Brk, a Breast Tumor-derived Non-receptor Protein-tyrosine Kinase, Sensitizes Mammary Epithelial Cells to Epidermal Growth Factor*†

(Received for publication, February 5, 1996, and in revised form, September 3, 1996)

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brk (breast tumor kinase) shows homology to the src family of non-receptor protein-tyrosine kinases and is expressed in breast carcinomas. In order to investigate the role of brk in breast tumor development, we have examined the growth and transformation properties of human mammary epithelial cells engineered to overexpress Brk. Interestingly, like c-Src, overexpression of Brk leads to sensitization to EGF, and also results in a partially transformed phenotype. Further investigation of the latter activity was attempted by mutational analysis, targeting key residues known to affect tyrosine kinase activity in Src-like kinases. Mutation of amino acid residue Lys-219 to Met, by analogy to Src, abolished both kinase activity and transformation capacity. Mutation of amino acid residue Tyr-447 to Phe, however, resulted in a decrease in transforming potential without affecting kinase activity. These results suggest that while Src and Brk share some functional properties, they act differently during transformation. These differences are discussed in the context of the mechanisms underlying breast cancer development.

The most prevalent type of cancer found in western women is that of the breast, with an approximate probability of one in nine women developing the disease during the course of their lifetime (Kelsey and Berkowitz, 1988). Approximately 10% of all breast carcinomas are due to two recently isolated familial predisposition genes, BRCA1 (Miki et al., 1994; Futreal et al., 1994) and BRCA2 (Wooster et al., 1995). However, the vast majority of breast carcinomas are sporadic and have a complex accumulation of molecular and cellular abnormalities that constitute the malignant phenotype. There is, hence, a considerable drive to identify, at a molecular level, factors that contribute to the normal development of the mammary gland, as well as alterations that might contribute to the progression from normal growth through malignancy to metastasis. To this end a number of somatic gene alterations, such as loss of expression of specific tumor suppressor genes have been found to occur in primary human breast tumors (Borg et al., 1992; Eeles et al., 1993). Additionally, there is increasing evidence that genetic alterations in growth factor signaling pathways can contribute to human breast malignancies. In this regard, activation of different protooncogenes have also been found in primary human breast tumors (Salomon et al., 1990; Berns et al., 1992; Klijn et al., 1994; Gullick et al., 1990; Borg et al., 1991).

Peptide growth factors have been shown to play a significant role in proliferation and differentiation of human mammary epithelial cells through potential autocrine, juxtacrine, and paracrine pathways mediated by their receptors (Niranjan et al., 1995; Yee et al., 1990; Daniel and Silberstein, 1990; Liu et al., 1995). Specifically, epidermal growth factor (EGF) and transforming growth factor α (a member of the EGF superfamily of proteins; Derynck (1988)) have been shown to be important in the development of the mammary gland (Zwiebel et al., 1986; Connolly and Rose, 1988; Liscia et al., 1990; Coleman et al., 1988; Vanderhaar, 1987). Additionally, transforming growth factor α and other members of the rapidly expanding EGF family of proteins have been implicated in the pathogenesis of breast carcinomas (Normanno et al., 1994). Moreover, it is now well established that members of the EGF receptor family of tyrosine kinases are also involved in neoplastic transformation of breast epithelial cells, where their overexpression present an inverse correlation with patient survival (reviewed by Rajkumar and Gullick (1994)).

Increasing evidence demonstrates that ligand-activated receptor protein kinases transduce their signal through association with a number of specific cytoplasmic target proteins that contain Src homology 2 (SH2) domains. SH2 domains have been found in a diverse group of proteins, some containing enzyme activity, i.e. phospholipase C-γ-1 (Margolis et al., 1989; Meisenhelder et al., 1989), RasGAP (Kaplan et al., 1990), and phosphatidylinositol 3′ kinase (Coughlin et al., 1989), while others lack any apparent enzyme activity, i.e. Grb2 (Lowenstein et al., 1992), Nck (Hu et al., 1992), and Shc (Pelicci et al., 1992). Activated receptor tyrosine kinases have also been shown to associate with cytoplasmic non-receptor tyrosine kinases such as Src and Src-like proteins (Kypta et al., 1990; Luttrell et al., 1994; Muthuswamy and Muller, 1995a). To date a number of non-receptor protein-tyrosine kinases have been identified. Of these, approximately half demonstrate oncogenic potential, while others could be classified as antioncogenes (reviewed by Bolen (1993)). In this context, recent studies have shown the protein-tyrosine kinase activity in the cytosolic and membrane fractions of most malignant human breast tissue to be significantly higher than that from benign or normal breast tissue (Lower et al., 1993; Henipman et al., 1989; Ottenhoff-Kalf et al., 1992). Moreover, the increased cytosolic protein-tyrosine kinase activity was found to be a prognostic indicator of decreased disease-free survival (Bolla et al., 1993).

* This work was funded by GlaxoWellcome. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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As part of a study aimed at identification of novel protein-tyrosine kinases, and examination of their potential involvement in human breast tumor development, we have recently reported the isolation and characterization of a cDNA from a human metastatic breast tumor, representing a novel non-receptor protein-tyrosine kinase, brk (breast tumor kinase) (Mitchell et al., 1994). Analysis of the putative Brk protein sequence revealed it to be almost exclusively composed of three domains defined on the basis of sequence homology to other signaling molecules. From the N terminus, these are: an SH3, an SH2, and a catalytic domain. This arrangement and amino acid sequence homology (particularly in the catalytic domain) relate Brk most closely to the Src family of protein-tyrosine kinases. However, Brk differs from the Src-like kinases in several respects, most notably in its lack of an N-terminal extension and predicted myristylation site (Mitchell et al., 1994).

In order to further investigate the role of Brk in breast cancer, we have examined the effect of Brk overexpression in human mammary epithelial cells as well as murine embryonic fibroblasts. Here we report that Brk overexpression mitogenically sensitizes human mammary epithelial cells to EGF, and that Brk is partially transforming not only in these cells, but also in mouse embryonic fibroblasts. Additionally, we demonstrate that Brk can interact directly with the EGF receptor. Our results provide an insight into the molecular mechanisms by which Brk may contribute to the progression of breast tumor development.

MATERIALS AND METHODS

Cell Lines

HB4a, an SV40-immortalized, but not transformed, human mammary luminal cell line (Stamps et al., 1994) was maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), insulin (5 µg/ml), hydrocortisone (5 µg/ml), and cholera toxin (100 ng/ml). MCF-10A, a spontaneously immortalized human mammary epithelial cell line (Soule et al., 1990) was maintained in Dulbecco’s modified Eagle’s medium/F-12 medium supplemented with 5% equine serum, hydrocortisone (0.5 µg/ml), insulin (5 µg/ml), cholera toxin (100 ng/ml), and EGF (10 ng/ml). NIH3T3, embryonic mouse fibroblasts (Jainchill et al., 1989) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. All cells were grown at 37 °C in a humidified 5% CO2 atmosphere.

Antibodies

A rat monoclonal antibody specific for the SV40 T-antigen, PAb416 (Harlow et al., 1981), was a gift from Dr. L. Crawford (Imperial Cancer Research Fund, Cambridge, United Kingdom). Mouse monoclonal antibody against the Brk receptor (E3138) was purchased from Sigma.

Growth Curves

2 x 10^4 cells were seeded in 24-well dishes in 1% FCS and left overnight to settle. The medium was then changed to test medium containing various amounts of EGF (mouse submaxillary, receptor grade, from Sigma). At indicated time intervals, the cells were trypsinized and cell numbers determined using a Coulter counter (Coulter electronics). In all growth curves, the medium was changed every 3 days.

Expression of Brk in Human Mammary Epithelial Cells and Fibroblasts

The brk cDNA was excised from clone λ2 (Mitchell et al., 1994) using the KpnI and BamHI sites in the cloning vector (pBluescript SK-, Stratagene), blunted with Klenow polymerase, and ligated into SmaI digested pBluescript SK+. In one orientation, this resulted in the situation of a HindIII site at the 5’ end of the cDNA. Digestion at this site and HindIII site in the 3’-untranslated region of the brk cDNA produced a coding fragment that was ligated, in the sense orientation, into the HindIII sites of pRcCMV and pREP8 (Invitrogen). A coding cDNA fragment was isolated from the brk-pRcCMV expression vector by digestion with EcoRI, and ligated into the EcoRI site of pDNA (Morgenstern and Land, 1990) in the sense orientation. Point mutations were introduced into brk cDNA using the T7-Gen kit (United States Biochemical Corp.).

Stable Brk expression in HB4a and NIH3T3 cells was achieved by calcium phosphate transfection followed by drug selection. Briefly, 1 x 10^6 cells were seeded into 10-cm dishes and left to settle overnight. The cells were then given fresh medium and, approximately 5 h later, transfected using 20 µg of DNA. Approximately 12 h post-transfection, the cells were shocked with 15% glycerol for 2.5 min. Forty-eight hours post-shock, drug selection was applied and maintained thereafter. The transfections generated on average 20–50 drug-resistant colonies/dish. NIH3T3 cells were transfected in triplicates and selected under 1 mg/ml Geneticin (G418), while HB4a transfectants were transfected in duplicates and selected under 1 mM Histidinol in histidine-free medium supplemented with dialyzed fetal calf serum, insulin, hydrocortisone, and cholera toxin. In all cases, all drug-resistant colonies generated in a transfection, were pooled to generate a single population of cells, for the respective cell lines, in order to avoid clonal variability and artifacts arising through selective cloning pressures.

Stable Brk expression in MCF10-A cells was achieved by retroviral infection. Briefly, brk cDNA was cloned into the retrovirus vector pINA, a derivative of the vector pagsneoSVR, in which the β-actin promoter replaces the SV40 promoter (Morgenstern and Land, 1990). In this vector the neo gene is expressed under the control of the retrovirus long terminal repeat promoter and the brk gene under the control of the β-actin promoter. Using amphotropically packaged virus (1 x 10^6 colony-forming units/ml), 5 x 10^5 cells were infected in the presence of 8 µg/ml Polybrene and selected in the presence of 2 mg/ml G418. Amphotropically packaged empty pINA vector was used as control retrovirus.

Transformation Assays

Transformation of the cells was assessed by their ability to demonstrate anchorage-independent growth. To this end, drug-resistant transfected cells were grown in medium containing 0.4% agar at a density of 1 x 10^6 in six-well dishes. After 4–6 weeks of growth, the colonies were visualized by staining with 0.33% iodonitrotetrazolium violet (Rosenthal et al., 1986). All assays were performed in duplicate. Clones to be recovered from soft agar were picked before staining.

Immunocytochemistry

Cells were seeded on to glass coverslips in 24-well dishes and left to grow. Exponentially growing cells were washed in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde for 20 min. Free aldehyde groups were then quenched with 0.1 M glycine for 10 min and the cells permeabilized using 0.5% (v/v) Triton X-100 in PBS for 5 min, washed extensively in PBS, and exposed to a monoclonal anti-Brk antibody (1:10). Fluorescein isothiocyanate-conjugated secondary antibody (Sigma) was used to visualize the primary antibody. The preparations were mounted in glycerol containing 1,4-diazabicyclo[2,2,2]octane (DABCO) as an antifade agent (Johnson et al., 1982) and visualized on a fluorescence microscope.

Immunoblots

Whole cells lysed in Laemmli lysis buffer were subjected to electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels (Laemmli, 1970) and electrothermally transferred to nitrocellulose membrane (Towbin et al., 1979). The nitrocellulose membrane was then blocked with 20% milk powder in PBS and probed with the primary antibody. Immunoreactive proteins were visualized using horseradish peroxidase-conjugated immunoglobulins (DAKO) and enhanced chemiluminescence (ECL, Amersham).

Immunoprecipitation

1 x 10^6 cells were lysed in ice cold lysis buffer (10 mM CHAPS, 50 mM Tris, pH 8, 150 mM NaCl, 2 mM EDTA, 1 mM Na2VO3, 1 mM phenyl-methylsulfonyl fluoride, 50 µg/ml leupeptin, and 10 µg/ml aprotinin) for 20 min. The cell lysates were cleared by spinning at 10,000 x g for 20 min at 4 °C, and then incubated with antibody-coated Protein G for 18 h at 4 °C. The beads were then washed three times (150 mM NaCl, 50 mM Tris, pH 8, and 5 mM EDTA) and finally resuspended in Laemmli lysis buffer. The immunoprecipitated material was resolved by SDS-PAGE electrophoresis, blotted, and probed as detailed above.
Receptor number per cell and the dissociation constant of the ligand-receptor complex (K_d) were estimated using Scatchard analysis (Scatchard, 1949).

Radioiodination of EGF—EGF (20 μg) was radiolabeled with carrier-free Na[125]I (2 mCi) using IODOGEN (Pierce), Briefly, 60 μg of IODOGEN in 60 μl of chloroform was dried on to a polypropylene vial under nitrogen flow. EGF and 125I were then added in PBS, pH 7.4. The reaction was allowed to proceed for 15 min, then the mixture was transferred to another vial and left to stand for 10 min. Carrier bovine serum albumin was added at 1 mg/ml in PBS, pH 7.4, and the labeled ligand separated from free Na[125]I using IODOGEN (Pierce). Briefly, a 60-μl aliquot of 7:1 (v/v) mixture of dibutylphthalate: mineral oil was overlaid with 175 μl of binding medium containing twice the desired 125I-EGF concentration. 5 × 10^6 cells were then added in another 175 μl of binding medium. Following incubation for approximately 12 h at 4°C, the cells were separated from the binding medium by sedimentation through the oil layer at 10,000 × g for 5 min. The tube tip containing the cells was then cut off and cell-bound radiolabel counted in an Innorot gamma counter. The binding experiments were performed in triplicate using 200-fold excess of unlabeled EGF in controls.

RESULTS
Expression of Brk in Human Mammary Epithelial Cells
In order to study the biological functions of Brk in human mammary epithelial cells, we have expressed Brk in two existing cell lines, MCF10-A and HB4a. These cell lines were chosen as good representatives of this cell type since, unlike most other breast cell lines, their origin is unequivocally known to be normal breast epithelia. Following stable expression of Brk in HB4a and MCF10-A cells, we found no effect on the morphology of the cells. Additionally, using a monoclonal anti-Brk antibody, raised to recombinant Brk protein, we have found that Brk is uniformly expressed in the cytoplasm of the cells (Fig. 1). The apparent nuclear staining observed in Fig. 1A is nonspecific, since similar staining is observed in the vector transfected control cells, which do not inherently express Brk as demonstrated by Western blotting using the same antibody (Fig. 1B).

Biological Characterization of Brk

Brk Overexpression Sensitizes Mammary Epithelial Cells to EGF—The ability of stable brk-transfected HB4a cells to respond to mitogenic stimuli was examined by assessing the growth of the cells after exposure to a variety of growth factors, over a period of time. We found that fibroblast growth factors (a and b), nerve growth factor, PDGF-BB, insulin, and macrophage colony stimulating factor had no effect on the growth of these cells (data not shown). Interestingly, the cells respond mitogenically to EGF over a concentration range of 1–20 ng/ml. The data presented in Fig. 2, demonstrate that HB4a cells transfected with brk, HB4a-Brk, and HB4a cells transfected with vector only, HB4a-vector, fail to grow in medium with reduced serum (1% FCS). However, EGF at 1 ng/ml increases the proliferation of HB4a-Brk cells by more than 10-fold over a period of 7 days. HB4a-Brk cells respond to EGF optimally at 5–10 ng/ml, improving their growth by approximately 20-fold over that seen at 1% FCS. Notably, HB4a-vector control cells also begin to respond to this growth factor as the EGF concentration is increased from 1 to 5 ng/ml. However, the magnitude of the response of HB4a-vector cells to EGF remains constant and consistently lower than HB4a-Brk cells, over a range of
5–20 ng/ml EGF. In comparison to the vector controls, Brk overexpression improves the growth of HB4a cells by 2–3-fold in response to 1–10 ng/ml EGF.

A similar mitogenic response to EGF was observed in MCF10-A cells stably expressing Brk, where, in comparison to vector-transfected controls, Brk overexpression improved the growth of MCF10-A cells by 35% in response to 1–5 ng/ml EGF (Fig. 3).

**EGF Receptor Expression and Affinity in HB4a Transfectants**—Scatchard analysis was used to determine the EGF receptor density and affinity in HB4a-Brk and vector-only transfected cells, in order to examine the possibility that the differential mitogenic response of these cells to EGF may be a consequence of different EGF receptor density/affinity in the two cell populations. Hence, EGF receptor density and affinity was determined for three separate populations of cells derived from three independent transfections. Fig. 4 represents a typical Scatchard plot obtained. We have found the EGF receptor density in both HB4a-Brk and HB4a-vector cells to be $3.7 \times 10^5$ receptors/cell. Additionally, both cell populations display only one class of receptor, with an affinity of $29 \times 10^{-9}$ M. Furthermore, the steady state levels of EGF receptor protein, as judged by Western blotting, are the same in both cell populations (data not shown).

**Association of Brk with the EGF Receptor**—In an attempt to elucidate the role of Brk in the EGF-induced proliferation of Brk overexpressing mammary epithelial cells, we sought to examine Brk’s ability to directly associate with the EGF receptor in these cells. To this end, co-immunoprecipitations of Brk with the EGF receptor were performed. The results, presented in Fig. 5, demonstrate that Brk interacts directly with the EGF receptor in Brk-overexpressing MCF10A cells even in the absence of exogenous EGF. Similar results were obtained for HB4a-Brk cells (data not shown).

**Transforming Potential of Brk**

The transforming potential of Brk and structurally altered forms of Brk, was evaluated in HB4a and NIH3T3 cells, respectively, by examining the ability of transfected cells to escape contact inhibition when grown on tissue culture plastic together with their capacity to support anchorage-independent growth when grown in agar. Following stable transfection of brk and brk mutants in HB4a and NIH3T3 cells, respectively, we have found no effect on the morphology of these cells. Additionally, in both NIH3T3 and HB4a stable transfectants, we were unable to detect differences in the total amount of tyrosine-phosphorylated proteins (data not shown). Furthermore, overexpression of Brk and Brk mutants does not allow HB4a or NIH3T3 cells to escape from contact inhibited growth. However, in comparison to the vector-transfected cells, overexpression of Brk in HB4a cells results in a significant increase in the number and size of the colonies formed in soft agar (Fig. 6). This phenomenon was also observed in NIH3T3 cells (Table I). Additionally, overexpression of Brk Y447F in NIH3T3 cells appears to reduce the ability of these cells to grow in soft agar when compared with Brk, while the K219M mutation appears to abolish the ability to support growth in soft agar altogether (Table I).

In order to confirm that the growth of NIH3T3 cells in soft agar is indeed due to Brk overexpression, colonies growing in soft agar were picked from empty vector (considerably smaller than those in NIH3T3-Brk) and brk-transfected populations. The clones were then expanded and examined for Brk protein expression by Western blotting. Fig. 7 demonstrates that while Brk expression was easily detectable in cells derived from NIH3T3-Brk foci, no Brk protein was detectable in the NIH3T3 cells derived from NIH3T3-vector foci.
Normal human mammary luminal cells therefore provide an ideal environment for studies of molecules that are thought to be involved in the development of human breast tumors. However, since primary luminal cells have a severely limited life span in culture, we have chosen two human mammary cell lines (MCF10-A and HB4a) with defined normal mammary epithelial origins, as the best available alternative for our studies. Specifically, MCF10-A cells arose from spontaneous immortalization in a culture of normal human organoids (Soule et al., 1992). Although MCF10-A cells exhibit features of mammary luminal cells, they also express markers characteristic of mammary myoepithelial cells such as keratin 14 and common acute lymphoblastic leukemia antigen, CALLA/CD10. Therefore, MCF10-A cells cannot definitively be classified as wholly luminal or myoepithelial. In this context, HB4a cells provide a better example of mammary luminal cells since they were derived by immortalization of a highly enriched population of normal luminal cells (Stamps et al., 1994). Additionally, despite their immortalization, HB4a continue to exhibit characteristics of human mammary luminal cells (Kamalati et al., 1996).

In experiments to assess the transforming potential of Brk, we have found that in vitro overexpression of Brk in HB4a cells potentiates anchorage-independent growth. Conventionally the transforming potential of Src and Src-like kinases has been evaluated in mouse embryonic fibroblasts (NIH3T3); in order to more directly compare the transforming potential of Brk with Src, we have also expressed Brk in these cells. Our results show that Brk expression can also partially transform mouse fibroblasts. The ability of Brk to support anchorage independent growth of these cells is in contrast to those reported for the Src family of protein-tyrosine kinases in that expression of Src, Lck, Hck, FynB, or FynT in NIH3T3 cells did not induce anchorage-independent growth (Cartwright et al., 1987: Amrein and Sefton, 1988: Abraham and Veillette, 1990: Ziegler et al., 1989; Amrein et al., 1990; Ziegler et al., 1989; Davidson et al., 1994). However, it has been suggested that Src, FynB, and FynT can transform NIH3T3 cells when expressed at high levels and in an appropriate environment (Kmieciak and Shalloway, 1987; Lin et al., 1995; Kawakami et al., 1988). Since brk was cDNA-cloned from a breast tumor, the

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**Assessment of Potential Mechanisms for Regulation of Brk Kinase Activity**

In our earlier work, we have demonstrated that Brk is a non-receptor protein-tyrosine kinase capable of autophosphorylation (Mitchell et al., 1994). Since the deduced Brk protein product shows highest overall amino acid homology to members of the Src family of non-receptor tyrosine kinases (Mitchell et al., 1994), we sought to establish whether Brk activity is regulated by similar mechanisms that regulate Src activity. To do this, we have generated two variants of Brk harboring mutations, which, in members of Src family of proteins, result in kinase-inactive or a constitutively active kinase in vivo (Jove et al., 1987; Davidson et al., 1994; Twamley et al., 1992). Specifically, lysine 219 (analogous to lysine 295 in p60src) was substituted with methionine (K219M), destroying the putative ATP binding site of Brk and thereby potentially abolishing its kinase activity. Additionally, tyrosine 447 (possibly analogous to tyrosine 527 in p60src) was substituted with phenylalanine (Y447F) to potentially produce a constitutively active kinase in vivo.

In order to establish the effects of the above point mutations on Brk autophosphorylation activity, the structurally altered Brk mutants were expressed in bacteria as fusion proteins with glutathione S-transferase and examined for tyrosyl phosphorylation. Fig. 8 demonstrates that in comparison to Brk, the K219M mutation interferes with Brk's intrinsic protein-tyrosine kinase activity, while the Y447F mutation appears to have no effect.

**DISCUSSION**

Recently, we have identified a novel non-receptor tyrosine kinase, brk, from a human metastatic breast tumor and have shown that human breast tumors and tumor cell lines express brk transcript (Mitchell et al., 1994). In this study, we have aimed to elucidate the molecular mechanisms by which brk may contribute to tumor pathogenesis, and present biological characterization of Brk activity in human mammary epithelial cells as well as mouse embryonic fibroblasts.

**Transforming Potential of Brk**—Since brk was originally derived from a metastatic mammary tumor, we sought to examine the transforming potential of Brk in human mammary epithelial cells. It is well established that the majority of human breast carcinomas originate from the luminal rather than the myoepithelial cells of this gland (Gusterson et al., 1982). Normal human mammary luminal cells therefore provide an

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8 T. Kamalati, unpublished observation.
Human mammary epithelial cells, currently analyzing the genomic sequence of which it was cloned. To address this possibility, we are currently analyzing the genomic sequence of **brk** in normal human tissue.

**Regulation of Brk Activity**—Although Brk is not classified as a member of any of the existing families of non-receptor protein-tyrosine kinases, it does show significant homology to the Src family of proteins. However, certain features of Brk differ substantially from the members of this family. Specifically, the sequence C-terminal of its catalytic domain diverges significantly from that of Src-like kinases. Nonetheless, similar to Src, Brk has a tyrosine residue in its C terminus (Tyr-447) that could potentially have a regulatory role (Mitchell et al., 1994).

Studies of the contribution of specific well conserved amino acid residues to the regulation of the catalytic activity of the Src-like family of proteins have revealed extensive information regarding the mechanisms involved in controlling the kinase activity and hence transforming ability of this family of kinases. It is now well documented that the catalytic activity of Src family tyrosine kinases is negatively regulated through phosphorylation of a conserved tyrosine residue (Tyr-527) in the C-terminal tail, by c-Src kinase, Csk (Courtneidge et al., 1993b; Liu and Pawson, 1994). Phosphorylation of this residue induces intramolecular interactions with the SH2 domain and a consequent inhibition of the catalytic activity. Moreover, mutation or deletion of this residue results in increased kinase activity and transforming ability. In order to determine whether Brk's activity is regulated by a similar mechanism as that of Src-like kinases, we have mutated Brk tyrosine 447 to Phe results in a significant increase in Brk's capacity to induce anchorage-independent growth. This is in contrast to what is known about the Src-like family of proteins, where analogous mutations result in increased kinase activity and transformation potential (Kmieciik and Shalloway, 1987; Piwnica-Worms et al., 1987; Cartwright et al., 1987; Amrein and Sefton, 1988; Abraham and Veillette, 1990; Ziegler et al., 1989; Davidson et al., 1994). The possibility exists that protein interactions may contribute to the transforming capacity of Brk. In this context, the Y447F mutation may alter Brk's ability to participate in interactions integral to its transformation process. We have found that as in Src, lysine 219 of Brk (homologous to Src Lys-295) is an indispensable catalytic residue (Jove et al., 1987; Davidson et al., 1994; Twamley et al., 1992). Replacement of Brk Lys-219 with methionine abolishes kinase activity and cell transformation. We therefore conclude that the catalytic activity of Brk is required for its growth deregulation of fibroblasts and that phosphorylation on protein-tyrosine residues is a necessary component of this transforming process.

**Brk Sensitizes Human Mammary Epithelial Cells to EGF**—In order to evaluate whether Brk is involved in the cellular response to external stimuli, we have examined the effect of exogenous growth factors on **brk**-transfected human mammary epithelial cells. Our data show that from a broad spectrum of growth factors tested, the cells respond mitogenically only to EGF. Specifically, in response to EGF, HB4a **brk** transfecants display 2–3-fold improved growth over the control cells carrying the vector only. MCF10A cells transfected with **brk** also exhibit an improved mitogenic response to EGF. Brk, therefore, appears to sensitize mammary epithelial cells to EGF. Using Scatchard analysis we have demonstrated that Brk expression does not alter the affinity or number of EGF receptors expressed on the surface of the transfected HB4a cells. It is therefore likely that the growth advantage of the Brk-expressing cells is achieved by alterations in the signaling cascade or cell cycle progression elicited by EGF in these cells.

Involvement of non-receptor tyrosine kinases in signal trans-
duction, through direct association with activated receptor tyrosine kinases, has been described before. Specifically, association of c-Src and Src-like kinases with platelet-derived growth factor receptor and macrophage colony-stimulating factor receptor upon ligand stimulation is well documented (Kypa et al., 1990; Courtneidge et al., 1993a). Fibroblasts overexpressing c-Src have been reported to be hypersensitive to EGF-mediated growth stimulation as assessed by enhanced DNA synthesis (Luttrell et al., 1988; Wilson et al., 1989). EGF has been shown to activate and translocate c-Src to the cytoskeleton in glioblastoma cells (Oude Weernink and Rijksen, 1995). Additionally, microinjection of antibodies that recognize c-Src, c-Yes, and c-Fyn have been shown to inhibit EGF-induced entry of fibroblasts into S phase (Roche et al., 1995). Studies examining the mode of EGF-dependent Src kinase activation have demonstrated that, as with PDGF and CSF receptors, Src can associate directly with the EGF receptor (Sierke et al., 1993; Luttrell et al., 1994; Maa et al., 1995; Oude Weernink et al., 1994) and that the EGF activation of Src family kinases is dependent on the levels of EGF receptor displayed by the cells (Osherove and Levitzki, 1994). In this light, having established that the mammary epithelial cells used in this study do possess appropriate levels of functional EGF receptor, we sought to examine Brk’s ability to directly associate with the EGF receptor. Our results demonstrate that Brk interacts directly with the EGF receptor even in the absence of exogenous EGF. The precise molecular mechanism by which Brk overexpression elicits an enhanced mitogenic response to EGF, is currently under investigation in our laboratory. The possibility exists that EGF receptor-associated Brk may become phosphorylated in the presence of EGF and hence generate new docking sites for adaptor and/or effector molecules, resulting in an amplified response to EGF.

Proteins that bind activated receptors are frequently substrates for its kinase activity, and often the inherent activity of the substrate protein is altered as a result of binding and/or tyrosine phosphorylation. In this context, we were unable to detect gross changes in the levels of tyrosine phosphorylated proteins in cells transfected with brk, implying that Brk substrate(s) may not be highly expressed or that their phosphorylation is not stoichiometric (data not shown). Additionally, since we have not detected obvious changes in the phosphorylation status of the EGF receptor in brk-transfected cells, pre- or post-EGF stimulation, we conclude that it is unlikely that Brk phosphorylates the EGF receptor.

Members of EGF receptor protein-tyrosine kinase family are frequently implicated in human cancers (Hayman and Enrietto, 1991). Specifically, in human breast cancer, expression of the EGF receptor c-erbB-1 and overexpression of c-erbB-2 (Holmes et al., 1992) have been shown to be associated with reduced patient survival and with failure to respond to chemotherapy (Gusterson et al., 1992) and endocrine therapy (Tripathy and Henderson, 1985; Klijn et al., 1995). Additionally, overexpression of c-erbB-3, a third member of the EGF receptor family, has been found to bear a significant association with nodal metastasis (Lemoine et al., 1992). Additionally, a direct involvement of overexpression of the protooncogene Neu (rat c-erbB-2) in mammary tumorigenesis has been demonstrated in a murine transgenic model (Guy et al., 1992).

Recent analysis of primary human breast cancers have revealed that a high proportion possess elevated c-Src kinase activity (Jacobs et al., 1983; Rosen et al., 1986; Ottenhoff-Kalff et al., 1992). Moreover, enhanced c-Src activity has been reported in both c-erb B2-induced human and Neu (rat homolog of c-erb B2)-induced murine mammary tumors (Muthuswamy et al., 1994). Recently, members of the c-Src family have been found to associate with c-erb B2/Neu in c-erb B2/Neu-overexpressing murine mammary tumors (Muthuswamy et al., 1994; Muthuswamy and Muller, 1995b), human breast carcinoma cell lines (Luttrell et al., 1994) and rat fibroblasts (Muthuswamy and Muller, 1995a, 1995b). The association of c-Src-like kinases with c-erb B2/Neu requires c-erb B2/Neu to be phosphorylated and has been shown to be stimulated by EGF (Muthuswamy and Muller, 1995a, 1995b). As EGF cannot directly bind Neu (Normanno et al., 1994), the observed tyrosine phosphorylation of Neu is likely the result of transphosphorylation by activated EGF receptor (Wada et al., 1990). The ability of the EGF receptor family to interact with one another and form heterodimeric complexes is now well established (Lemmon and Schlessinger, 1994). Clearly such a mechanism for cross-talk has the potential to increase diversity of response elicited by either receptor. Therefore, studies of associations of Brk with cellular components should examine a spectrum of interactions inducible by EGF.

In summary, our results demonstrate that Brk is partially transforming in fibroblasts as well as mammary epithelial cells, and that Brk expression sensitizes mammary epithelial cells to EGF. Given the existing knowledge of the role of EGF family of receptors and ligands in breast cancer, it is conceivable that Brk may act as a potentiator in the development of breast tumor pathogenesis. In studies to assess the role of Brk in human breast cancer, we have found Brk to be expressed in a significant proportion of human breast tumors. Studies to evaluate the EGF receptor family status of Brk-overexpressing breast tumors, together with an evaluation of the possible correlation between the two, will help elucidate the potential molecular mechanisms by which Brk may contribute to breast tumor development and progression.

Acknowledgments—We thank Dr. L. Buluwa and B. Niranj for much help and constructive discussion, and Dr. M. O’Hare for H64a cells.

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M. R. Crompton, manuscript in preparation. 3

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