Role of Src Kinases in Neu-Induced Tumorigenesis: Challenging the Paradigm Using Csk Homologous Kinase Transgenic Mice

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Abstract
Amplification of the HER-2/neu (ErbB2) gene is observed in ~30% of human breast cancers, correlating with a poor clinical prognosis. Src kinases are also involved in the etiology of breast cancer, and their activation was suggested to be necessary for Neu-induced oncogenesis. To address whether Src activity is essential for Neu-mediated tumorigenesis, we used a physiologic inhibitor of Src kinase activity, the Csk homologous kinase (CHK), expressed as a mammary tissue-specific transgene. Our data, using a physiologic inhibitor of Src activity (CHK), showed that blocking of Neu-induced Src activity without altering Src expression levels had no significant effects on Neu-mediated mammary tumorigenesis in vivo. This contradicts the current paradigm that activation of Src kinases is essential for Neu-induced oncogenesis. This study is the first to distinguish between the kinase-dependent and kinase-independent actions of Src and shows that its kinase-dependent properties are not requisite for Neu-induced tumorigenesis. (Cancer Res 2006; 66(11): 5757-62)

Introduction
Neu is a member of the epidermal growth factor receptor family of class I receptor tyrosine kinases (RTKs; ref. 1). The Neu gene has been found to be amplified and overexpressed in ~30% of human breast cancers (2). Patients with Neu-overexpressing breast cancer have substantially lower survival rates and shorter times to relapse than patients without Neu overexpression. Moreover, overexpression of Neu may lead to increased breast cancer metastasis (3).

Activation of the Neu tyrosine kinase leads to autophosphorylation of tyrosine residues in its COOH-terminal domain. The phosphorylated tyrosine residues then function as docking sites for HER2 downstream signaling molecules. However, the overall picture of how Neu exerts its function through these signaling molecules and the signaling events responsible for the Neu-mediated malignant transformation of mammary cells remain elusive. Results derived from in vitro and ex vivo investigations suggest that activation of Src is a necessary step for tumor induction by Neu (for review, see ref. 4). Although previous data have clearly documented that c-Src is required for polyomavirus middle T (PyMT)-mediated tumorigenesis (4), the requirement for Src in the Neu-induced tumorigenesis model remains to be firmly established using genetically modified mouse models.

The function of Src has been derived mostly from studies of cellular transformation either by v-Src or by the activated c-Src proto-oncogene (5). Src kinase is activated by a variety of signaling pathways, including stimulation of growth factor receptors, G-protein-coupled receptors, oxidative and UV stress, and integrin-mediated signal transduction (5). Targeted gene disruption of c-Src in mice leads to only one major phenotype: severe osteopetrosis with thickened teeth, decreased marrow space, and a failure of bone eruption (6). The Src−/− phenotype is due to an intrinsic defect in osteoclasts (6). The severe morbidity and increased mortality resulting from osteopetrosis have prevented the analysis of Src function in other tissues and organ systems in these mice.

A connection between Src and breast cancer was first revealed by several animal model systems. Increased Src kinase activity was found in murine breast carcinomas induced by the transgenic expression of PyMT and Neu (7–10). However, mouse mammary tumor virus (MMTV)–regulated transgenic Src expression resulted in breast hyperplasia, whereas progression to carcinomas was rare (4). These results support the role of expressed Src as a contributor to malignant conversion rather than a causal oncogene.

Most Src studies have focused on the deregulation of Src kinase activity in breast cancer in vitro, whereas the kinase-independent properties of the Src molecule have been left undefined. The defective phenotype of Src knockout mice is to a great extent rescued by transgenic expression of the kinase-inactive Src molecule (11). Moreover, signaling from integrin receptors involves Src family kinases (SFKs) but may not require Src's kinase activity (11–13). In these pathways, proper protein interactions may be more critical for Src function than its kinase activity. Thus, in integrin signaling, Src seems to more closely resemble an adaptor molecule that may function by recruiting or activating other tyrosine kinases.

Establishing the role of Src in vivo and distinguishing between the kinase-dependent and kinase-independent actions of Src are of great importance in breast cancer because inhibition of Src kinase activity has been proposed as a promising strategy for anticancer treatment in this malignancy. Indeed, generation of inhibitors of Src kinase activity for clinical applications is being pursued actively, including for breast cancer treatment. Here, we designed a novel model of Csk homologous kinase (CHK) transgenic mice to address the contribution of the functions of Src in Neu-mediated oncogenesis in vivo.

Materials and Methods
Animals. FVB/N-Tg(MMTVneu)202 Mul/J (The Jackson Laboratory, Bar Harbor, ME), FVB/NJ (The Jackson Laboratory), and FVB-Tg(MMTV-ErbB2)1Led (Charles River Laboratories, Wilmington, MA) were used throughout all of the experiments. All mice were kept under aseptic...
conditions in the Harvard Institutes of Medicine local animal facility (Association for Assessment and Accreditation of Laboratory Animal Care accredited).

**Generation of MMTV-CHK transgenic mice.** The HindIII restriction site from CHK cDNA was deleted (without altering the amino acid sequence) using site-directed mutagenesis, and the resulting CHK (HindIII−) was subcloned into the MMTV-SV40-BSSK plasmid (a generous gift from Dr. Philip Leder, Harvard Medical School, Boston, MA). The MMTV-SV40-BSSK-CHK construct was linearized (Fig. 1A), resolved on an agarose gel, and purified using the Qiagick gel extraction kit (Qiagen, Inc., Valencia, CA). DNA was filtered, microinjected into pronuclear stage zygotes, and transferred into pseudopregnant recipients at the Transgenic Core Facility located at the Beth Israel Deaconess Medical Center. Founder mice were crossbred with the wild-type FVB mice. Strains with germ line transmission were bred for further use.

**PCR.** Two microliters of purified DNA were subjected to amplification using an iCycler (Bio-Rad, Hercules, CA); 1.2 units of Taq polymerase (New England Biolabs, Beverly, MA) were used in each reaction. The Neu and MMTV primers were obtained from The Jackson Laboratory. The primers for the CHK transgene were designed using DNASTAR software (DNASTAR, Inc., Madison, WI) and obtained from Integrated DNA Technologies (Coralville, IA): CHK-TR4F, 5′-GGCTGGAGAACAAGAGC-3′; CHK-TR3R, 5′-GGCTCAGA-CACCTCTTTCC-3′. The reaction was carried out at 1 × 95°C for 30 seconds and 36 × (95°C for 30 seconds, 63°C for 45 seconds, 72°C for 1 minute).

**Southern blotting.** Tail clips were digested with proteinase K (Roche, Basel, Switzerland). DNA was purified using the phenol/chloroform extraction method. Ten micrograms of DNA were digested with EcoRI and HindIII or BamHI (New England Biolabs), resolved on agarose gel, denatured, and transferred onto nylon membrane (Hybond N+, GE Healthcare, Piscataway, NJ). Human CHK cDNA was labeled radioactively using the Megaprime kit (Roche, Basel, Switzerland). Human CHK cDNA was labeled radioactively using the Megaprime kit (Roche, Basel, Switzerland). Hybridization was carried out for 16 hours at 55°C in Church buffer. Membranes were exposed to radiographic film (Kodak, New Haven, CT).

**Histologic and morphologic (mammary gland whole mount) evaluation.** Complete autopsies and both gross and microscopic examinations were routinely done. To evaluate the physiologic development of the mammary tissue, five mice from each stage (virgin animal, 4 weeks of age; during pregnancy, 16.5 days after coitum; lactating, 1.5 days after delivery; and glands during involution after lactation) of the MMTV-CHK transgenic mice and from the nontransgenic (wild-type) FVB/N strain were analyzed. The inguinal mammary fat pads were excised, fixed in Bouin’s solution, blocked in paraffin, sectioned at 5 μm, and then stained with H&E. Mammary whole mount preparations were prepared from the inguinal mammary fat pads, as described previously (14).

**Protein extract preparation.** Tissue samples (tumors or mammary glands from control animals) and cells were fresh-frozen and lysed in TNE lysis buffer.

**Src kinase assay.** One milligram of protein extracts (tumors or mammary glands from control animals) was immunoprecipitated using antibodies against Src (clone GD11, Upstate Biotechnology, Charlottesville, VA). The immunoprecipitates were washed and resuspended in 30 μL of kinase buffer containing 0.25 mg/mL poly(Glu/Tyr)4:1 or acid-denatured enolase (both from Sigma, St. Louis, MO) and 10 μCi of [γ-32P]ATP, as described (15).

**Immunoprecipitation and Western blotting.** For the immunoprecipitation analyses, 300 μg of protein extracts were incubated with appropriate antibodies for 12 hours. Sepharose G beads (GE Healthcare) were added for 1 hour, washed thrice, and subsequently resolved on polyacrylamide gel. For the Western blot analyses, 60 μg of protein extracts were separated on 10% to 12.5% polyacrylamide–SDS gels, transferred to nitrocellulose membrane (Bio-Rad), and probed with antibodies against CHK, Csk, FAK, and anti-phosphotyrosine (PY99; Santa Cruz Biotechnology, Santa Cruz, CA); actin (Chemicon International, Temecula, CA); c-Src (clone GD11), phospho-Src ( Tyr416), 94A6; Upstate, Chicago, IL); and phospho-Src (Tyr537), paxillin, phospho-p130cas, and phospho-paxillin (118B; Cell Signaling Technologies, Beverly, MA). Densitometric analyses were done using ImageQuant TL software (GE Healthcare). The percentage of phosphorylated protein was calculated according to the formula: [(phosphoVol – Bg) / (Vol – Bg)] × 100%, where Bg represents the background, phosphoVol represents the volume of the phosphorylated protein band, and Vol represents the volume of the given protein band.

**Terminal deoxynucleotidyl transferase–mediated nick-end labeling assay, immunofluorescence, and immunohistochemical staining.** All stainings were done according to the protocols by Debnath et al. (16).

**Tumor growth analysis.** Each experimental group consisted of ~20 animals. All mice were observed daily for tumor appearance. The tumor-free days in each experimental group were counted. To analyze the difference in morbidity between the single and bitransgenic strains, Kaplan-Meier plots were used. Statistical analysis was assessed with the Mann-Whitney U test.

**Results**

SFK activity is inhibited by phosphorylation of the conserved, COOH-terminal tyrosine (17). The protein tyrosine kinase responsible for this phosphorylation is Csk (18). We and others identified

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the strains and it, most probably, could be assigned to the several weeks. This phenotype was not observed in any other of defective, as it gave birth only one time, and the litters died within (strains 4 and 7) were used for further experiments. Strain 6 was (strains 4, 6, and 7) gave germ line transition, only two of them transgene using Southern blotting (Fig. 1C). Positive litters were confirmed for the presence of the CHK transgene (human isoform) in the mammary glands of the transgenic mice was confirmed using Western blotting (Fig. 1F).

**Development of mammary glands in the Tg(MMTV-CHK) mice.** Whole mounts of mammary glands were prepared from the CHK transgenic mice [Tg(MMTV-CHK)]. Mammary glands were examined at several stages of development. Four time points were chosen: 8-week-old adult mice, mice at 16.5 days of pregnancy, lactating mice, and mice during involution (isolated 3 days after weaning). No alterations were observed in the overall patterning of the mammary tree at any time point (Fig. 2). Terminal buds were well developed and did not differ from those in the mammary glands of the wild-type mice. During progression to pregnancy and lactation, we observed robust growth of the mammary tree with a progression in the number and shape of the terminal buds (Fig. 2). These animals did not have any problems feeding their fetuses. Milk was found in the alveoli of microscopic sections taken from the lactating animals.

**Biochemical characterization of mammary glands in the Tg(MMTV-CHK) mice.** Protein extracts from the mammary glands were analyzed for the expression and activity of Src kinases. The activity of c-Src kinase was measured using a kinase assay (Fig. 3). As expected, the activity of this kinase was abrogated in the mammary glands of CHK transgenic mice. Of note, we did not detect hyperplasia or tumors in the mammary glands of the MMTV-CHK transgenic mice during the whole period of observation, which lasted for >2 years.

**Tumor development in bitransgenic mice carrying single oncogenes of Neu or activated Neu.** Newly created animals a second member of the Csk family, CHK, previously referred to as Matk, Ctk, Hyl, Ntk, Lsk, or Batk (19–27). CHK and Csk structurally share 53% amino acid identity overall and 59% amino acid identity within the catalytic domain. Like Csk, CHK phosphorylates the inhibitory COOH-terminal tyrosine of several SFKs, including Lck, Fyn, c-Src, and Lyn. Unlike Csk, which is ubiquitously expressed, the expression of CHK is limited to neuronal and hematopoietic cells. In addition, unlike Csk, CHK is capable of binding to some activated RTKs, including Neu (28, 29), resulting in inhibition of the RTK-induced activation of Src kinases. Our previous *in vitro* studies also showed extensive elevation of Src kinase activity upon heregulin stimulation of breast cancer cells, and that overexpression of CHK completely inhibited this activity (15, 28, 29). Based on these results, we aimed to generate CHK transgenes (expressed in a mammary tissue-specific manner) that would inhibit Neu-induced Src activity and then study the effects of this inhibition on mammary tumor formation *in vivo*.

**Generation of FVB-Tg(MMTV-CHK) mice.** We have generated CHK transgenic mice (Fig. 1A). Founders were backcrossed with wild-type FVB mice, and strains with germ line transmission were bred for further analysis. From the transgenic progeny (F₀), we obtained 10 animals that were positive for the CHK transgene as determined by PCR (Fig. 1B) and Southern blot analysis. All of the positive mice were crossed with the wild-type FVB/N mice. All litters were then screened for the CHK transgene using PCR. Positive litters were confirmed for the presence of the CHK transgene using Southern blotting (Fig. 1C). Although three strains (strains 4, 6, and 7) gave germ line transition, only two of them (strains 4 and 7) were used for further experiments. Strain 6 was defective, as it gave birth only one time, and the litters died within several weeks. This phenotype was not observed in any other of the strains and it, most probably, could be assigned to the transgene insertion site in this strain. As the MMTV promoter also gives expression in tissues other than the mammary epithelium, we examined the expression of CHK in various tissues. The expression of CHK was observed in the spleen, seminal vesicles, skin, thymus, and epididymis (Fig. 1D).

In addition, a detailed microscopic evaluation of several tissues was done. We found no significant differences between the wild-type animals and the MMTV-CHK transgenic mice in any of the analyzed tissues (data not shown). Expression of the CHK transgene (human isoform) in the mammary glands of the transgenic mice was confirmed using Western blotting (Fig. 1F).
harboring CHK under a breast tissue-specific promoter were crossed with MMTV-Neu or MMTV-NeuT transgenic mice, resulting in the generation of CHK/c-Neu and CHK/NeuT double transgenic mice as shown in Fig. 4A. The double transgenic offsprings were observed for tumor occurrence over a period of 54 weeks (Fig. 4B).

Interestingly, despite previous reports of CHK inhibiting the action of Neu in in vitro models (29), we did not detect any inhibitory effect of CHK on tumor appearance in the oncogene-expressing mice in vivo (CHK/NeuT and CHK/c-Neu double transgenic mice compared with the NeuT and c-Neu mice; Fig. 4B). Furthermore, our ex vivo experiments showed that there were no differences in the number of apoptotic cells in tumors (median of 5.92% versus 5.39%) that arose in the single oncogene-expressing mice versus the tumors in mice expressing the oncogene along with CHK. Similar observations were made when the proliferation marker [proliferating cell nuclear antigen (PCNA)] was assessed by immunohistochemistry in the single versus bitransgenic animals (Fig. 4C and D).

Src kinase activity and phosphorylation of c-Src targets in CHK and CHK/NeuT transgenic mice. Src kinase activity was significantly elevated in tumors developing in the NeuT transgenic mice, whereas tumors from the CHK/NeuT bitransgenic mice had drastically reduced Src kinase activity (Fig. 5A). This observation was confirmed in 10 different tumors developing in the CHK/NeuT bitransgenic mice compared with 10 different tumors derived from the NeuT mice (data not shown). Thus, CHK down-regulated pp60c-Src activity in the mammary tumors of the double transgenic mice. Consequently, the phosphorylation status of c-Src targets (FAK, p130cas, and paxillin) was largely abrogated in the samples derived from mammary tumors developing in the CHK/NeuT bitransgenic mice (Fig. 5B and C).

Taken together, our results show that while the activity of c-Src kinase was greatly reduced in tumors derived from the double transgenic mice (expressing the oncogene and CHK in their mammary tissue), these double transgenic mice developed tumors similar to those in the single transgenic NeuT or c-Neu mice (Fig. 4B). These experiments indicate that, at least in the mouse model, the activity of c-Src kinase is not essential in the signal transduction originating from the Neu oncogene, which leads to Neu-mediated oncogenesis.

Discussion

Neu (also known as ErbB2) amplification and elevated expression have been implicated in the etiology of human breast cancer and correlate with a poor clinical prognosis in these cancer patients (3). In addition, anti-ErbB2 antibodies show efficacy for the treatment of breast cancer patients with elevated Neu levels. Direct evidence supporting a role for Neu in mammary tumorigenesis is derived from observations made with transgenic mice expressing oncogenic forms of Neu. Mammary epithelial cell-specific expression of wild-type Neu or constitutively active Neu results in the induction of mammary tumors, which histologically resemble human adenocarcinomas. Although ErbB2/Neu plays a role in mammary tumorigenesis, the molecular mechanism by which Neu transforms cells and confers metastatic potential is not well elucidated.

Another tyrosine kinase involved in the etiology of breast cancer is the Src kinase. The overexpression or increased activity of cellular Src (c-Src) is frequently detected in human breast cancer (9, 10, 30, 31). However, the signaling pathway for the abnormal up-regulation of the protein expression or catalytic activity of c-Src in breast cancer remains undefined.
oncogenesis and did not alter the growth rate of the Neu-dependent tumors.

Inhibition of pathways mediated by the ErbB receptor family, especially Neu, is currently one of the most attractive areas of study in the development of novel therapeutic strategies for breast cancer. Although anti-Neu antibodies are now being introduced into antitumor regimens for advanced breast cancers overexpressing Neu, the effectiveness of this anti-Neu therapy is limited. Hence, other therapeutic approaches potentiating the capacity of anti-Neu therapies are needed. In vitro and ex vivo studies suggest that activation of Src kinase is necessary for Neu to induce breast cancer growth. Following this concept, Src kinase inhibitors are being developed for clinical applications, including breast cancer treatment. To validate the necessity of Src activation for the Neu-induced formation of breast cancer, we have generated transgenic mice expressing CHK (a natural inhibitor of Src activity) under the MMTV promoter. Overexpression of CHK in the mammary tissues of these transgenic mice did not significantly alter the development or function of their mammary tissue. However, when the MMTV/CHK transgenic mice were crossed with the MMTV/Neu or MMTV/NeuT transgenic mice, which are known to develop mammary tumors (3, 32–34), overexpression of CHK along with Neu significantly inhibited Neu-induced Src kinase activity in the developing mammary tumors, whereas no effects of CHK expression on the dynamics of tumor occurrence or tumor growth were noted. Noteworthy, recent data suggest that the MUC1 molecule can modify Src-mediated tumorigenesis in breast cancer in vivo by solely modifying the protein-protein interactions of the Src molecule, without altering the activity of Src (35). Interestingly, similar observations have recently been made for chronic myelogenous leukemia (CML; ref. 36).

Taken together, our results indicate that down-regulation of Src kinase activity by CHK overexpression has no effects on ErbB2/Neu-mediated oncogenesis, and that Src kinase expression and not Src kinase activity may play a role in the oncogenesis mediated by ErbB2/Neu. Although we can not exclude the possibility that the CHK-dependent COOH-terminal phosphorylation of Src affects its abilities as an adaptor protein, we strongly suggest that Src enzymatic activity is not a requisite for Neu-induced tumorigenesis.

Acknowledgments


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Because the necessity of SFK activation for Neu-induced breast tumor formation had not been confirmed using genetically modified mouse models, we used CHK as a natural inhibitor of Src activity following Neu activation (15) and generated transgenic mice expressing CHK in their mammary tissue. As shown in Fig. 3, we successfully inhibited Neu-induced Src activity in CHK-expressing animals. Consequently, we expected CHK to inhibit mammary tumor formation or at least slow down the growth of Neu-induced tumors. However, in our experiments, we did not observe any significant effects of CHK expression on the dynamics of tumor occurrence or growth (Fig. 4). Moreover, tumors that developed in any group had comparable levels of PCNA or apoptotic cells within their mass (Fig. 4). Thus, our results showed that down-regulation of SFK activity by CHK overexpression had no significant effects on Neu-mediated

Figure 5. A, Src kinase activity and the expression of c-Src protein in mammary tumors versus mammary glands in the MMTV-NeuT and MMTV-CHK/NeuT transgenic strains. B, Western blot analysis of the phosphorylation status of the c-Src protein kinase targets: paxillin, p130cas, and FAK. Total cell lysates were prepared from tumors developing in MMTV-NeuT transgenic mice and the MMTV-CHK/NeuT double transgenic mice. The lysates were immunoprecipitated with FAK antibody, and then the immunocomplexes were analyzed by SDS-PAGE and Western blotted with either phospho-Py99 or FAK antibodies. In addition, total cell lysates were analyzed by SDS-PAGE and Western blotted with either phospho-paxillin antibody or with paxillin antibody. Bottom, phosphorylation of p130cas using phospho-p130cas antibody. Expression of CHK in the double transgenic mice, as indicated. Actin was used in both transgenic mice to confirm equal loading. C, densitometric analyses of phosphorylated c-Src targets (paxillin and FAK). The Student’s t test was applied for the statistical analyses.

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