide. Subsequently, HeLa cell lysates were added to each tube for 30 min at 4°C. The beads were washed extensively, and the bound Sam68 was quantitated by immunoblotting. Binding of Sam68 with the GST-Sik SH3 fusion protein was efficiently competed with the P3 peptide.

major proline motif in Sam68, P3, that is neither a type I nor a type II proline motif (13).

Sik inhibits the RNA binding ability of Sam68. Tyrosine phosphorylation of Sam68Δ1–67 negatively regulates its ability to bind to homopolymeric RNA (45). To determine whether Sik can regulate the ability of Sam68 to bind RNA, HeLa cells were transfected with Myc-Sam68Δ1–67 or cotransfected with Myc-Sam68Δ1–67 and Sik Y-F or Fyn. The expression of Fyn served as a positive control, as we have shown previously that Fyn can negatively regulate Sam68Δ1–67 homopolymeric RNA binding (45). HeLa cell lysates were divided equally and incubated with either poly(U) immobilized to agarose or agarose

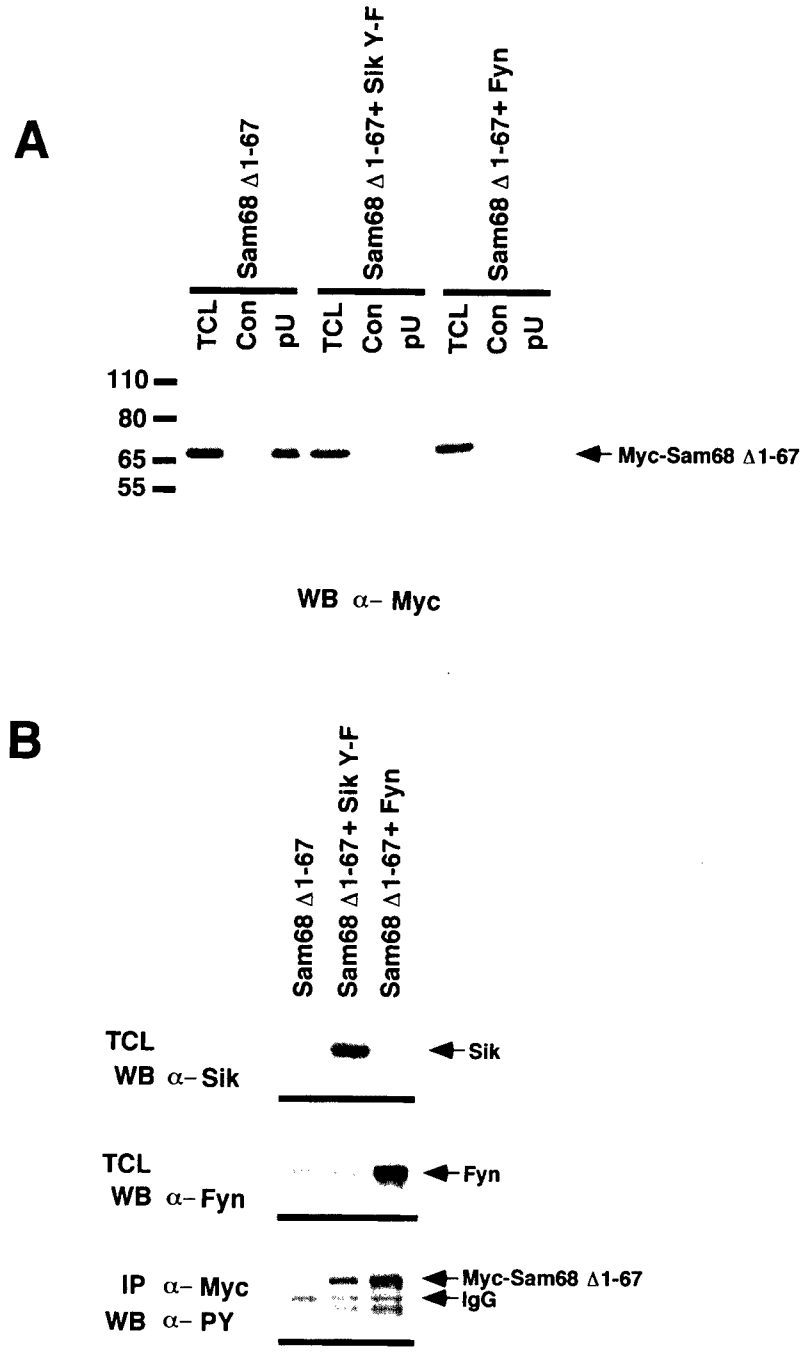

FIG. 7. Sik negatively regulates the ability of Sam68 to bind RNA. (A) HeLa cell lysates from cells either transfected with Myc-Sam68Δ1–67 alone or cotransfected with Myc-Sam68Δ1–67 and Sik Y-F or Fyn were divided equally and precipitated with agarose (Con) or poly(U)-agarose (pU), followed by anti-Myc immunoblotting. An aliquot of total-cell lysate (TCL) was also included to monitor expression of Myc-Sam68Δ1–67. The ability of Myc-Sam68Δ1–67 to bind poly(U) agarose was inhibited in cells transfected with either Sik Y-F or Fyn. (B) Sik and Fyn are efficiently expressed and phosphorylate Myc-Sam68Δ1–67 in transfected cells. Cell lysates from transfected cells were immunoblotted with anti-Sik and anti-Fyn antibodies to demonstrate that these kinases were efficiently expressed. Immunoprecipitation of Myc-Sam68Δ1–67 followed by immunoblotting with antiphosphotyrosine confirmed that Myc-Sam68Δ1–67 was tyrosine phosphorylated by both Sik and Fyn.

alone. Sam68 bound poly(U) homopolymeric RNA, when expressed alone (Fig. 7A). However, little or no RNA binding was detected when Sam68 was coexpressed with Sik Y-F or Fyn (Fig. 7A). The reduction of bound Myc-Sam68 was not due to poor expression of Myc-Sam68 (Fig. 7A). Total-cell lysates were immunoblotted with anti-Sik, anti-Fyn, and anti-phosphotyrosine antibodies, and we found that Sik Y-F and Fyn were both overexpressed and Myc-Sam68 was tyrosine phosphorylated (Fig. 7B). These data suggest that Sik is able to negatively regulate the ability of Sam68 to bind RNA.

Sam68 has been shown to function as a cellular homologue of Rev in transporting HIV RNA (27). We examined whether Sik could regulate this nuclear function of Sam68 by determining if Sik could modulate RRE-directed reporter gene expression (Fig. 8). COS7 cells were transfected with an RRE-CAT reporter plasmid in the presence of Sam68 and increasing

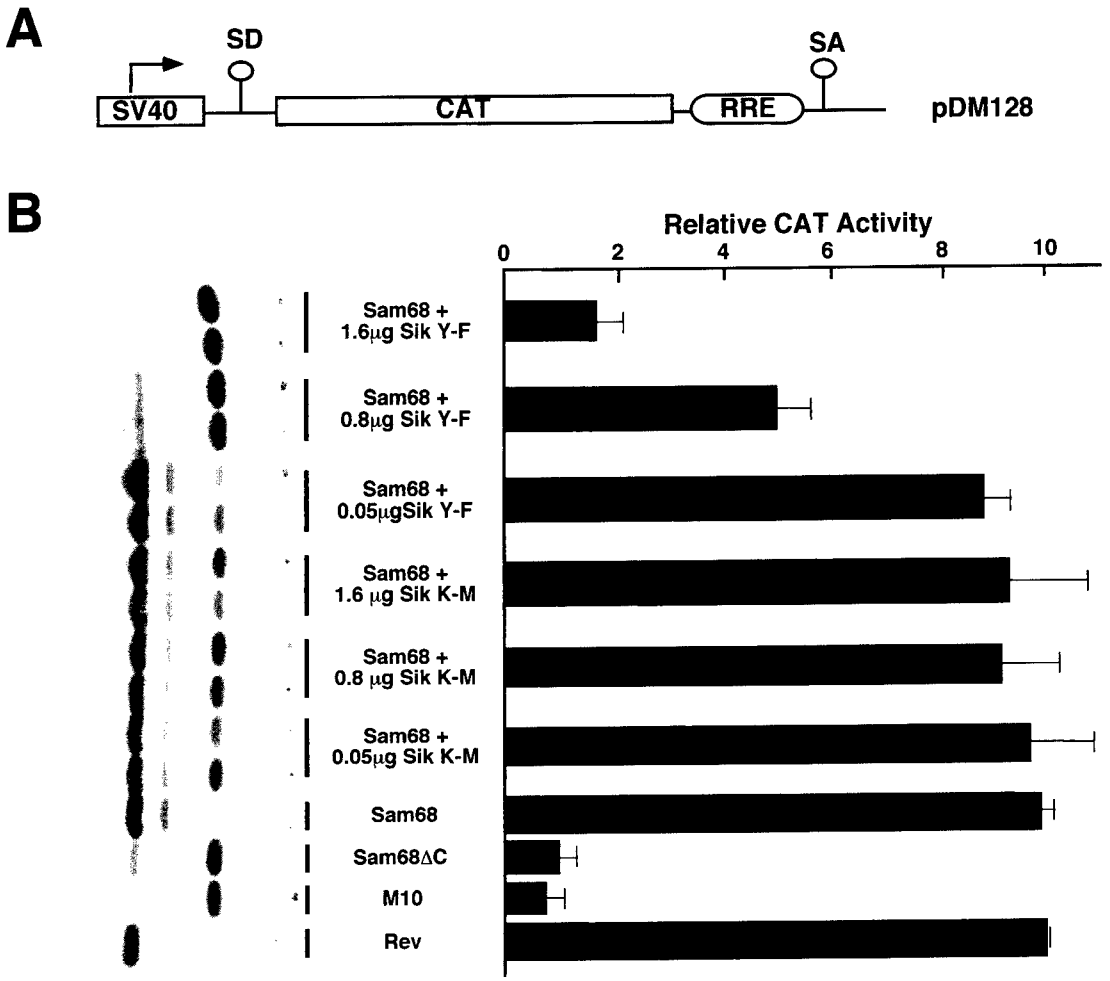

FIG. 8. Sik negatively regulates the ability of Sam68 to function as a cellular Rev homologue. (A) A schematic diagram of the Rev responsive element reporter CAT construct is shown. The splice acceptor and donor sites are indicated as SA and SD. CAT indicates the chloramphenicol acetyltransferase cDNA, and RRE is the HIV Rev responsive element. (B) COS7 cells were transfected with the RRE-CAT reporter plasmid in the presence of the indicated expression vectors and pCH110. CAT activity was normalized to β-galactosidase activity. Each bar represents CAT activity from at least eight samples from at least three separate experiments and the standard deviation indicated. The autoradiogram shown on the left represents a typical experiment: the two rows of dots on the left represent monoacetylated [^{14}C]chloramphenicol and the row on the right represents unacetylated [^{14}C]chloramphenicol.

amounts of kinase-active Sik Y-F or kinase-inactive Sik K-M. The transfection of Sam68 or Rev with the RRE-CAT reporter resulted in a 10-fold increase in CAT activation in comparison to Sam68ΔC and RevM10, two proteins shown to be inactive in Rev function. These data are consistent with previously published data (27). The cotransfection of Sam68 with Sik Y-F, but not Sik K-M, decreased CAT activity in a dose-dependent manner. These findings indicate that Sik can regulate a nuclear function of Sam68 and that its kinase activity is required.

DISCUSSION

The tyrosine kinases represent a large family of diverse proteins that play important roles in the regulation of growth and differentiation. Thus far, only a small number of nuclear tyrosine kinases have been identified, including Abl, Rak, Fes, Fer, and the dual-specificity kinase Wee1 (reviewed in references 26 and 44). In these studies, we showed that Sik and BRK are present in the nucleus. The mechanisms by which Sik and BRK localize to the nucleus are unknown, as they lack a clear nuclear localization signal (41). It is possible that Sik and BRK are transported to the nucleus through association with other proteins containing nuclear localization signals. Like Abl, Sik and BRK are not nuclear specific. In addition to being present in the nucleus, BRK was detected in the cytoplasm of HT29 cells (Fig. 1). Sik protein and kinase activity were also detected at the membrane of transfected NMuMG cells (Fig. 5A), consistent with the earlier observation that Sik can associate with a GAP-associated protein (37).

We demonstrate that the RNA binding protein Sam68 is a substrate for Sik within the nucleus and that Sik can inhibit the ability of Sam68 to bind RNA. RNA binding proteins may regulate gene expression by a number of mechanisms (reviewed in reference 32). They may alter RNA structure to regulate interaction with *trans*-acting factors or provide localization or targeting signals. Although its cellular function is unknown, Sam68 has been shown to be able to functionally substitute for the HIV-1 Rev protein, which plays an essential role in the nuclear export of unspliced and partially spliced viral transcripts and export of the HIV genome (27). We show that Sik kinase activity can negatively regulate the ability of Sam68 to function as a cellular homologue of Rev. These data

strengthen the possibility that signaling cascades can regulate the RNA function of GSG domain-containing proteins or 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 Sik within the nucleus. Sam68 may also serve as an adaptor for Sik, bringing it into proximity of other, as of yet unidentified, substrates.

Sik and its human orthologue BRK have only 80% amino acid sequence identity (24). Nevertheless, the genes have been mapped to regions of the mouse and human genomes that share conservation of synteny, and we have found that the mouse and human proteins are expressed in similar patterns in differentiated epithelial tissues (24). Here we show that the mouse and human proteins both localize to the nucleus and that they both associate with Sam68. These data provide further evidence that the functions of Sik and BRK in the two species are conserved.

NMuMG cells were isolated from the mammary glands of Namru mice and have epithelial growth characteristics, and they do not form malignant lesions when introduced into nude mice (18). Sik localization is diffuse within the nuclei of immortalized NMuMG cells, while BRK appears in SNBs in the HT29 colon adenocarcinoma cell line (Fig. 5). These data complement earlier studies by Chen et al. (4), who found that SNBs were predominant in transformed cells. SNBs are novel unique dynamic structures that disassemble when transcription is inhibited with actinomycin D (4). When GFP-Sam68 and wild-type Sik are introduced into HT29 cells, they localize to the SNBs, which become tyrosine phosphorylated (Fig. 5C). Tyrosine phosphorylation of Sam68 by Sik does not appear to alter its localization because Sam68 coexpressed with active Sik Y-F is retained in SNBs (Fig. 5).

We have shown that Sik can bind Sam68 through both its SH3 and SH2 domains. It was previously shown that Sam68 also binds SH3 and SH2 domains of Src kinases (14, 29, 39, 46). The binding affinities of specific SH2 domains are influenced by sequence context. For example, Src family members prefer the sequence pYEEI, while the SH2 domains of p85 and PLC-γ select the general motif pY-hydrophobic-X-hydrophobic (35, 36). Using a technique employing degenerate phosphopeptide libraries to predict the specificity of individual SH2 domains, it was determined that the Sik SH2 domain may bind to phosphorylated proteins with p-YEEY, YEDY, YDEY, and YDDY motifs (Z. Songyang and L. C. Cantley, personal communication). Interestingly, Sam68 contains the sequence YEDY in its carboxy terminus, and this is a putative binding site for the Sik SH2 domain. This sequence may also be the target of Sik, as we show here that Sik can phosphorylate the carboxy terminus of Sam68.

Sam68 is the first substrate identified for the Sik kinase. Related KH domain-containing proteins have been shown to play important roles in development, and these include human FMR1 (fragile X mental retardation syndrome) (9); mouse Qk1 (quaking), required for myelination (12); *Caenorhabditis elegans* GLD-1, required for germ cell differentiation (19); and *Drosophila* Who/How, required for muscle differentiation (1). The tissue- and differentiation-specific expression and activation of Sik (41, 42) suggest that Sik is involved in regulating epithelial cell differentiation. Sik is unique in that it is the only known tyrosine kinase that can phosphorylate Sam68 within the nucleus, where it can modulate its RNA binding ability and perhaps the pattern of gene expression associated with epithelial cell differentiation. Since Sam68 has also been shown to be involved in cell cycle regulation (3), overexpression of Sik and BRK may contribute to the development of epithelial cancers by altering the ability of Sam68 to regulate cell growth.

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J.J.D. and S.R. contributed equally to this work.

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BRK/Sik Expression in the Gastrointestinal Tract and in Colon Tumors[1]

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ABSTRACT

Clones encoding the breast tumor kinase BRK were isolated from a normal human small intestinal cDNA library that was screened with the cDNA encoding the mouse epithelial-specific tyrosine kinase Sik. Although BRK and Sik share only 80% amino acid 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, the location of the *BRK* gene. BRK expression was examined in the normal gastrointestinal tract, colon tumor cell lines, and primary colon tumor samples. Like Sik, BRK is expressed in normal epithelial cells of the gastrointestinal tract that are undergoing terminal differentiation. BRK expression also increased during differentiation of the Caco-2 colon adenocarcinoma cell line. Modest increases in BRK expression were detected in primary colon tumors by RNase protection, *in situ* hybridization, and immunohistochemical assays. The BRK tyrosine kinase appears to play a role in signal transduction in the normal gastrointestinal tract, and its overexpression may be linked to the development of a variety of epithelial tumors.

INTRODUCTION

Sik is an intracellular tyrosine kinase that was identified in a screen for tyrosine kinases in intestinal epithelial cells (1). Although it is related to the SRC family and contains SH2 and SH3 domains, it has a very short unique NH_2 terminus and is not myristoylated (2). Expression of Sik is restricted to epithelial cells and has been detected in the skin and in all linings of the alimentary canal. Transcription of Sik is initiated as cells migrate away from the proliferative zone and begin the process of terminal differentiation. Sik expression is first detected at mouse embryonic day 15.5 in the differentiating granular layer of the skin (2).

The role of Sik in differentiation was examined in mouse keratinocytes (3). The addition of calcium to cultured mouse keratinocytes induces a terminal differentiation program and a cascade of tyrosine phosphorylation. Sik was activated within 2 min after calcium addition to keratinocytes. It was found to bind a rapidly phosphorylated M_r 65,000 GAP[5]-associated protein (GAP-A.p65) through its SH2 domain. Overexpression of Sik in the embryonic mouse keratinocyte cell line resulted in increased expression of the differentiation marker filaggrin during calcium-induced differentiation. This suggested that Sik, the only known tyrosine kinase activated in keratinocytes within minutes after calcium addition, is involved in a signal transduction pathway that may promote differentiation.

Here we demonstrate that the breast tumor kinase BRK is the human orthologue of Sik. A portion of the BRK catalytic domain was initially 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. The full-length BRK cDNA was isolated from the MCF-7 and T-47D breast cancer cell lines (4). BRK has been detected in breast tumors and in a number of breast tumor cell lines, but not in normal breast, liver, placenta, pancreas, or other tissues (4, 5). Of 41 primary breast tumor samples quantified by Western blotting relative to cytokeratin 18, BRK was overexpressed 5-fold or more in 27% of the samples and overexpressed 2-fold or more in 61% of the samples as compared to normal breast tissue. One tumor expressed 43-fold higher levels of BRK protein (5).

BRK has also been cloned from melanoma cells and named PTK6 (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, and may be expressed in 10% of primary melanoma and melanocyte cultures (7).

Using human/hamster somatic cell hybrids, *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

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[5] The abbreviations used are: GAP, GTPase-activating protein; UTR, untranslated region; RT-PCR, reverse transcription-PCR; nt, nucleotide; o/e, observed/expected.

three primary breast carcinomas (9). In addition to being amplified 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). We discuss the relationship between *BRK* and *Sik* and the possible role that this kinase may play in epithelial cell cancers.

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones. A human small intestine (duodenum) 5′ Stretch cDNA library prepared in the λgt11 vector (Clontech) was screened using a ^{32}P-labeled 562-bp *Sst*I fragment of the Sik cDNA (2). cDNA clones were sequenced by the dideoxynucleotide chain termination method (14), using Sequenase (USB).

Tissue and Blood Samples. Tissues for *in situ* hybridization experiments were surgically resected, fixed in neutral buffered formaldehyde, and embedded in paraffin. Tissues for protein expression studies were obtained through pinch biopsies obtained during endoscopic procedures performed in the GI suite at the University of Illinois Hospital (Chicago, IL). All samples were collected after informed consent was obtained in accordance to the previously approved institutional review board protocol.

Southern Blot Analyses. Human genomic DNA was prepared from a blood sample obtained from a healthy volunteer. Mouse genomic DNA was extracted from the liver of a CD1 mouse. Ten μg of human and mouse DNA were digested with *Bam*HI, *Hin*dIII, *Sst*I, and *Bam*HI-*Hin*dIII; subjected to electrophoresis through a 0.8% agarose gel; and transferred to a nitrocellulose membrane. Probes for hybridization included a 562-bp purified *Sst*I fragment of Sik cDNA and an 884-bp *Sac*I-*Eco*RI fragment of human BRK cDNA. 100 ng of each fragment was used for random primer labeling with 50 μCi of [^{32}P]dCTP. Membranes were washed in 0.1% SDS and 0.1× SSC at 65°C for the corresponding species probe and at 55°C for the interspecies hybridizations and exposed to film.

Mapping of Mouse Sik. *Sik* is closely linked to *Eef1a2* and was found on BAC clones containing this gene. Mapping of *Eef1a2* was carried out using DNA from The Jackson Laboratory Interspecific Backcross BSS panel (15). This panel is made up of 94 N2 offspring derived from the cross (C57BL/6J × SPRET/Ei)F_1 × SPRET/Ei. Over 3310 loci have been mapped in this cross. *Eef1a2* mapped to within the most distal group of markers on mouse chromosome 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 containing an insert of approximately 65–70 kb. Sequencing indicated that each clone contained 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 Fig 3*B* include mouse sequences *Pltp:* basepairs 67-1548 of GenBank locus U37226 (NCBI accession 1051265); *Eya2:* 166-1587 of U81603 (1816530); *Gnas:* 20-1204 of Y00703 (51127); *Pck1:* 1-546 of EST sequence AI037119, 470-539 of AA562908, 1-59 of AI021099, 18-478 of AA080172, 458-569 of AA286042, 9-537 of AA106463, 118-515 of AA110781; *Lama5:* 5906-10820 of U37501 (2599231); *Eef1a2:* 134-1525 of L26479 (1220409); *Col9a3:* 1-333 of X91012 (975686), 1-104 of AA027742 (1493761); *Sik:* 286-1641 of U16805 (847794); human sequences *PLTP:* 88-1569 of L26232 (468325); *EYA2:* 196-1617 of Y10261 (1834488); *GNAS:* 69-1253 of X04408 (31914); *PCK1:* 122-1990 of L05144 (189944) excluding 589-609 (for which mouse EST sequence was unavailable); *LAMA5:* 1-4930 of AB011105 (3043589); *EEF1A2:* 84-1475 of X70940 (38455); *COL9A3:* 1564-2012 of L41162 (1196420); *BRK:* 814-2169 of X78549 (515025).

Immunoprecipitation and *in Vitro* Kinase Assays. Proteins extracted from mouse tissues were incubated for 1 h at 4°C with anti-Sik (C-17) antibody (Santa Cruz Biotechnology). After the addition of 40 μl of protein A-Sepharose (Pharmacia, Piscataway, NJ), samples were incubated for 1 h at 4°C. Complexes were extracted several times in 50 mM HEPES (pH 7.0), 0.15 M NaCl, and 0.1% NP40 and resuspended in kinase buffer [50 mM HEPES (pH 7.0), 1% NP40, and 10 mM MnCl]. Autophosphorylation reactions were performed by incubation of immunoprecipitated protein with 20 μCi of [γ-^{32}P]ATP (Amersham) for 15 min at 30°C. Reactions were stopped by adding EDTA to 20 mM, and samples were resuspended in 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 10% glycerol. Denatured proteins were separated on a 10% SDS-polyacrylamide gel, which was then treated for 2 h with 1 M KOH at 55°C, dried, and exposed to X-ray film.

RNase Protection Assays. Expression of *Sik* and BRK was analyzed by RNase protection assay, as described previously (1, 16), 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 *Stu*I-*Apa*I fragment of the BRK cDNA was subcloned into pBlueScript SK II−. This plasmid was linearized with *Hin*dIII, 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 or human cyclophilin (pTRI-cyclophilin-mouse and pTRI-cyclophilin-human; Ambion).

Total RNA from mouse tissues was prepared by homogenization in guanidine thiocyanate solution with 2-mercaptoethanol followed by CsCl gradient centrifugation (17). Total RNA from human colon tumors and adjacent normal colon tissue was a generous gift from Robert M. Lee and N. O. Davidson (University of Chicago). Twenty μg (mouse) or 10 μg (human) of each total RNA sample or an equal amount of baker's yeast tRNA was precipitated with ethanol and resuspended in 30 μl of hybridization buffer containing 2 × 10^5 cpm of probe. The concentration and quality of the RNA were confirmed on stained 4-morpholinepropanesulfonic acid/formaldehyde gels.

Semiquantitative RT-PCR. The SuperScript Preamplification System (Life Technologies, Inc.) and 2 μg of total RNA were used for synthesis of cDNA. PCR was performed in 20 μl containing 80 ng of cDNA, 50 pmol of each primer, 0.2 mM deoxynucleotide triphosphate mix, 1.2 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), and 50 mM KCl. PCR was done using the following parameters: (*a*) denaturation, 45 s at 94°C; (*b*) an-

nealing, 45 s at 67°C; and (*c*) extension, 1 min at 72°C. For each combination of primers, the kinetics of PCR amplification was studied, and the number of cycles corresponding to the plateau were determined. PCR was performed at an exponential range (29 cycles for BRK; 25 cycles for keratin 8). A 224-bp BRK PCR product was generated using primers Brk-1 (5′- ATCCAGGCCATGAGAAGC-3′) and Brk-2 (5′- TGGATGTAATTCTGCGACTCC-3′), corresponding to nt 706–724 and nt 929 to 909 of the Brk sequence. A 110-bp K8 PCR product was generated using primers K8-305 (5′-TTGCCTCCTTCATAGACAAGG-3′), corresponding to nt 305–326, and K8–415 (5′-TGTTGTCCATGTTGCTTCG-3′), corresponding to nt 396–415 of the human K8 gene sequence. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Bands corresponding to each specific PCR product were quantitated using NIH Image.[6]

Western Blot Analyses. Total protein was isolated from pinch biopsies or cultured cells. Samples were lysed in radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS). Twenty μg (tissue) or 30 μg (cell lines) of protein per lane were subjected to electrophoresis through a 10% SDS-polyacrylamide gel and transferred to Immobolin P membranes. Filters were blocked for 1 h in 5% nonfat dry milk in buffer containing 10 nm Tris-HCl (pH 7.5), 500 mM NaCl, and 0.1% Tween 20 and then incubated for 1 h with BRK (C-17) antibody (Santa Cruz Biotechnology), or they were blocked with buffer containing 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20 and then incubated with β-antibody for 1 h. (Sigma Chemical Co., St. Louis, MO). The higher NaCl substantially reduced background obtained with the commercially available BRK polyclonal antibody. Subsequently, the membranes were stained with appropriate horseradish peroxidase-conjugated secondary antibodies, and antibody binding was detected using the SuperSignal ULTRA chemiluminescence substrate (Pierce). In control experiments, the immunogenic peptide specifically competed out BRK antibody binding, confirming the specificity of the signal.

***In Situ* Hybridizations.** BRK mRNA expression in the human intestine was examined using *in situ* hybridization techniques as described previously (2). The cRNA probe was prepared from a template consisting of an 884-bp *Sac*I-*Eco*RI fragment of human BRK cDNA cloned into pBluescript II SK. The *in vitro* transcription product was labeled with ^{35}S-labeled UTP and hydrolyzed to fragments of an average size of 150 nt in length for better tissue penetration. After hybridization, slides were treated with RNase A for excess probe removal, washed with increasing stringency to 0.1% SSC at 55°C, and dehydrated in graded ethanols diluted with 0.3 M sodium acetate. Sections were coated with autoradiographic emulsion and exposed for 8–11 days at room temperature.

Indirect Immunohistochemistry with Tyramide Amplification. Paraffin-embedded biopsy samples sectioned at 5–8 μm thick were deparaffinized, hydrated, and preincubated in block buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20; 1:50 normal goat serum] for 40 min. Sections were then incubated with 0.2 μg/ml BRK antibody (Santa Cruz Biotechnology) in block buffer overnight at 4°C, washed, and incubated with 1:250 biotinylated goat antirabbit antibody (Vector Laboratories) in block buffer for 30–60 min. After washing, the TSA-Indirect Kit (DuPont New England Nuclear) was used according to the manufacturer's instructions. Briefly, sections were treated with strepavidin-horseradish peroxidase, reacted for 5 min with biotinyl tyramide reagent, visualized with 1:500 FITC-avidin DCS (Vector Laboratories), and mounted with Vectashield mounting medium (Vector Laboratories). For control sections, 4 ng/ng BRK antibody of the peptide from which the BRK antibody was raised (Santa Cruz Biotechnology) were added to block buffer, 10–15 min before use.

RESULTS

The Breast Tumor Kinase BRK Is the Human Orthologue of Mouse Sik. To isolate the human orthologue of Sik, we screened a normal human small intestine cDNA library (Clontech) with mouse Sik cDNA. From 10^6 recombinant phage, three positive clones were isolated. These partial cDNA clones shared sequence identity with the nonreceptor tyrosine kinase BRK that was isolated from a human metastatic breast tumor (4).

Like Sik, BRK is also a 451-amino acid protein with SH2 and SH3 domains and putative regulatory tyrosines at the activation loop and COOH terminus. Unlike members of the SRC family, Sik and BRK each lack consensus myristoylation motifs at the NH_2 terminus. Sik and BRK also have the sequence HRDLAARN in their catalytic domains, in contrast to the HRDLRAAN sequence shared by members of the SRC family. The amino acid sequences of Sik and BRK are aligned in Fig. 1. Whereas the functional domains of BRK and Sik are conserved, Sik and BRK share only 80% amino acid identity and 83% nt identity.

The Sik and BRK mRNAs each contain repetitive elements in their 3′ noncoding regions. In Sik mRNA, a B1 repeat precedes the observed polyadenylation site by approximately 350 bp, whereas the related Alu repeat (18, 19) comprises the last 300 bp of the known BRK cDNA. The Sik B1 element is in an antisense orientation, occurs in a truncated form, and contains sequence motifs closely matching the family of mouse B1.m-B repeats (19), which is estimated by mutation rates of CpG and non-CpG positions to have been amplified in the mouse genome 4.4 ± 3.1 Myr ago (20). The BRK Alu element occurs in a sense orientation, terminating the known BRK sequence with its polyadenylation sequence, and displays hallmarks of the modern human Alu dimer Alu-Sb, the most recently amplified family of Alu repeats (21). Coincidental integrations are not improbable because Alu elements preferentially insert into regions of high GC content (22). Such repetitive elements may have regulatory effects on the mRNAs in which they are incorporated (23).

Because of the relatively low level of sequence identity (80%) between Sik and BRK, we performed Southern hybridization experiments to confirm that Sik and BRK are true orthologues. ^{32}P-labeled BRK and Sik probes were hybridized with restricted mouse and human genomic DNA. Stringent hybridization with probe from the same species indicated the

[6] NIH Image is available at http://rsb.info.nih.gov/nih-image/.

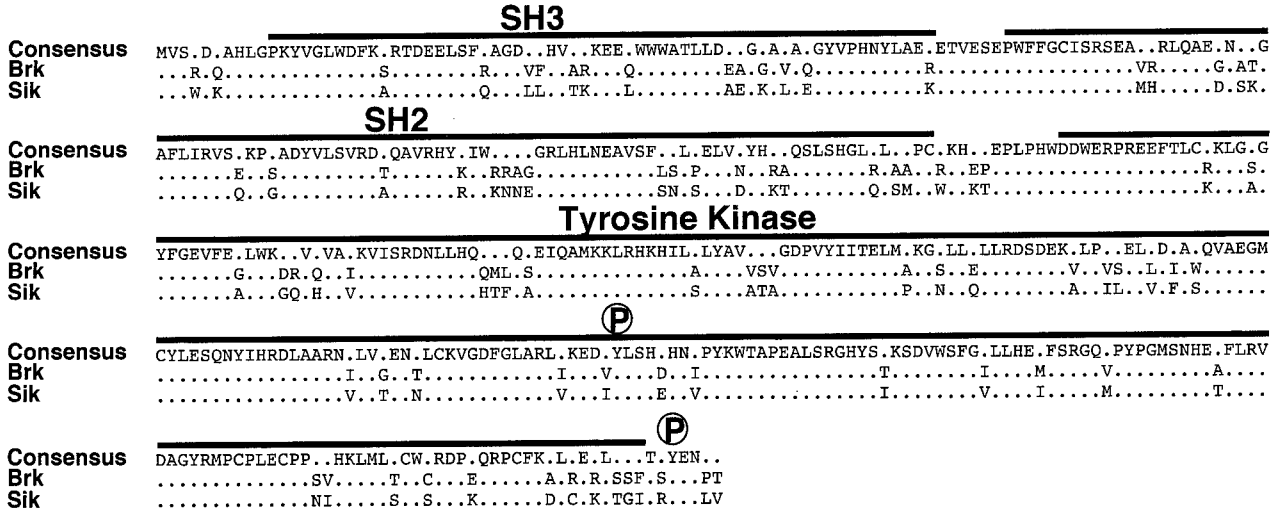


Fig. 1 BRK and Sik protein share an 80% sequence identity. The amino acid sequences of Sik (2) and BRK (4) are aligned, and the differences between the sequences are distributed throughout the different domains. The *first circled P* (phosphate) corresponds to the phosphorylated tyrosine at the ATP binding site, and the *second circled P* corresponds to the putative regulatory tyrosine in the COOH-terminal region. The SH3 and SH2 domains of Sik and BRK are marked.

position of bands for each gene. If the probe from the other species recognized a gene of equal or greater homology, an additional set of bands would be visible; however, both the mouse Sik probe and the human BRK probe detect the same fragments in mouse and human genomic DNA as shown in Fig. 2, confirming that *Sik* and *BRK* are the closest homologues of one another.

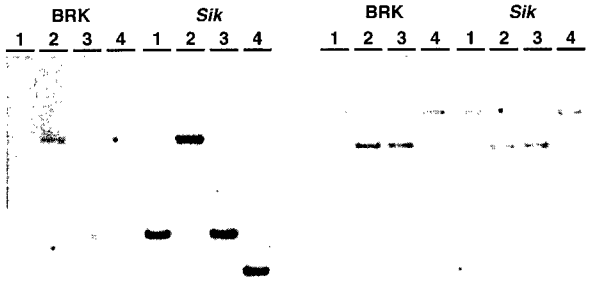
Comparison of the nt sequences encoding *Sik* and *BRK* indicated no apparent insertions or deletions; sequence differences largely reflect changes in the GC content of the DNA code. Of 231 differences within the coding sequence, 159 are positions where G or C is present in the human sequence, whereas the corresponding mouse nt is A or T. Many alterations in the protein sequence seem to be due to mutations of CpG dinucleotides. Of 86 CpG dinucleotides in the human, 18 are conserved with mouse sequence, which has 24 in total. Throughout the known promoter and mRNA sequence, the o/e CpG ratio of *Sik* averages 0.23 and does not exceed 0.3 within a 500-bp window, whereas *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 (24). Loss of *Sik* CpG content is evenly distributed throughout promoter and coding sequence, with *BRK* always having at least double the *Sik* o/e CpG ratio.

The mouse *Sik* gene is linked to the gene *Eef1a2* that encodes translation elongation factor α-2. A BAC library constructed from 129/Sv ES cell DNA was screened with primers corresponding to *Eef1a2* (25). Two independent clones were isolated, each containing a 65–70-kb insert. Each was found by sequencing to contain the coding and 3′-UTR sequence of the *Sik* gene in addition to *Eef1a2*. One clone also contained all of the 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 (Fig. 3). Because *BRK* maps to human chromosome 20q13.3 (8), this provides further evidence that these two genes are orthologous. In wasted mice, the *Eef1a2* gene is deleted (25), but the *Sik* gene is intact and appropriately expressed.[7]

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 that forms 3% of the human genome and contains 28% of human genes (26). 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 (27, 28). Although overall values for G + C% and 5-methylcytosine in human and mouse are nearly identical (24), the mouse genome lacks the very GC-rich H3 isochore (29, 30). Accordingly, the mouse *Sik, Eef1a2*, and *Lama5* genes contain 14–19% less G + C in the third codon position. 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 (31). The case of *BRK* and *Sik* suggests that this can occur by effects on the isochore level that increase CpG loss uniformly on a megabase scale. Thus, the conserved synteny of these genes 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.

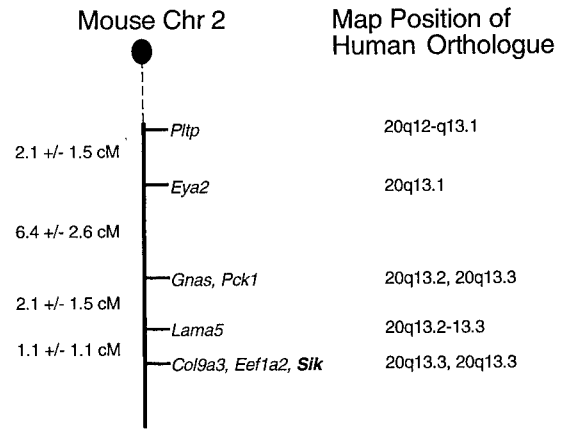
Tissue-specific Expression of Sik in the Mouse. Sik mRNA expression has only been detected in regenerating epithelia such as that lining the gastrointestinal tract, as well as in the liver and skin. Little or no mRNA expression was detected

[7] C. A. Abbott, unpublished data.



A B

Fig. 2 BRK is the human orthologue of mouse *Sik*. Mouse (*A*) and human (*B*) genomic DNAs were digested with *Bam*HI (*Lanes 1*), *Hin*dIII (*Lanes 2*), *Bam*HI/*Hin*dIII (*Lanes 3*), and *Sst*I (*Lanes 4*); electrophoretically separated; transferred to nitrocellulose membranes; and hybridized with Sik and BRK ^{32}P-labeled probes. The Sik and BRK probes hybridize with the same fragments in human and mouse DNA. Because no additional bands appear, we can conclude that no other sequences of equal or greater homology are present in either genome.



A

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

Fig. 3 *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 of the markers is available on the World Wide Web at http://www.jax.org/resources/documents/cmdata. Distances between markers are given to the *left* of the chromosome as cM ± SE. *B*, isochore G + C differences in distal mouse chromosome 2 and human chromosome 20q. Known sequences for the syntenic genes above are analyzed for total G + C content, third codon position G + C content, and o/e (*Obs/Exp*) CpG ratio for human (*H*), mouse (*M*), and the degree to which the human exceeds mouse content (Δ). The percentage of nt sequence identity (with <0.6% gaps) is also indicated (*Cons*). 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. The *asterisk* indicates that mouse *Pck1* sequence was assembled from mouse EST fragments and is less reliable. The segments examined are restricted to the coding sequence and usually include all of the coding sequence, but segments of *Lama5* and *Col9a3* compared use only the 3′ sequence because the 5′ sequence is unknown.

in the kidney, lung, spleen, testis, uterus, diaphragm, or brain (1, 2).[8] We examined Sik mRNA expression in the mouse small and large intestine (Fig. 4*B*) by RNase protection. Levels of the Sik protected fragment increase from the duodenum to the ileum, with the ileum having peak levels of Sik mRNA expression.

We could not detect Sik protein in total tissue protein extracts using standard Western blot analysis with a rabbit polyclonal anti-Sik antibody, perhaps due to its low differentiation-specific expression. Immunoprecipitations were performed, followed by *in vitro* kinase assays, with total proteins from brain, spleen, tongue, ileum, liver, and muscle (Fig. 4*C*). Active mouse Sik, capable of autophosphorylating itself with ^{32}P, was detected in total protein from the ileum of the mouse small intestine. No autophosphorylated Sik was detected when Sik peptide was added to the immunoprecipitation reaction, indicating that the signal is specific. At longer exposures, some autophosphorylated Sik was also detected after immunoprecipitation with total tongue protein. These protein data are consistent with the earlier mRNA expression studies, which indicated that Sik mRNA was present in the ileum and the tongue (2).

Sik expression was also examined in the mammary gland. Mammary gland differentiation is regulated hormonally and requires pregnancy for the establishment of terminal differentiation (for a review, see Ref. 32). During involution after weaning, a number of morphological and biochemical changes occur. We examined Sik expression in the mammary glands of virgin, pregnant, and lactating mice and at different times after weaning. At no time did we detect expression in the normal mammary gland. In contrast, significant levels of Sik expression were detected in the skin and small intestine (Fig. 4*A*). Sik expression was also not detected in the mammary gland at these stages using *in situ* hybridization and immunohistochemistry (data not shown), ruling out the possibility that Sik expression was induced in a small subset of the cells. We have also not detected Sik expression in the NMuMG normal mouse mammary gland cell line (Ref. 33; data not shown).

BRK Expression in Colon Tumor Cell Lines. BRK expression was examined in the human colon carcinoma tumor cell lines SW480, HT29, T84, and Caco-2. Caco-2 cells, which differentiate spontaneously after becoming confluent, provide a model system for studying enterocyte differentiation. As these cells are maintained in culture, they polarize, form microvilli, and express increasing levels of brush border enzymes, such as

[8] M. S. Serfas and A. L. Tyner, unpublished data.

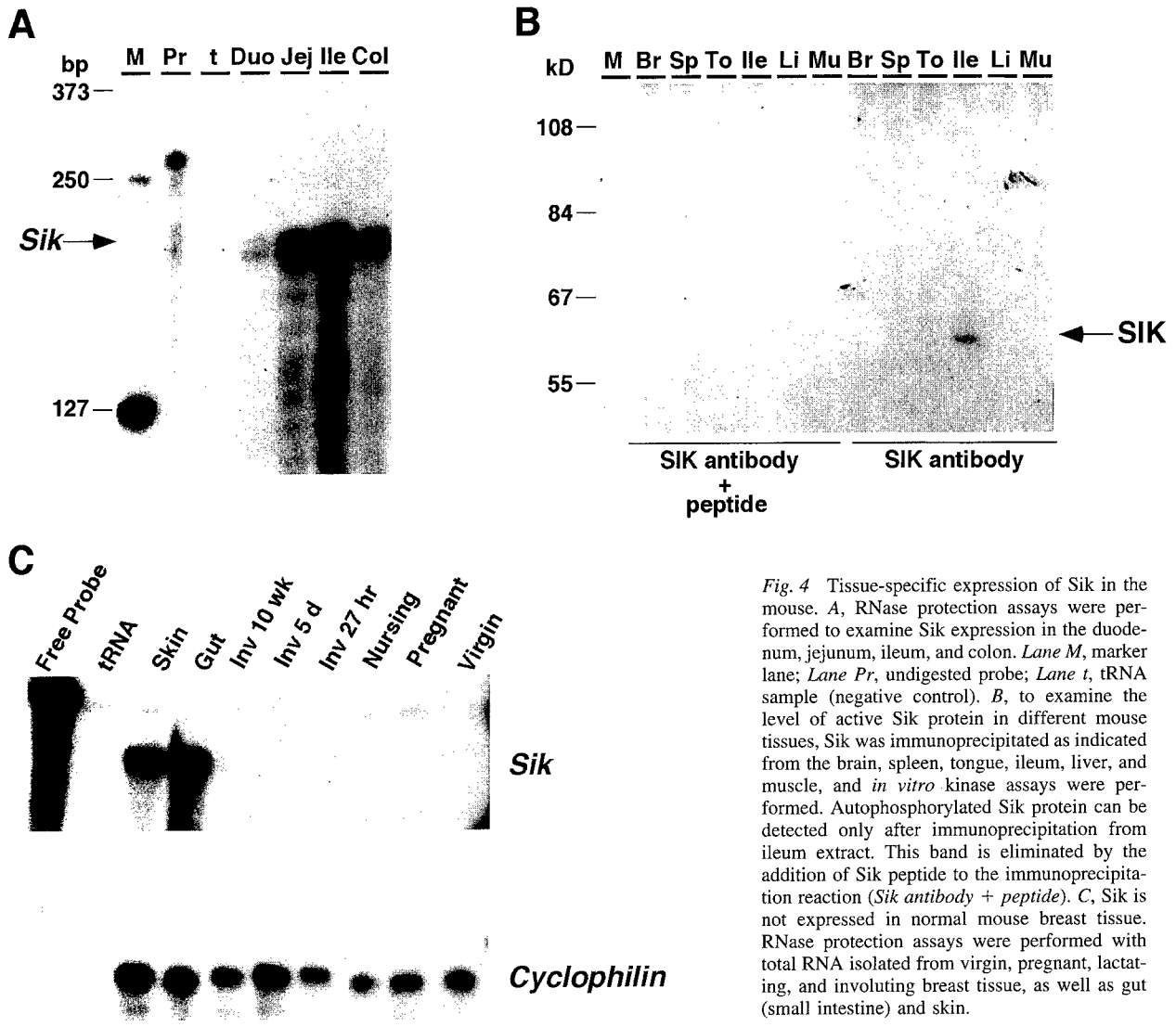


Fig. 4 Tissue-specific expression of Sik in the mouse. *A*, RNase protection assays were performed to examine Sik expression in the duodenum, jejunum, ileum, and colon. *Lane M*, marker lane; *Lane Pr*, undigested probe; *Lane t*, tRNA sample (negative control). *B*, to examine the level of active Sik protein in different mouse tissues, Sik was immunoprecipitated as indicated from the brain, spleen, tongue, ileum, liver, and muscle, and *in vitro* kinase assays were performed. Autophosphorylated Sik protein can be detected only after immunoprecipitation from ileum extract. This band is eliminated by the addition of Sik peptide to the immunoprecipitation reaction (*Sik antibody + peptide*). *C*, 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 gut (small intestine) and skin.

sucrase isomaltase (34). Caco-2 cells are poorly tumorigenic in nude mice when compared with SW480 and HT29 cells (35). Using RNase protection assays, high levels of BRK mRNA were detected in SW480 and HT29 cells (Fig. 5*A*), but low levels were present in Caco-2 and T84 cells. BRK mRNA levels increased 4-fold as Caco-2 cells differentiated, with peak levels appearing at 14 days after plating. This increase in BRK expression levels was detected in different stocks of Caco-2 cells as well as in total and polyadenylated RNAs. Expression of BRK was not detected in the Hep3B liver hepatoma cell line.

Levels of BRK protein were examined by Western blotting. SW480 and HT29 cells express high levels of BRK protein, equal to or exceeding that of the breast tumor cell line MCF-7 (Fig. 5*B*). Although peak levels of BRK mRNA were detected at day 14 after plating in Caco-2 cells, peak levels of BRK protein were found at 7 days after plating, suggesting BRK posttranscriptional regulation. A BRK doublet in the Caco-2 and HT29 cells indicates that a modified form of the protein is also expressed in these cell lines.

BRK Is Expressed in the Normal Human Gastrointestinal Tract and in Colon Tumors. Biopsy samples from esophagus, stomach, duodenum, and colon epithelia were obtained, and total proteins were extracted. The pinch biopsy samples were composed primarily of surface epithelial tissue. Using a rabbit anti-BRK polyclonal antibody, BRK protein expression was detected in all human gastrointestinal tissues that we examined (Fig. 6*A*). Significant levels of Sik expression have also been detected in the epithelium of the mouse fetal stomach (2) and adult stomach and esophagus.[8]

Levels of BRK mRNA expression in five human intestinal tumors were compared with expression in adjacent normal mucosa by RNase protection (Fig. 6*B*). BRK expression in three moderately differentiated colon tumors (*T1, T2*, and *T5*) was 2–3.5 times higher than in adjacent normal tissue (*N1, N2*, and *N5*). No difference in BRK levels was detected

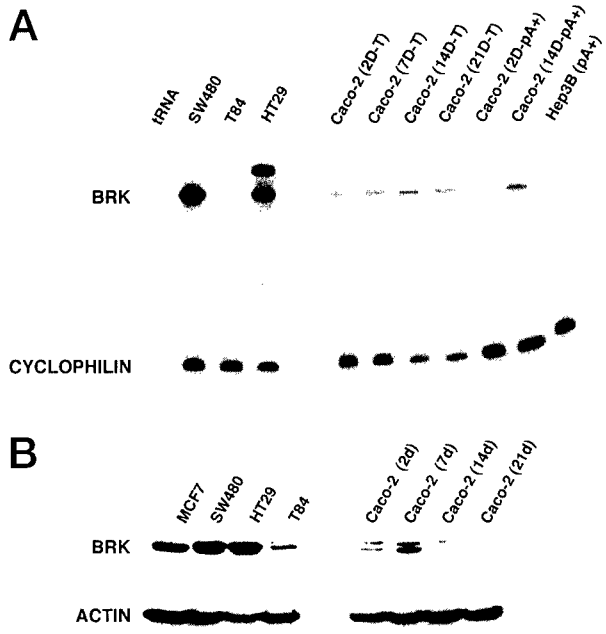


Fig. 5 BRK expression in colon carcinoma cell lines. *A*, RNase protection assays were performed with total RNAs isolated from SW480, T84, HT29, and Caco-2 cells, which were derived from adenocarcinomas of the colon, and the Hep3B hepatoma line. SW480 and HT29 cells express high levels of BRK mRNA. Levels of BRK mRNA increase approximately 4-fold during differentiation of the Caco-2 cell line. Results are shown for total and polyadenylated enriched RNAs from two different stocks of Caco-2 cells. Hep3B cells do not express BRK. *B*, Western blotting was performed with total protein extracts from MCF-7, SW480, HT29, T84, and Caco-2 cells. SW480 and HT-29 cell extracts contain the highest levels of BRK, whereas Caco-2 cells contain too low a level of BRK to be visible in the 10-min exposure at the *left*. In the 5-h exposure at the *right*, BRK protein levels can be seen to increase during early differentiation of Caco-2 cells, but the peak protein expression (7 days after plating) does not correspond with the peak mRNA expression (14 day after plating in *A*). The membrane was stripped and probed with anti-β-actin antibody as a control (exposure, 2 min).

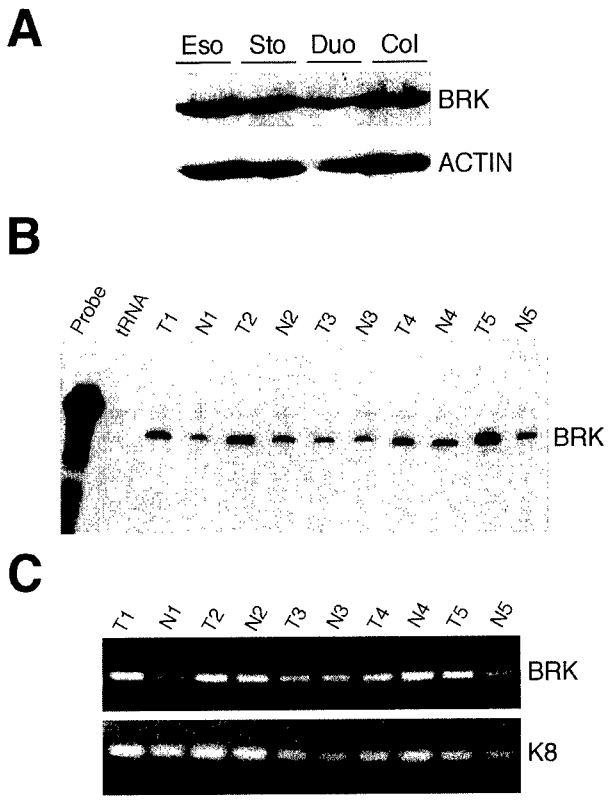


Fig. 6 BRK is expressed throughout the normal human gastrointestinal tract and in colon tumors. *A*, Western blot analysis was performed with total human proteins from pinch biopsies from the esophagus, stomach, duodenum, and colon. Identical filters were incubated with BRK antibody (*top panel*) or with β-actin antibody (*bottom panel*) as a control for protein levels. *B*, RNase protection assays were performed with 10 μg of total RNA from five colon tumor samples and adjacent normal tissue. Increased BRK expression was detected in total RNA from three tumor samples (*T1, T2,* and *T5*) when compared to adjacent normal tissue. *C*, RT-PCR was used to examine BRK and keratin 8 mRNA expression in the five colon tumor samples and adjacent normal tissue. After normalization to the amount of epithelial keratin in each sample, a 2-fold increase in BRK levels was detected in tumors T1 and T5.

in a moderately differentiated rectal tumor (*T3* and *N3*) or in one tubular adenoma from the cecum (*T4* and *N4*). Expression of cyclophilin, our standard control, also increased approximately 2–3-fold in the tumor samples in a pattern very similar to that observed with BRK (data not shown). A 2–3-fold increase in cyclophilin expression in tumor samples has been reported previously (36). Other controls such as β-actin and glyceraldehyde-3-phosphate dehydrogenase also exhibit similar levels of increased expression in tumor samples (37). We performed semiquantitative RT-PCR to examine BRK expression in the paired samples and the expression of keratin 8, an epithelial specific marker expressed in the colon. When levels of BRK were normalized to the amount of keratin 8 expressed, 2-fold increases in BRK expression were observed for tumors T1 and T5 (Fig. 6*C*).

We examined BRK expression in the normal human colon and colon tumors using *in situ* hybridization and immunocytochemistry. BRK mRNA can be detected at the highest levels in the upper crypts in cells that are exiting the cell cycle and undergoing terminal differentiation in the normal colon (Fig. 7*A*). These data coincide with the findings obtained for the murine orthologue Sik (2). Higher levels of hybridization grains were apparent over the disorganized cells of moderately differentiated human colon adenoma (Fig. 7, *C* and *D*, *open arrow*) than in adjacent normal epithelium (*closed arrow*). A higher magnification of a portion of the tumor is shown in Fig. 7, *E* and *F*, which shows variability in the levels of BRK RNA expressed within the tumor.

We examined BRK expression in additional 12 archival colon tumor samples by immunohistochemistry. Several samples stained strongly positive for BRK expression specifically in the epithelial cells of the tumor. A statistical analysis was not possible because some of the samples obtained were of poor quality and did not stain with BRK or various control antibodies, and no conclusion about BRK expression could be made from those samples. BRK expression in epithelial cells of three archival tumors is shown in Fig. 8. BRK protein in the normal colon and in the tumor tissue appears to be primarily cytoplasmic.

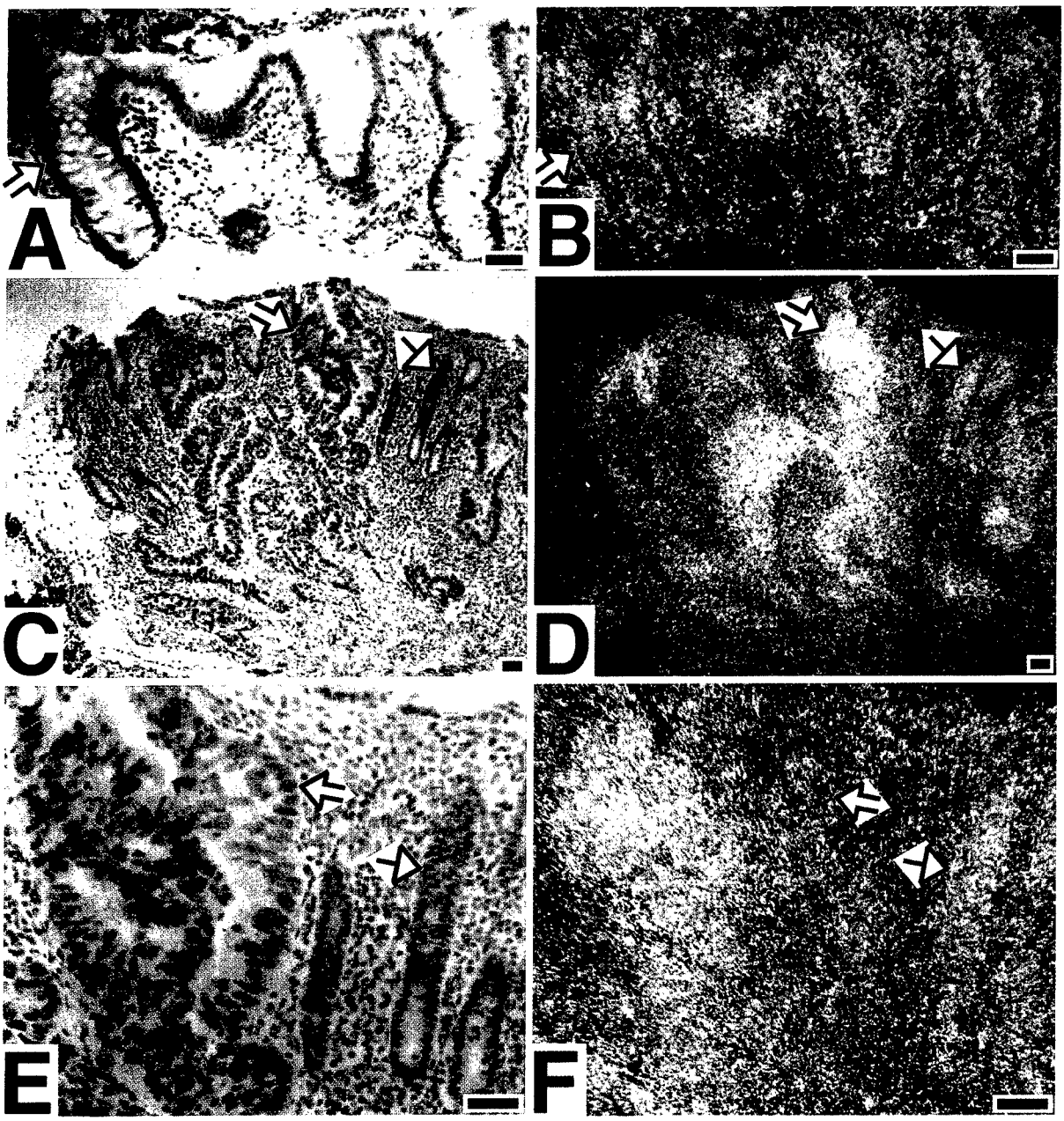


Fig. 7 BRK mRNA is expressed in an epithelial-specific and differentiation-specific manner in normal human colon and at increased levels in a colon tumor. *In situ* hybridizations were performed with ^{35}S-labeled sense and antisense cRNA probes that correspond to a fragment of the BRK cDNA. Bright-field (*A, C,* and *E*) and dark-field (*B, D,* and *F*) views of the tissue and emulsion silver grains are shown. In normal colon (*A* and *B*), epithelium hybridized strongly and specifically with BRK probe, particularly at and above the cuff region at which colonocytes undergo terminal differentiation (*arrow*). In colon adenocarcinoma (*C* and *D*), BRK signal is present in normal colonic crypts (*closed arrow*) and transformed tissue (*open arrow*). At high magnification (*E* and *F*), Brk signal over the upper crypt (*closed arrow*) appears to exceed that of some regions of the tumor (*open arrow*), but is itself exceeded by the strong signal present in a large portion of the main tumor mass. The sense probe hybridization produced no signal over background (data not shown). *Bars*, 50 μm.

DISCUSSION

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, in which 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 a 45% homology and six of seven introns with Dsrc41, a Src-like gene from *Drosophila*. Nonetheless, BRK is highly diverged, with nearly equivalent homology to the proto-oncogenes

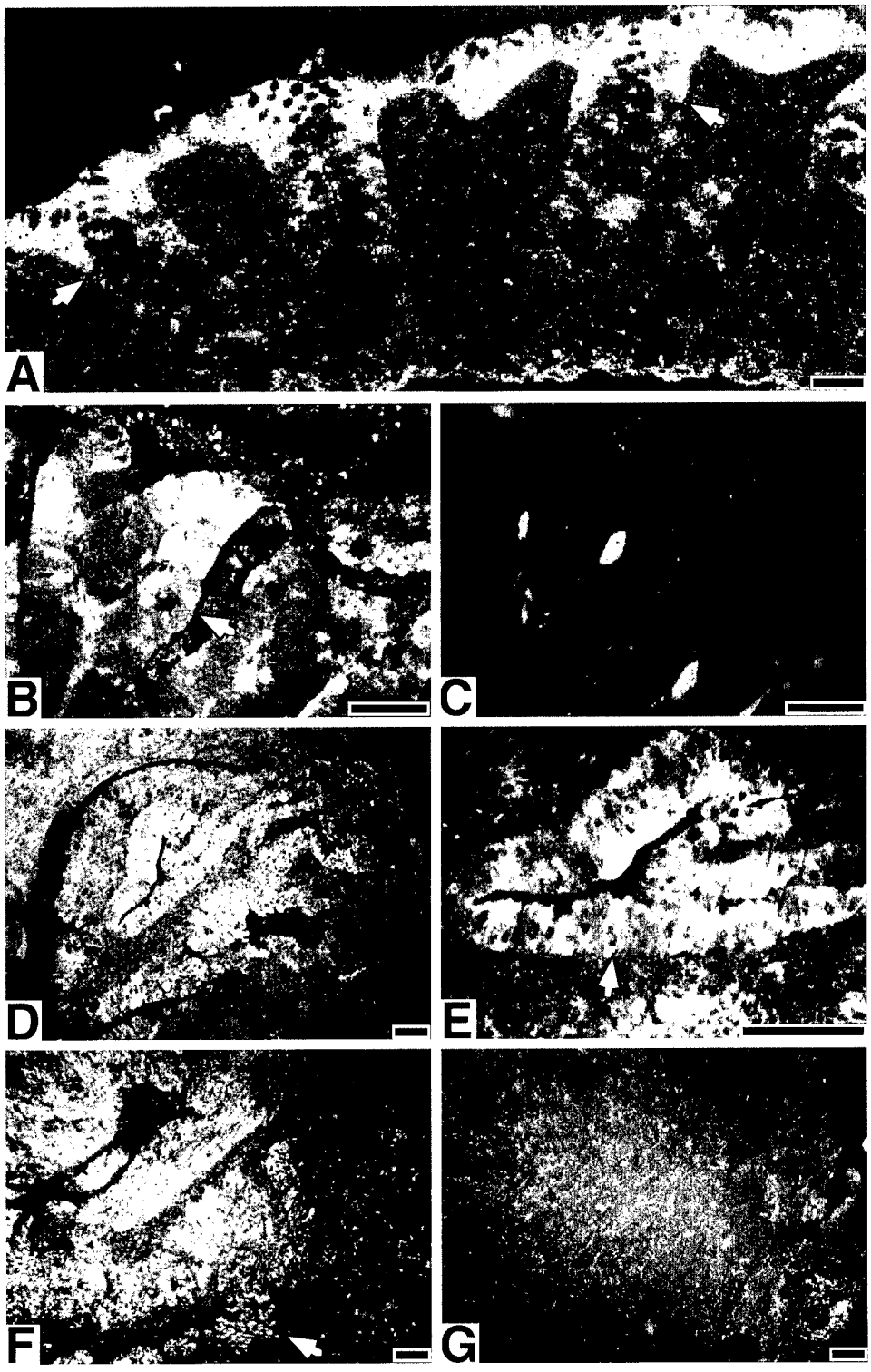


Fig. 8 Immunohistochemical detection of BRK expression in normal human colon tissue and archival human colon tumor samples. *A*, in the normal human colon, BRK protein expression is induced in the upper crypt epithelium (*arrows*) and is present in the surface epithelium. BRK is also expressed in epithelial cells of three different colon tumors (*B, D, E,* and *F*). Specificity is indicated by the lack of signal in the controls in which BRK antibody was preincubated with the synthetic peptide used for immunization (*C* and *G*); similar control results were obtained for the samples shown in *A* and *D* (data not shown). *Arrows* in *B* indicate a transition between high and low levels of BRK in a villus-like region of colon adenocarcinoma, one of several such regions in this tumor; *E,* two isolated BRK-expressing cells in a monolayer with many transitions in BRK status (this disorganized monolayer represents part of a large adenocarcinoma that does not contain detectable BRK in most regions); *F*, a transition between sporadic and widespread BRK expression with a granule-specific intracellular distribution within a disorganized colon adenocarcinoma. *Bars*, 50 μm.

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 a 44–45% protein identity. The *BRK* gene has only two intron boundaries conserved with the SRC family members (38, 39), further suggesting that BRK and Sik represent a distinct family of nonreceptor tyrosine kinases.

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% amino acid 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. 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 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.

Because BRK was initially cloned from metastatic breast tumor RNA, we examined its expression during breast development in the mouse to determine whether it plays a role in normal differentiation. 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, has suggested a role for BRK expression in carcinomas. BRK was found to be expressed at appreciable levels in approximately two-thirds of the breast tumors examined (5). Overexpression of BRK in the HB4a human mammary cell line mitogenically sensitizes these cells to epidermal growth factor. 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 (40).

We determined that BRK is expressed in tumor cell lines derived from adenocarcinomas of the colon. Using different methodologies, we also detected moderate increases in BRK RNA and protein expression in primary human colon tumor samples. Whereas the BRK colon tumor data are only of preliminary statistical significance, they consistently favor the notion of a modest increase in BRK in colon tumor tissue, which falls short of the more dramatic induction reported for breast carcinomas. The increase detected in colon tumors may not be related to factors such as gene amplification, which would be expected to yield larger increases. The activity of BRK in colon tumors has not been examined, and it is possible that increases in BRK expression do not reflect increased kinase activity. Mutations of the *BRK* gene in tumors have not been reported. The sequence of BRK isolated from tumor cells and normal cells appears to be identical thus far (38, 39), suggesting that BRK overexpressed in tumor cells is the normal protein.

Several studies suggest that the related SRC family tyrosine kinases participate in the development of colon cancer. SRC tyrosine kinase activity was found to be increased in human colon tumor tissue and in a variety of colon carcinoma cell lines when compared with normal adjacent tissues and normal colonic epithelial cells (41–44). SRC activity was found to progressively increase as adenomas become carcinomas, and the highest levels of SRC activity were found in metastatic lesions in the liver (45). Recently, an activating mutation in SRC codon 531 was identified in 12% of advanced colon tumors examined, providing the first genetic evidence for a role for SRC in colon cancer (46). A significant increase in the activity of YES has also been observed in colon tumors and in colon carcinoma cell lines (47, 48), although no increase in the activities of some other SRC family members such as LCK, FYN, HCK, or FGR was detected (47).

We found that BRK is present throughout the normal human gastrointestinal tract, in the esophagus, stomach, duodenum, and colon. 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. We also found that BRK expression increased during the early stages of Caco-2 cell differentiation *in vitro*. These data support the hypothesis that BRK may play a role in a signal transduction pathway associated with differentiation. In previous studies, we found that mouse Sik is expressed in a differentiation-specific manner in regenerating epithelial linings. Sik was shown to associate with a GAP-binding protein, possibly linking it to the Ras pathway. It will be important to determine the role of this epithelial-specific tyrosine kinase during normal differentiation and to unveil its potential relationship to the development of breast and colon cancers.

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Ryk is expressed in a differentiation-specific manner in epithelial tissues and is strongly induced in decidualizing uterine stroma

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Ryk is a ubiquitously expressed tyrosine kinase-like receptor of unknown activity and associations. We examined ryk expression in adult mouse epithelial tissues and during embryonic development at the histological level. Ryk RNA is present at greatly increased levels in cells at particular stages of epithelial differentiation: the basal layer of skin and tongue epithelia, the intervillous layer and some crypt bases of the intestine and the lower matrix region of the hair follicle. Although ryk RNA is expressed at similar levels in a variety of tissues from embryonic day 10.5 to 18.5, specific induction of ryk RNA can be seen by *in situ* hybridization in the basal layer of skin and hair follicle at day 15.5–16.5, and protein staining localizes to the hair follicle by immunohistochemistry. At day 4.5 and 6.5, little if any ryk is present in the blastocyst, but it is transiently induced at a high level in mature decidual cells of the uterine stroma. We review a number of independent isolations of ryk, including fruit fly and nematode members of the ryk family. Because ryk is induced in epithelial cells seeking a final place in a differentiated tissue, or during remodeling of the endometrium, and a homologous gene, derailed, is known to regulate muscle and nerve target seeking in *Drosophila*, ryk may also be involved in cellular recognition of appropriate context.

Keywords: Ryk; tyrosine kinase; differentiation; epithelia

Introduction

Ryk, a molecule related to receptor tyrosine kinases, is expressed widely and at relatively high levels in comparison with other tyrosine kinase family members. Its widespread expression has led to its cloning by PCR-based strategies (Wilks, 1989) from the K-562 chronic myelogenous leukemia cell line (clone 'JTK5', Partanen *et al.*, 1990); day 17.5 fetal mouse cerebellum and limb bud (clone 'PTK1', Stark *et al.*, 1991); a peritoneal macrophage cDNA library (Hovens *et al.*, 1992); cell line T10, a subclone of the murine plasmacytoma T1165 ('nyk-r', Paul *et al.*, 1992); the NB41 neuroblastoma cell line ('ntbk-1', Maminta *et al.*, 1992); 12.5 day post-coitum mouse placenta ('vik', Kelman *et al.*, 1993); highly enriched erythroid progenitors ('MRK', Yee *et al.*, 1993); hematopoietic stem cells (clone 'stk-4', Iwama *et al.*, 1994); intestinal crypt epithelium (Siyanova *et al.*, 1994); day 8 fetal yolk sac and day 14 fetal liver (clone '9B4', Larsson-Blomberg and Dzierzak, 1994); low density bone marrow cells (Simoneaux *et al.*, 1995); E-5 mouse thymus medullary epithelial cells (Potworowski and Beauchemin, 1996); the SKOV-3 human epithelial ovarian cancer cell line (Wang *et al.*, 1996); a primitive neuroectodermal tumor (Weiner *et al.*, 1996); and perinatal developing kidney (Kee *et al.*, 1997).

As a result of these independent isolations, the tissue distribution of ryk at the RNA level has been extensively studied (Table 1). Ryk has been detected in almost every tissue examined. Among cell lines, expression is more variable, with reports of a variety of nonexpressing and overexpressing cell lines of many tissue types (Partanen *et al.*, 1990, Paul *et al.*, 1992, Maminta *et al.*, 1992, Yee *et al.*, 1993, Larsson-Blomberg and Dzierzak, 1994, Wang *et al.*, 1996). Expression in neuroectodermal tumors does not appear to be correlated with progression of disease or c-myc amplification (Maminta *et al.*, 1992), but in stroma and epithelium of ovarian tumors ryk expression increases with tumor progression (Wang *et al.*, 1996).

Comparison of six mouse ryk sequences verifies that the nyk-r sequence (Paul *et al.*, 1992; Genbank accession L02210) agrees with the consensus throughout the entire region in which three or more homologous sequences can be compared (nearly the entire length), and hence can be regarded as definitive, the sole exception being the 'TA' at bp 2125–2126 of the published sequence, which is absent in two other publications of the same region. The MRK sequence (Yee *et al.*, 1993) extends another 31 bp in the 5′ direction, and has been shown by primer extension to include all but 51 base pairs at the 5′ end of the gene. Mouse ryk RNA hybridizations have given two transcripts of equal intensity in all tissues examined, and two bands are seen in rat intestinal mRNA (Park and Tyner, unpublished data), but human ryk has only one strong transcript of 3.0–3.4 kb (Wang *et al.*, 1996, Tamagnone *et al.*, 1993). Mouse transcripts with five different polyadenylation sites have been reported (Paul *et al.*, 1992, Kelman *et al.*, 1993, Yee *et al.*, 1993, Simoneaux *et al.*, 1995), as well as an alternately spliced transcript which diverges from the others only 4 bp after the stop codon (Simoneaux *et al.*, 1995). The human Ryk protein is 93% identical to mouse Ryk (564/610 amino acids), and surprisingly the 3′ noncoding sequences for mouse and human are also highly conserved, with 78% (436/557) nucleotide identity after the stop codon.

Discussions of ryk homologies have concluded that ryk is a highly diverged member of the receptor tyrosine kinase family. Alignment using the PCR-

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Table 1 Compilation of Ryk Northern blot hybridization data

Ryk expression	++	+	−
8d yolk sac			F[1]
8d embryo			F
9d, 10d, 11d yolk sac		F	
9d, 10d, 11d embryo		F	
13d embryo	A		
14d embryo		B	
14d fetal brain		B	
12d, 13d, 14d fetal liver		BF	
12d, 13d, 19d placenta	AC		
placenta [term]	***GH***[2]		
ovary	AE		
testis		ACDE	
uterus	E	F	
tongue		E	
salivary gland		A	
stomach		D	
duodenum		E	
intestine		D	
pancreas		***GH***	
liver		ABCDEF***GH***	
bone marrow		BF[3]	
thymus		AF	
spleen	E	AD	F
lymph node		F	
lung	ADE***G***	CF***H***	
kidney	DE***H***	ACF***G***	
skeletal muscle	C***GH***	ADF	
heart	D***G***	ACFE***H***	
brain	E	ACF***GH***	
eye		C	

A: Hovens, 1992; B: Paul, 1992; C: Kelman, 1993; D: Yee, 1993; E: Siyanova, 1994[4]; F: Larsson-Blumberg, 1994[5]; G: Tamagnone, 1993; H: Wang, 1996. Categorization of expression levels is based on comparison of the data in the original articles, which must be consulted for more precise comparisons. Italicized references (G, H) represent experiments with human probe and RNA samples; otherwise, the experiments used mouse probe and tissue. Notes: (1) ryk was cloned from this tissue but could not be detected by Northern hybridization. (2) Additional, higher molecular weight transcripts were observed (Wang, 1996). (3) Paul (1992) tested bone marrow after treatment with 5-fluorouracil. (4) Siyanova *et al.* (1994) measured expression by RNase protection of total RNA samples. (5) Larsson-Blomberg (1994) **(Larsson-Blomberg and Dzierzak, 1994) includes tissue results in + or − format

amplified fragment (Partanen *et al.*, 1990) placed the human ryk clone's divergence at or before the divergence of met, sea, igf1r, insulin receptor, trk, ros and sevenless. A subsequent alignment of the complete catalytic domain (Stacker *et al.*, 1993) indicates a later divergence of ryk and met; nonetheless, many characteristics of kinases other than met have been proposed for ryk. While the catalytic domain resembles met and v-sea, weak homology has been proposed between a fragment of the extracellular domain and trk (Maminta *et al.*, 1992). Two other kinase-related molecules, klg and CCK4, define a novel subclass of the receptor tyrosine kinases with weak (39% for klg) homology to v-sea (Chou and Hayman, 1991, Mossie *et al.*, 1995). These kinases, like ryk, are mutated in the GXGXXG and DFG motifs and lack kinase activity in standard assays; however, their sequence and organization, with seven extracellular Ig repeats, bear no exceptional similarity with ryk. Kelman *et al.* (1993) find homology and a potential structural similarity between vik and the insulin receptor: a sequence KRRK, found between the only two cysteines in the mouse sequence, is reminiscent of the proteolytic cleavage site between alpha and beta chains of insulin receptor precursor. All six residues are conserved between mouse and human sequences. Tamagnone *et al.* (1993) propose that human ryk resembles ros and sevenless in spanning the plasma membrane twice, with the 5′ end remaining in the cytoplasm. Alternatively, a downstream CTG could be used for initiation.

Because ryk contains divergent motifs, several groups have sought to measure its catalytic activity. Immunoprecipitation of Ryk from Triton-100 extracts of mouse NIH3T3 cells, followed by *in vitro* kinase reaction, yields several potassium hydroxide resistant labeled bands, yet phosphoamino acid analysis indicates that they represent phosphoserine and phosphothreonine, suggesting that they are produced by an associated serine-threonine kinase (Hovens *et al.*, 1992). Fusion protein containing the intracellular domain of Ryk fails to phosphorylate poly (Glu, Tyr) or acid-denatured enolase *in vitro*, nor does it cause detectable tyrosine phosphorylation when expressed in bacteria. Immunoprecipitation and autophosphorylation of human ryk using several antisera also failed to demonstrate tyrosine kinase activity (Wang *et al.*, 1996, Stacker *et al.*, 1993).

Comparison of Ryk immunoprecipitations by several groups indicates a strong degree of posttranslational modification. *In vitro* transcription and translation of human Ryk with T7 polymerase and reticulocyte lysates demonstrates an approximately 70 kD protein product, predicted by sequence (Stacker *et al.*, 1993). Expression of cDNA constructs in COS cells yields a single 85 kD band (Yee *et al.*, 1993). Immunoprecipitation of human ryk from MCF-7 cells (Stacker *et al.*, 1993) or mouse ryk from NIH3T3 cells (Hovens *et al.*, 1992) yield approximately 90 kD products, while immunoprecipitation of human ryk from CHO cells yields a 100 kD product and from 41 M cells yields two bands around 116 kD (Wang *et al.*, 1996). Additionally, bands can be seen at 35–45 kD in each case, possibly indicating a cleavage fragment. The substantial increase in Ryk protein size likely reflects the presence of five predicted N-linked glycosylation sites in the extracellular segments of mouse and human Ryk (Paul *et al.*, 1992, Stacker *et al.*, 1993).

Despite these data, Ryk remains a ubiquitous, orphan receptor of unknown catalytic activity, associations, and biological function. In this paper we present a survey of its expression on the RNA and protein levels during embryonic development and in adult epithelial tissues, seeking to infer a function from the details of its distribution.

Results

Ryk expression occurs in a differentiation-specific manner in several epithelia

We initially cloned ryk as one of nine tyrosine kinases amplified from cDNA prepared from a crypt-enriched epithelial fraction of the mouse small intestine (Siyanova *et al.*, 1994). In the adult small intestine, the stem cells and a rapidly dividing transit population located within the crypts continually repopulate the epithelium, producing differentiated enterocytes, goblet cells and enteroendocrine cells which emerge and

migrate along the villi which extend into the lumen, as well as the Paneth cells which migrate to the base of the crypts (Cheng and Leblond, 1974). Examination of the distribution of ryk expression by *in situ* hybridization indicates that the RNA is expressed most highly in the lower villi (Figure 1a and b), but intriguingly, there is also a patchy distribution of positively expressing crypts (Figure 1c–d). This may represent expression in small groups of Paneth cells or precursors which are missed in the plane of section of some crypts, but which participate in general differentiation-associated ryk expression.

Other adult epithelial tissues yielded a variety of striking expression patterns in which epithelial cells of particular differentiation states produce far more ryk mRNA than the remainder of the tissue. However, ryk expression in other epithelia does not match the more advanced state of differentiation of the intestinal cells, but instead coincides with replicating populations. In the adult mouse tongue (Figure 2a–d), ryk expression localizes to the basal layer of the stratified squamous epithelium, where the dividing cells are located, giving rise to upward-migrating layers of differentiated cells, and also to the columns of proliferating cells which give rise to the filiform papillae of the upper surface of the tongue (Figure 2a and b) (Hume and Potten, 1976). In the hair follicle, stem cells are present at the bulge region of the outer root sheath, and produce a transit amplifying population which moves downward to the base of the bulb, where the cells contact the dermal papilla and form the layers of hair, cuticle, and inner root sheath which subsequently move upward with hair growth (Rochat *et al.*, 1994). Ryk is present specifically at the base of the bulb, and appears absent from the upper region of the bulb, where cells of both lesser and greater degrees of differentiation are present (Figure 2e and f).

Non-epithelial structures, such as the muscles and arteries of the tongue, or subcutaneous fat of the skin, consistently hybridized much more weakly and evenly than the epithelial structures. Although the studies cited in Table 1 suggest that ryk is expressed at equal or higher levels in skeletal or cardiac muscle as in duodenum and intestine, *in situ* hybridization stresses epithelial expression. An explanation for this is suggested by tongue hybridization (Figure 2c and d). Expression of ryk in the muscle at top is a fraction of that present in the basal epithelium, but the high-expressing basal layer is only one cell thick, whereas the entire mass of muscle expresses homogenously. While the filiform papillae and hair follicles express higher levels, they represent only a small fraction of the overall tissue mass.

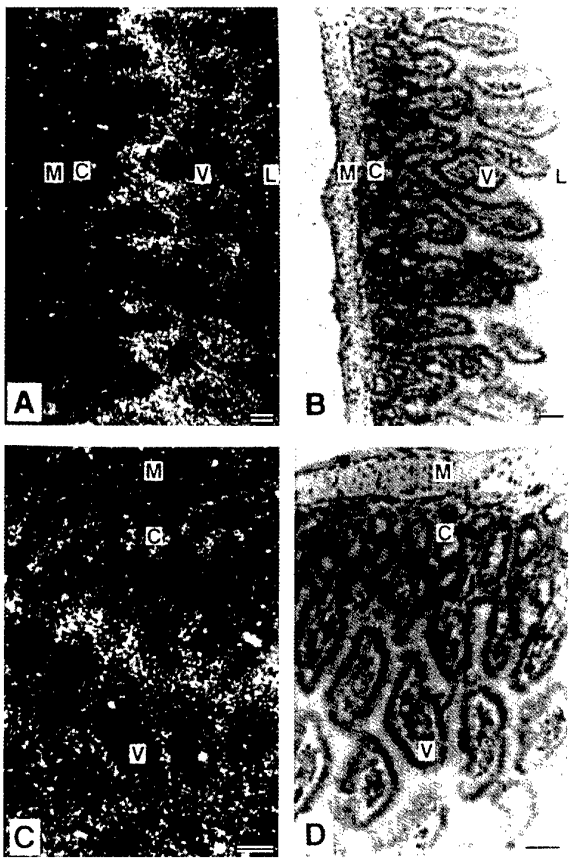

Figure 1 *In situ* hybridization of ryk cRNA probe indicates differentiation stage specific expression in adult mouse small intestine. Autoradiography of hybridized probe yields reflecting silver grains seen in darkfield views (**a,c**). In brightfield views of the same tissue sections (**b,d**), hematoxylin and eosin staining is visible. Most regions (**a,b**) include the predominant intervillus epithelium localization, while some regions (**c,d**) emphasize a sporadically visible hybridization at the base of the crypts. M, muscle; C, crypt; V, villus. L, intestinal lumen. All bars represent 50 micrometers

Ryk expression occurs throughout embryonic development, and at high levels in the primary decidual zone of uterine stroma

Highest levels of ryk expression were generally observed in structures that differentiate late in embryonic development from simple monolayers. However, when we examined ryk levels by RNase protection in total RNA extracts from a range of embryonic time points from day 10.5–18.5, and from day 18.5 intestine (Figure 3), overall ryk expression varied little. As in the adult, it appears that the uniform overall expression of ryk overwhelms the effect of specific induction in the epithelium.

When RNA was examined *in situ* at embryonic day 16.5, hair follicles and the basal layer of the developing skin produce a strong signal in a pattern similar to that seen in adults (Figure 4a and b). In the tail of a day 15.5 embryo, basal localization of ryk in the newly stratifying skin appears only slightly enhanced and ryk expression is uniformly present at approximately equal levels in most tissues other than developing bone (Figure 4c and d). At day 9.5 and 11.5 (data not shown), a strong, homogenous hybridization occurs throughout embryonic and extraembryonic fetally derived tissues, while maternal tissues can be distinguished by a lower level of ryk expression.

Immunohistochemistry of the day 16 embryo confirms that the high level of ryk RNA expression in newly developing hair follicles of the whisker pads translates into a strong protein localization (Figure 5). The protocol used here was unable to detect Ryk staining in other embryonic structures, but yielded a specific signal in hair follicles that could be eliminated

through preincubation of the antibody with control peptide (data not shown).

At embryonic day 4.5, some ryk mRNA appeared to be present in the inner cell mass (Figure 6c–f), a result consistent with previous detection of ryk in embryonic stem cells (Yee *et al.*, 1993), but this is overshadowed by a dramatic pattern of induction surrounding the blastocyst, corresponding to the decidualization of maternal uterine stroma in response to implantation (Figure 6a and b). Within this region, induction appears to be sharply delimited at a point within the predecidual region, and increases to a maximum in the most strongly differentiated deciduocytes near the embryo. By contrast, ryk is much more weakly expressed in the uterine epithelial monolayer immediately surrounding the blastocyst, which undergoes apoptosis and becomes detached by the evening of day 4.5 post-coitus, as well as in the region of primary decidual epithelium immediately adjacent to the embryo, which is fated to undergo subsequent involution and phagocytosis by trophoblastic giant cells (Abrahamsohn and Zorn, 1993). In regions of the uterus far from an implanted blastocyst, there appeared to be low, uniform induction of ryk in the uterine epithelium and adjacent uterine stroma. Ryk induction continues through day 6.5, surrounding the embryo in an asymmetric manner reflecting the antimesometrial progression of decidualization (Figure 7a and b). Weaker induction is visible in the surrounding region, except in the uterine glands. The epithelium of the uterine glands and the uterine lumen, as well as a region located mesometrially to the embryo, express very little if any ryk. Because a central region of this tissue corresponding to the secondary decidual zone no longer expresses ryk as it had at day 4.5, and maternal induction was not detected at later time points, the strong ryk expression appears to persist for approximately two days in individual stromal cells, presumably moving outward from the embryo in association with the induction of

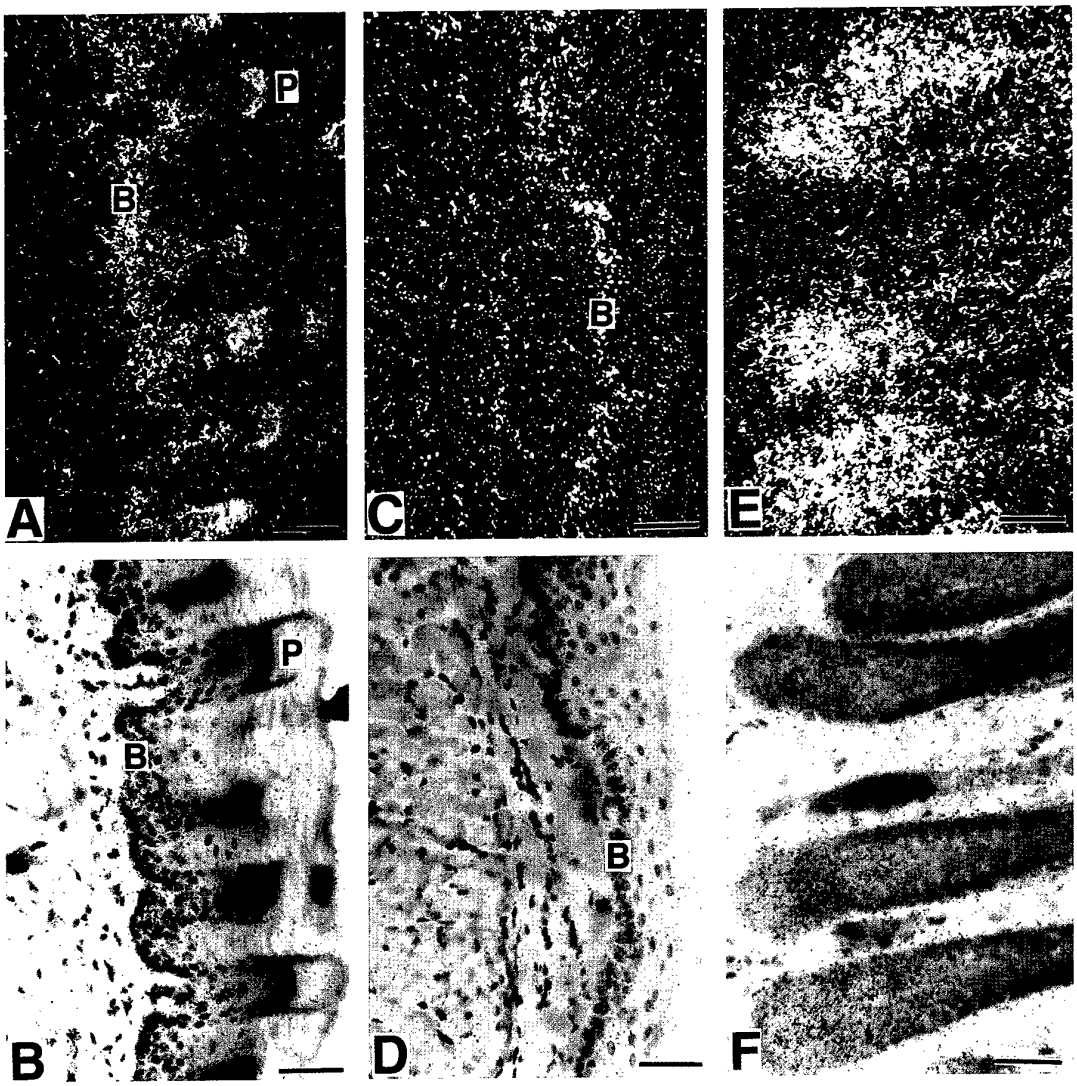

Figure 2 *In situ* hybridization indicates specific ryk RNA expression in the basal layer and replicating papillar columns of the dorsal tongue (**a,b**), the basal layer of the ventral tongue (**c,d**) and the lower, dividing bulb region of the hair follicle (ELF). B, basal layer; P, filiform papilla. Yellow autofluorescence seen in the strongly eosinophilic trichohyaline-containing regions of the filiform papillae (**a,b**) should be ignored. All bars represent 50 micrometers

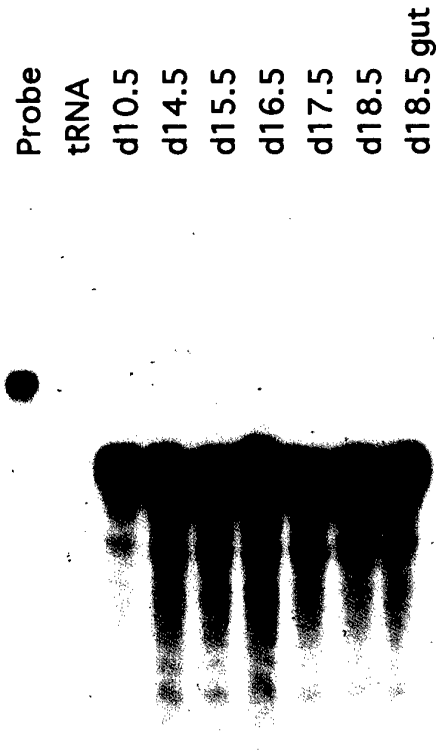

Figure 3 Ryk RNA levels vary little during late embryonic development. Total RNA from embryos at times post-conception, or intestine dissected from day 18.5 fetuses, show high, nearly equal levels of ryk hybridization by RNase protection assay. Interestingly, although intestinal ryk expression shows regions of strong specificity, the total level does not differ from that in the embryo as a whole

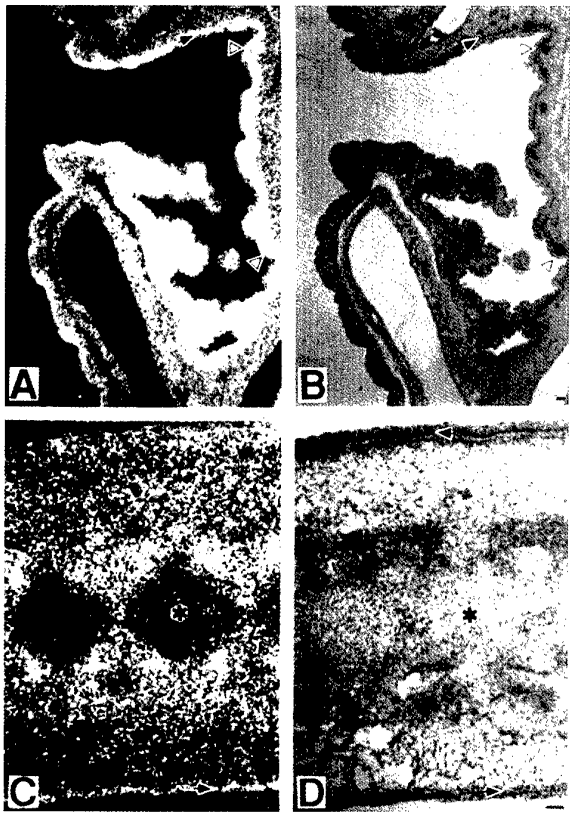

Figure 4 Ryk expression patterns reach peak levels in basal epithelia in an oblique section of paraffin-embedded day 16.5 post-coitus embryonic skin epithelium (**a,b**), and of a cross-section of frozen day 15.5 embryonic tail (**c,d**). In (**a,b**), solid arrows mark the basal layer, which shows stronger hybridization throughout the section, while outlined white arrowheads mark two of the developing hair follicles. In (**c–d**), developing tail is marked with solid arrows at the basal epithelial layer, while an asterisk indicates the nonhybridizing bone tissue. All bars represent 50 micrometers

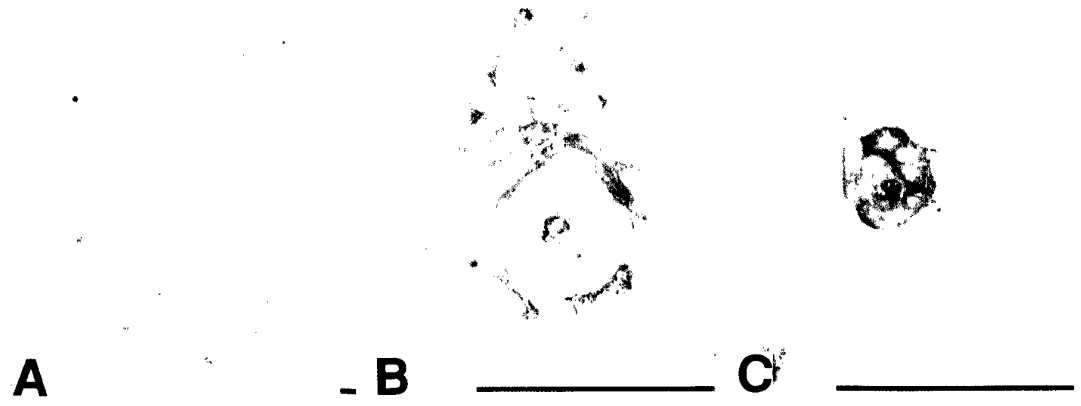

Figure 5 Immunohistochemistry with ryk antibody in day 16 embryo shows strong staining only in the developing hair follicles. A low magnification view of the embryonic whisker pad (**a**) and two higher magnification views of individual follicles (**b,c**) show that ryk is present with membrane localization, peaking in an intermediate layer of cells in the follicle and increasing with approach to the surface. All bars represent 50 micrometers

decidualization, and ceasing expression in the decidуocytes which will subsequently undergo apoptosis.

Discussion

Every organ and tissue type examined appears to express ryk at some stage of differentiation and ryk is expressed continuously throughout the development of the embryo. In this study, we examined epithelial ryk expression in more detail, and show that there is a clear pattern of positive regulation of ryk transcription in cells at some stage of differentiation, with subsequent shut-off of the gene in many of the most differentiated cell types. Our data is complemented in this regard by another *in situ* panel (Wang *et al.*, 1996) in which human ryk was localized to epithelium and stroma of the colon, mammary epithelium, epithelium of developing alveoli of the fetal lung and at lower levels in airway epithelium and the cuff of smooth muscles, and specifically in the neogenic zone of the cortex of the fetal kidney. Most interestingly, while ryk was found to be absent from normal ovary, it is expressed at low levels in the stroma of benign or borderline ovarian tumors, and becomes most strongly expressed in epithelial tissue of malignant ovarian tumors (Wang *et al.*, 1996). Taken together, these data and ours indicate a fundamental relationship between epithelial growth or differentiation processes and regulation of the ryk promoter.

Expression of ryk in the female reproductive tract is of special interest due to its inducibility in proximity to the implanting embryo and in advanced ovarian cancer. Ryk generally is expressed at high levels in mouse placenta, ovary, and uterus (Table 1), and is induced surrounding the embryo at d4.5 and d6.5. In independent experiments using Clontech human Multiple Tissue Northerns, placental ryk signal appeared once at a moderate level with the usual transcript size (Wang *et al.*, 1996), and once at an extremely high relative level with high-molecular-weight transcripts

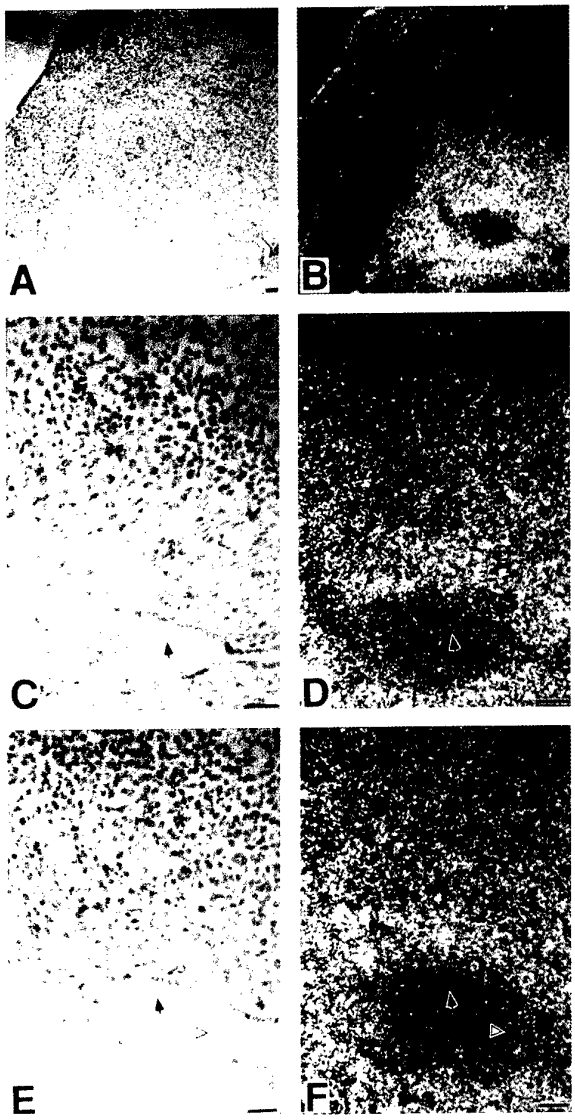

Figure 6 *In situ* hybridization of day 4.5 blastocyst and surrounding placenta reveals a strong maternal induction in decidualizing stromal cells surrounding the embryo. Low magnification (**a,b**) and high magnification (**c,d**) views of one section show hybridization in the mature decidual cells of the uterus, which are recognizable by their larger nuclei, and also in some of the predecidual cells surrounding the mature decidua, which have larger extracellular spaces. The uterine epithelium, and the irregular, smaller cells of the primary decidual zone, appear to express very little ryk. The solid arrow in (**c–d**) marks the trophectoderm, present as a ring within the ring of nonexpressing uterine epithelial cells. The trophectoderm is disrupted in a nearby section (**e–f**), but the positively expressing inner cell mass (outlined arrow) is visible in this section at the mesometrial end of the blastocyst. An intervening sense control section was used to help reconstruct the morphology (data not shown). All bars represent 50 micrometers

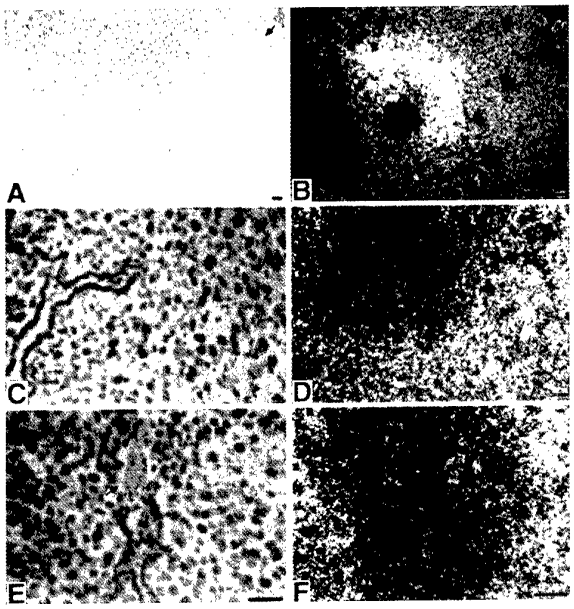

Figure 7 *In situ* hybridization of day 6.5 embryo and placenta shows continuing induction of ryk surrounding the embryo. In a low magnification view (**a,b**) the asymmetry of expression associated with the decidualizing antimesometrial stroma is evident. Scattered glands and a region immediately surrounding the embryo have little or no expression. The region surrounding the embryo is shown at greater magnification (**c,d**). In an adjacent section where trophectodermal tissue is present (**e,f**), there is little observable signal in the embryonic tissue

(Tamagnone *et al.*, 1993), suggesting that human ryk may also be strongly induced in maternal endometrium in contact with the placenta. Interestingly, another tyrosine kinase, neu/*erb*B2, an EGF receptor subunit whose overexpression correlates with poor prognosis in breast and ovarian cancers, has been shown to be induced in decidualizing stromal cells beginning with day 4.5 (Lim *et al.*, 1997), in a pathway which may relate to the release of TGF-alpha by blastocyst and uterine epithelium during implantation (Dey, 1996). Activation of these signal transduction pathways may reflect a general shift of the stromal cells from a fibroblast-like morphology to a more epithelioid character during decidual differentiation, with greater development of granular endoplasmic reticula, Golgi profiles and vesicles, abundant intermediate filaments and adherens junctions, and accumulation of glycogen and lipids (Abrahamsohn and Zorn, 1993, Welsh and Enders, 1985).

Some comparison may also be made between expression in epithelial differentiation and hematopoietic differentiation. Ryk is present in cells selected for immunofluorescence staining to antibodies for CD4, CD8, B220, GR-1, or Mac1, but is expressed at 1000-fold lower levels in the undifferentiated population sorted by lack of staining with these markers (Simoneaux *et al.*, 1995). Likewise, ryk could not be detected in B6SutA, FDCP1, or BAF3 cells, which have multipotential hematopoietic qualities, but was present in 11 of 14 of differentiated hematopoietic cell lines tested (Yee *et al.*, 1993). Hematopoietic cells arise from the yolk sac and liver, endodermal tissues associated with the primitive gut and continually regenerate from a stem cell population in the adult. Interestingly, the intestine and colon are also endodermally derived tissues in which ryk appears to be expressed at a later stage of differentiation. In all of the epithelia examined, ryk is never specifically localized only to stem cells or to the most differentiated or quiescent cells, but varies in expression preference for amplifying or terminally differentiated portions of particular regenerating tissues.

One explanation for the epithelial expression pattern can be made by analogy with the highest-scoring ryk homologs with Gapped BLAST (Altschul *et al.*, 1997). These are the Drosophila proteins doughnut (dnt) and derailed (drl), and the *C. elegans* predicted ORF C16B8.1, for each of which there is data suggesting a potential role in controlling specific cell-surface interactions recognizing an appropriate location within the organism. Each has weak but significant homology to ryk in the extracellular domain, a similarly placed transmembrane domain, and a tyrosine kinase-like catalytic domain which conserves some unique sequences more strongly than the invariant motifs for tyrosine kinases (Figure 8). Alignment of human ryk with Gapped BLAST yields highest scores and indicates significant extracellular domain homologies for mouse ryk, doughnut, derailed, and C16B8.1, which are 93, 36, 34, and 29% identical overall and 91, 33, 24, and 22% identical in the extracellular domain, respectively. The next matches

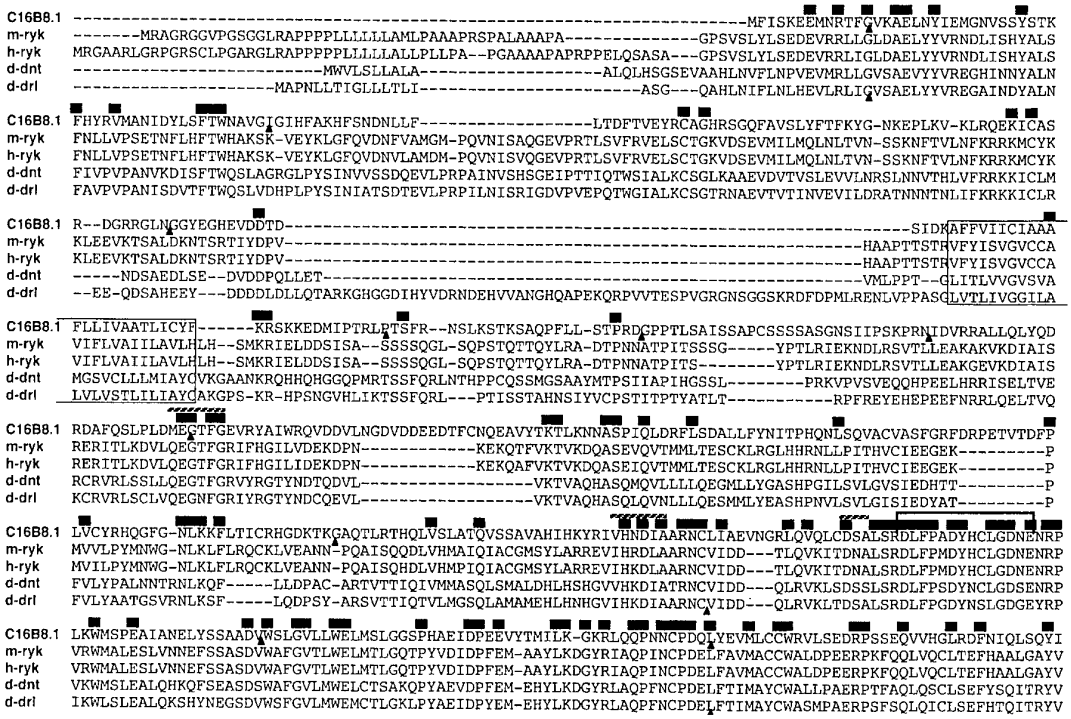

Figure 8 Alignment of ryk family members. *C. elegans* predicted ORF C16B8.1, mouse ryk, human ryk and Drosophila doughnut (dnt) and derailed (drl) are shown. Conserved amino acids are indicated by black boxes, and the conserved transmembrane domain is boxed. Arrowheads indicate splice junctions in the genomic sequences of C16B8.1 and derailed. Narrow shaded bars indicate the normally invariant tyrosine kinase motifs GXGXXG, VHRDLA and DFG, which are altered in a small group of tyrosine kinase homologs (Mossie *et al.*, 1995). The bracketed sequence corresponds to the activation segment portion that is crystallographically disordered in hck and src, coincides with sequence uniquely conserved among ryk family members, and includes the sequence specific context for possible phosphorylation at a conserved regulatory tyrosine. Accession numbers are GenBank U41031, L02210, X69970, AJ224361, L47260, respectively. The predicted C16B8.1 protein sequence is NIH 1098983

are viral and cellular sea, which are limited to tyrosine kinase domain homology, and which contain intact DFG motifs. Interestingly, although many of the conserved motifs of the tyrosine kinase domain are similarly variant in these molecules, a block of unique conserved sequence corresponding to crystallographically disordered regions of the Src and Hck activation segments (Xu *et al.*, 1997, Sicheri *et al.*, 1997) surrounds a tyrosine residue, suggesting that whatever their catalytic activity, that the ryk family members should be activated by a specific tyrosine phosphorylation.

The derailed gene was isolated by enhancer trap screening for neural defects in Drosophila, which identified it as a gene whose deficiency causes inappropriate targeting of interneurons as axons of specific bundles take random paths (Callahan *et al.*, 1995). Subsequently, derailed was shown to have a similar effect on a subpopulation of muscle fibers, which bypass their appropriate attachment sites in 20% of hemisegments (Callahan *et al.*, 1996). Derailed is expressed both in the muscle fibers and in the region of epidermis surrounding the attachment site, in an increasingly tight pattern as development progresses. Therefore, if ryk and derailed are members of a larger family and if we hypothesize that the protein interaction of the derailed in making or recognizing attachment with some external feature of a target cell is conserved within the family, then we can make an analogy with the epithelial systems. In each location where ryk is expressed, the epithelial cells are entering a final alignment for their differentiated function. In the basal layer of the skin, cells are not only dividing and distributing from stem cells, but establishing contacts with their neighbors and choosing a moment to end contact with the basal lamina. In the hair matrix, the cells are establishing cortex, medulla, and inner root sheath, layers which will then move outward in unison. In the intestine, intervillus epithelial cells which emerge from invaginated crypts must find and move onto extruding villi and establish a new monolayer. In the decidualizing uterine stroma, the cells surrounding the embryo phagocytose thin collagen fibrils and deposit a thicker type of collagen, while developing intermediate filaments and adherens junctions, and reducing the size of the extracellular compartment (Abrahamsohn and Zorn, 1993, Welsh and Enders, 1985). These situations suggest that both derailed and ryk could be responsible for recognizing an appropriate interaction, and signaling to the cell when it has, in effect, found its place. The recent submission of Drosophila doughnut sequence to GenBank (AJ224361), with the annotation that it is expressed in cells bordering sites of epithelial invagination during embryogenesis suggests that it will further contribute to this model.

The C18B6.1 cosmid and a shorter construct predicted to produce only ryk-homologous C18B6.1 protein have been shown to complement the lin-18 (e620) mutant in *C. elegans* (Katz, personal communication). Lin-18 mutants produce an incompletely penetrant phenotype (Ferguson and Horvitz, 1985), in which a single vulval precursor cell P7.p produces progeny whose asymmetric cell fates are reversed or duplicated and at elevated temperature the longitudinally dividing cells may fail to adhere to the cuticle (Ferguson *et al.*, 1987). Lin-18 affects the direction of process formation in some cells, and the relative positions of adherent and nonadherent cells. Lin-17, a homolog of the Drosophila frizzled seven-transmembrane receptor which affects P7.p progeny in a manner similar to lin-18 (Ferguson *et al.*, 1987), can completely abolish cuticle adhesion in the lin-17; lin-18 double mutant (Katz, personal communication). The role for lin-18 in precisely directed cell adhesion pathways is reminiscent of the function of derailed and supports a hypothetical role for ryk in epithelial and decidual morphogenesis.

The existence of ryk homologs in fly, nematode, and man suggests either that Ryk has a fundamental function that is shared among the widest diversity of animals, or else that a variety of related functions may be mediated by a family of as yet undiscovered ryk homologs. Derailed function is apparently unnecessary for 70% of muscle fibers to find their targets, which suggests the existence of other proteins with related functions. In mammals, ryk can be localized to a syntenic region of mouse chromosome 9 and human 3q22 (Kelman *et al.*, 1993, Simoneaux *et al.*, 1995, Wang *et al.*, 1996). An additional strong signal (ryk-2), which recognizes coding sequence probes but not untranslated sequence, can be mapped by *in situ* hybridization to human chromosome 17q13.3 (Stacker *et al.*, 1993) or RFLP analysis with linkage to mouse chromosome 12 (Gough *et al.*, 1995). Because isolations of ryk generally employed PCR-based methods for cloning tyrosine kinases, and Ryk homologs contain substantial variants of motifs which are normally invariant in the molecules shown to be active as tyrosine kinases, it is possible that other family members have eluded detection, though they might be amenable to cloning using the more strongly conserved motifs near the activation loop and the C-terminal portion of the kinase domain.

Our data have indicated specific transient induction of ryk in particular epithelia according to proliferation or differentiation stage, but which varies between tissues and correlates more generally with the establishment of final morphological relationships. Strong transient induction was also observed during differentiation of uterine stromal fibroblasts to 'epithelioid' mature decidua in response to implantation, which includes many changes in cellular contact with extracellular matrix and other cells. These circumstances resemble, in broad outline, those regulated by ryk homologs in fruit fly muscle and nerve fiber target recognition, and roundworm vulval epithelial cell adhesion and polarity determination. Further work characterizing ryk in these specific tissues, enlightened by continuing research of its known and unknown homologs, will allow us to gain an understanding of its function and associations at a molecular level.

Materials and methods

Ribonuclease protection assays

Fetuses were dated by time post-coitus: morning plugs are deemed to be day 0.5 (d0.5). Fetuses were dissected free of extraembryonic tissues and homogenized for total fetal RNA, or the intestines and caecum were removed for fetal

gut RNA. All tissues were homogenized in guanidine thiocyanate solution with 2-mercaptoethanol (Chirgwin *et al.*, 1979), and total RNA was isolated by CsCl gradient centrifugation.

Distribution of ryk was analysed by ribonuclease protection assay using an α-^{32}-CTP labeled antisense RNA probe transcribed from a linearized pBlueScript SK II$^-$ plasmid (Stratagene, La Jolla, CA, USA) containing the cloned 210 bp catalytic domain fragment and protocol described previously (Siyanova *et al.*, 1994).

In situ *hybridizations*

In situ hybridization of intestine, skin, tongue and embryo time points frozen in OCT was performed as described (Siyanova *et al.*, 1994). A ryk cDNA (Siyanova *et al.*, 1994) fragment 638–1677 of the nyk-r sequence (Paul *et al.*, 1992), in pBluescript KS II$^-$ was used for *in vitro* transcription of sense and antisense probes. The probes were hydrolyzed to 150 bp fragments in sodium carbonate buffer at 60°C for better tissue penetration.

For paraffin-embedded tissue blocks (used for embryos of d9.5 and above), an alternate *in situ* protocol was used (Lee *et al.*, 1992), which yielded better tissue morphology at the expense of somewhat higher background hybridization. Briefly, embryos were fixed overnight in 4% paraformaldehyde in PBS and embedded in Paraplast. Paraffin sections (8–10 μm) on 3-aminopropyl-triethoxysilane treated slides were hydrated through xylene and graded ethanols to PBS, post-fixed for 2 min in 4% paraformaldehyde in PBS, washed, incubated for 7.5 min with 0.3 μg/ml proteinase K in 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, washed, treated with 0.25% acetic anhydride in triethanolamine for 10 min, dehydrated and stored dry for up to 1 week prior to hybridization. Dry slides were prehybridized for 2–4 h with 0.6 M NaCl, 0.12 M Tris-HCl pH 8.0, 8 mM EDTA, 0.02% Ficoll-400, 0.02% polyvinylpyrrolidone, 0.1% BSA, 500 μg/ml sheared, denatured herring sperm DNA, 600 μg/ml yeast total RNA, 50% formamide. For hybridization, the probe was added to a final concentration of 2×10^5 c.p.m./μl in a volume of formamide, incubated at 80°C for 1 min, quenched and added to an equal volume of 2 × hybridization buffer and supplemented with 1/50 volume of 10% SDS and 1/50 volume of 1 M DTT. 1 × Hybridization buffer is 0.6 M NaCl, 0.12 M Tris-HCl pH 8.0, 8 mM EDTA, 0.02% Ficoll-400, 0.02% Polyvinylpyrrolidone, 0.1% BSA, 100 μg/ml sheared, denatured salmon or herring sperm DNA, 100 μg/ml yeast total RNA, 10% dextran sulfate. Slides were sealed with cover slips and rubber cement for hybridization.

After an initial wash in 50% formamide, 2 × SSC at room temperature, slides were transferred to 50% formamide, 2 × SSC, 10 mM 2-mercaptoethanol at 50°C for 30 min; 0.5 × SSC at room temperature for 30 min; 20 μg/ml RNAse A in 3.5 × SSC for 30–60 min; two washes in 3.5 × SSC at room temperature for 10 min each; and 1.5 liters of 0.1 × SSC for 2 h at 65°C. The 30% and 50% ethanol washes for final dehydration used 0.3 M NaOAc for the aqueous component to avoid denaturation of hybrid complexes. Dipping and developing was done as previously (Siyanova *et al.*, 1994).

Immunohistochemistry

The immunochemistry of paraformaldehyde-fixed, paraffin-embedded sections of d16.5 fetus was done using a Vectastain ABC Kit according to the manufacturer's instructions.

Acknowledgments

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