Csk Homologous Kinase, a Novel Signaling Molecule, Directly Associates with the Activated ErbB-2 Receptor in Breast Cancer Cells and Inhibits Their Proliferation*

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Sheila Zrihan-Licht, Bijia Deng, Yosef Yarden‡, Gina McShan, Iafa Keydar§, and Hava Avraham¶

From the Divisions of Experimental Medicine and Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts 02115, the ‡Department of Chemical Immunology, the Weizmann Institute of Science, Rehovot 76100, Israel, and the §Department of Cell Research and Immunology, Tel Aviv University, Ramat Aviv 69978, Israel

Substantial evidence exists supporting direct roles for ErbB-2/neu and Src kinase activation in breast cancer. The Csk homologous kinase (CHK) is a recently identified tyrosine kinase which, like Csk, phosphorylates the C-terminal tyrosine of Src kinases, resulting in inactivation of these enzymes. Recently, we observed that CHK is associated with the ErbB-2/neu receptor upon heregulin stimulation of breast cancer cells. Here, we report that CHK expression was observed in 70 out of 80 primary breast cancer specimens but not in normal breast tissues (0/19). Confocal microscopy analysis revealed colocalization of CHK with ErbB-2 in these primary specimens (6/6). In addition, we observed that the cytoplasmic domain of the ErbB-2/neu receptor is sufficient for its interaction with the CHKSH2 domain. Phosphopeptide inhibition of the in vitro interaction of CHKSH2 or native CHK with ErbB-2/neu, as well as site-directed mutagenesis of ErbB-2/neu, indicated that CHKSH2 binds to Tyr1253 of ErbB-2/neu. Interestingly, autophosphorylation at this site confers onco-sensitiveness to this receptor. Moreover, CHK was able to down-regulate ErbB-2/neu-activated Src kinases. Overexpression of CHK in MCF-7 breast cancer cells markedly inhibited cell growth and proliferative response to heregulin as well as decreased colony formation in soft agar. These studies indicate that CHK binds, via its SH2 domain, to Tyr1253 of the activated ErbB-2/neu and down-regulates the ErbB-2/neu-mediated activation of Src kinases, thereby inhibiting breast cancer cell growth. These data strongly suggest that CHK is a novel negative growth regulator in human breast cancer.

Breast cancer is the second leading cause of cancer death among women in the United States and is the leading cause of death among women aged 30 to 70 (1–3). The majority of breast carcinomas appear to be sporadic and have a complex accumulation of molecular and cellular abnormalities that constitute the malignant phenotype (4–5). In many cases, random onset of breast cancer has been correlated with increased ErbB-2/neu receptor expression and Src tyrosine kinase activity (6–12). Substantial evidence indicates that the c-Src proto-oncogene and ErbB-2/neu play important roles in breast cancer (7, 13). Src kinase activity is elevated in ErbB-2/neu (Neu) induced mammary tumors, and this elevated activity correlates with its capacity to physically associate with ErbB-2/neu (14–15). A common pathway linking the activation mechanisms in ErbB-2/neu amplification in breast cancer is increased tyrosine kinase activity, which leads to cellular transformation (16).

Four members of the ErbB (HER) family are presently known: p170ErbB-1 (epidermal growth factor receptor (EGF-R))1, p185ErbB-2, p180ErbB-3, and p180ErbB-4 (3, 17, 18). In particular, the overexpression of the p185ErbB-2 correlates with a poor clinical prognosis in breast cancer (9). ErbB-2/neu undergoes autophosphorylation on five tyrosine residues that are located on its non-catalytic C terminus (19, 20). The autophosphorylated tyrosine residues function as docking sites for proteins that contain SH2 domains (19, 20). The sequence homology between the human and rodent ErbB-2/neu is high, particularly in the C terminus (98%). The autophosphorylated tyrosine residue Tyr1253 of rodent neu (20, 21) or the human homologue Tyr1248 of ErbB-2 (21) is the most critical residue for onco-sensitiveness and the transforming potential of ErbB-2/neu. Although overexpression of the ErbB-2/neu gene products contributes to the aggressive behavior of various human adenocarcinomas, including breast cancer (12, 22, 23), the precise molecular mechanisms explaining this phenomenon are unknown.

Ligands that bind to and stimulate the kinase activity of ErbB family members have been identified and are classified as “EGF-like” ligands. Heregulin (HRG) and its rat homologue, neu differentiation factor (NDF), are a subfamily of neu-regulins that bind to and activate both ErbB-3 and ErbB-4 (22, 24–26). Recently, neuregulin-2, a new ligand of ErbB-3/ErbB-4, was characterized (27). Although none of these factors binds directly to ErbB-2/neu, both EGF and HRG induce its tyrosine phosphorylation, presumably by ligand-driven heterodimerization and cross-phosphorylation (22, 25, 27, 28).

The Csk homologous kinase (CHK), originally referred to as

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1 The abbreviations used are: EGF-R, epidermal growth factor receptor; HRG, heregulin; SH2 and SH3, Src homology domains 2 and 3, respectively; CHK, Csk-homologous kinase; GST, glutathione S-transferase; CHKSH2, GST fusion protein containing the SH2 domain of CHK; EGF, epidermal growth factor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.

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the megakaryocyte-associated tyrosine kinase, was identified in our laboratory (29–31). The kinase was also independently identified as Lsk (32), Hyl (33), Ctk (34), and Batk (35). CHK shares ~55% identity with Csk tyrosine kinase (29, 30) and consists of SH3, SH2, and tyrosine kinase domains. In contrast to Csk, which is widely expressed, CHK is highly restricted in its expression to brain and hematopoietic cells. Like Csk, CHK was also shown to phosphorylate pp60src in vitro on its C-terminal tyrosine (29, 36, 37). Studies in which CHK or Csk was expressed in Csk-deficient mouse embryo fibroblasts showed that murine p52 CHK was comparable to Csk in its ability to reduce the activity of the Src kinases Fyn and pp60src (37).

Recently, we found a specific interaction between CHK and the HRG-activated ErbB-2/neu receptor in MCF-7 and T47D breast cancer cell lines. The CHKSH2 and CHKSH2-SSH2 domains precipitated the tyrosine-phosphorylated ErbB-2/neu receptor upon stimulation with HRG. In vivo association of the tyrosine-phosphorylated ErbB-2/neu with CHK was also observed in co-immunoprecipitation studies using anti-CHK antibodies. This association of CHK with ErbB-2/neu occurred via the CHK-SH2 domain and appeared to be receptor-specific (e.g. ErbB-2/neu) and ligand-specific (e.g. HRG) (38).

In this report, we characterize the binding of the SH2 do- main of CHK to specific tyrosine-phosphorylated sites on ErbB-2/neu. Our results indicate that the CHKSH2 domain binds to the ErbB-2/neu receptor and that CHK directly associates with the activated ErbB-2/neu receptor. These findings further support the hypothesis that CHK down-regulates ErbB-2/neu activation and inhibits cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Recombinant human HRG (pHRG-β1, 177–244), rabbit polyclonal anti-ErbB-2/neu antibodies, and 3E8 monoclonal anti-ErbB-2/neu antibodies were generously provided by Dr. Geert A. Sliwkowski, Genentech (San Francisco, CA). Ab-4 anti-neu oncogene antibodies were purchased from Oncogene Science. Monoclonal anti-phosphotyrosine antibody (4G10) was kindly provided by Dr. Brian J. Druker (Division of Hematology and Medical Oncology, Oregon Health Sciences University, Portland). Monoclonal and polyclonal antibodies for EGF-R and polyclonal anti-CHK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GST monoclonal antibody was purchased from Pharmacia Biotech Inc. Rhodamine-conjugated anti-rabbit IgG antibody was obtained from Sigma. EGF was purchased from Collaborative Biomedical Products (Bedford, MA). Tyrosine-phosphorylated and non-phosphorylated synthetic peptides were obtained from the Dana Farber Cancer Institute Molecular Biology Core Facility (Boston, MA). Peptides were analyzed for purity by high pressure liquid chromatography, mass spectrophotometry, and amino acid analysis. T₇ polymerase vaccinia recombinant virus, the vaccinia wild-type virus, and the PTM-1 vector were generously provided by Dr. Bernard Moss (National Institutes of Health, Bethesda). CHKSH2 GST fusion protein was prepared as described previously (31, 38). The primers for the polymerase chain reaction were synthesized with an automated DNA synthesizer (Applied Bio-system model 384). Reagents for electrophoresis were obtained from Bio-Rad. Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Corp. All other reagents were purchased from Sigma.

**Cell Cultures**—The MCF-7, NIH3T3, HeLa, COS, BSC-1, and CV-1 cell lines were all obtained from ATCC (American Type Culture Collection, Rockville, MD). MCF-7 cells stably transfected with CHK-Flag pDNA250 or pDNA300 constructs, resulting in CHK-Flag-MCF-7 or CHK-MCF-7 cells, respectively, were previously described (38). MCF-7 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 5 μg/ml insulin (Sigma), 1 mM nonessential amino acids, and 1 mM sodium pyruvate. Prior to stimulation with HRG or EGF, cells were starved overnight in media containing 1% fetal bovine serum and then starved for 4 h in serum-free media. COS, HeLa, CV-1, and BSC-1 cells were grown in DMEM supplemented with 10% fetal bovine serum. NIH3T3 cells were grown in DMEM supplemented with 10% calf serum.

**Immunohistochemistry and Confocal Laser Scanning Microscopy Analysis**—Paraffin blocks from human primary breast carcinomas were serially sectioned at 5–6 μm. Tissue sections were stained with hematoxylin and eosin to identify tumor as well as normal breast tissue. Par- allel sections were deparaffinized, washed in PBS, blocked (1% normal donkey serum and 0.1% bovine serum albumin in PBS) for 1 h, and double-stained with rabbit anti-human CHK polyclonal antibody and mouse monoclonal antibody against ErbB-2 (Oncogene Science). Following washing, the cells were incubated with fluorescein isothiocya- natelabeled rabbit donkey anti-rabbit (1:50) and rhodamine-conjugated donkey anti-mouse antibodies (1:100) (The Jackson Laboratory, Bar Harbor, ME). Stained cells were analyzed using a Zeiss confocal laser scanning microscope. The Zeiss (Oberkochen, Germany) confocal laser scanning microscope 410 is equipped with a 25-megawatt krypton-argon laser and a 10-megawatt HeNe laser (488, 543, and 633 maximum lines); co-localization analysis was performed on simultaneously labeled samples using the Zeiss co-localization procedure. This procedure results in a graphic representation of the distribution of green (y axis) and red (x axis) fluorescence for each pixel. Images were printed using a Codonics NP 1600 printer (Codonics, Middleburg Heights, OH).

For confocal microscopy, CHK-Flag-MCF-7 cells were fixed and pre- pared as described previously (39). Fixed and permeabilized cells were incubated with CHK antibodies (1:100 dilution) (Santa Cruz, CA) for 1 h. Cells were washed in PBS, incubated with rhodamine-conjugated (rabbit IgG) antibodies for 1 h (Sigma), and examined using a Sarastro 2000 confocal laser scanning microscope (confocal laser scanning microscope, Molecular Dynamics, Sunnyvale, CA).

**Expression Vectors and Transfection**

NEC and TEC chimeric plasmids containing the extracellular domain of EGF-R and the transmembrane domain of the ErbB-2/neu oncogene were previously described (19, 26). The P₁ and Y1253F mutated constructs as well as the neu* construct were previously described (19–20). Transfections of NIH3T3 and COS cells were performed using LipofectAMINE reagents (Life Technologies, Inc.) according to the manufac- turer’s protocol.

**Precipitation with GST Fusion Proteins and Immunoprecipitation**

Approximately 5 × 10⁶ cells/plate TEC or NEC stable transfectants were starved for 4 h and then stimulated with 100 ng/ml EGF for 5 min at room temperature and analyzed as described previously (38). Lysates were precleared and then precipitated with GST fusion proteins coupled to glutathione-Sepharose beads (40). For the immunoprecipitations, polyclonal CHK antibodies (10 μl), monocular EGF-R (10 μg/ml) (Santa Cruz, CA), or ErbB2/neu antibodies (10 μg/ml) (Oncogene Science) (10 μg/ml) were used. The samples were analyzed by 5% or 10% SDS-PAGE as described (38). Bound proteins were immunoblotted with anti-phosphotyrosine (4G10), polyclonal EGF-R antibodies, or polyclonal ErbB-2/neu antibodies. The blots were developed using the ECL system (Amersham Corp.). Blots were stripped for 30 min at 55°C in stripping buffer according to the manufacturer’s protocol (Amersham Corp.).

**Peptide Inhibition of the CHK SH2 GST Fusion Protein-ErbB-2/neu Interaction**

Cell lysates from COS cells transiently transfected with the neu*, P₁, or Y1253F plasmids were added to the CHKSH2 GST fusion proteins (10 μg/incubation) that were preincubated with synthetic peptides as described previously (40). Lysates were incubated for 1 h at 4°C and then precipitated by the addition of glutathione 4B for 30 min at 4°C. The washed precipitates were separated on 7.5% SDS-PAGE.

**Association of CHKSH2 GST or Native CHK with Peptide Beads**

Phosphorylated and non-phosphorylated peptides were linked to Affi-Gel 15 as described previously (40). Peptide beads (15-μl bead volume) were incubated with 10 μg of CHKSH2 for 1.5 h at 4°C. The washed samples were separated on 10% SDS-PAGE. To test the binding of native CHK to the peptide beads, 15 μl of beads was incubated with 1.5 ml of MCF-7, CHK-Flag-MCF-7, or neo-1 cell extracts for 1.5 h at 4°C. After three washes, the precipitates were subjected to SDS-PAGE and Western blotting with anti-CHK antibodies.

**T₇ Polymerase Vaccinia Expression System**

The CHK cDNA (1.6 kb) was cloned into EcoRI sites in the PTM-1 vector. The NoI site that includes the ATG initiation codon was added to the CHK sequence by polymerase chain reaction using a 5’ sense...
primer from nucleotides 269 to 296 and a 3′ antisense primer from nucleotides 510 to 481 (30). The polymerase chain reaction product was introduced to the vector at the Ncol-BstEII sites (New England Biolabs, Beverly, MA). The construct was sequenced, and its expression was examined by transfection assay followed by Western blot analysis.

Generation of recombinant CHK vaccinia virus was performed using the method described previously (41). The CHK vaccinia recombinant virus was used along with the Tp vaccinia recombinant virus for the co-infection assay, as follows: approximately 5 × 105 cells/plate were seeded. One day later, the cells were infected with trypsinized recombinant viruses 10 × multiplicity of infection each, for 1–2 h in 2.5% fetal calf serum/DMEM at 37 °C. Next, 5 ml of 10% DMEM were added, and the plates were incubated overnight. The infected cells were harvested the day after infection. Infections of transiently transfected COS cells were performed 2 days after transfections, as described above.

**Cell Growth Assay**

Cells (105 cells/well) were spread in microtiter plates (96-wells), and the number of live cells was determined by using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide method (42) at the indicated days after spread.

**Transformation Assays**

Transformation of the cells was assessed by their ability to demonstrate anchorage-independent growth (42). MCF-7, neo-1, or CHK-Flag cells (1 × 103 in 6-well dishes) were grown in medium containing 0.4% agar. After 2 weeks of growth, the colonies were visualized by staining with 33% isodinitrotetrazolium violet. All assays were performed in duplicate.

**In Vitro Kinase Assay**

MCF-7 cells were co-infected with the Tp polymerase vaccinia virus and CHK recombinant virus as described above. One day post-infection, the cells were starved for 4 h in serum-free media and then stimulated with 10 nM HRG as described previously (38). Cell lysates were immunoprecipitated using anti-Src antibodies, and the washed precipitated proteins were submitted to kinase assay or analyzed by SDS-PAGE. In vitro kinase assays were performed by incubating washed Src immunoprecipitates with lysates from CHK-expressing or non-expressing cells in kinase buffer for 20 min at room temperature as described (14–15, 29). Proteins were separated by 7.5% SDS-PAGE under reducing conditions. The gels were dried and subjected to autoradiography.

**RESULTS**

**Expression and Localization of CHK in Breast Cancer—**Immunohistochemical studies demonstrated CHK protein expression in 70 of 80 breast adenocarcinoma specimens (stage I, 32/41; stage II, 34/35; stage III, 4/4) but not in normal or benign breast tissues (normal breast and fibroadenoma, 0/19). Confocal microscopic imaging in primary breast tumors 6/6 (Fig. 1A) and activated T47D cells (not shown) immunostained for CHK expressing or non-expressing cells in kinase buffer for 20 min at room temperature as described (14–15, 29). Proteins were separated by 7.5% SDS-PAGE under reducing conditions. The gels were dried and subjected to autoradiography.

**Association of the CHK SH2 Domain with NEC and TEC EGF-R/neu Chimeric Receptors—**To verify that the association of CHK with ErbB-2/neu is mediated exclusively by ErbB-2/neu and not by other members of the ErbB family, we used chimeric molecules composed of the extracellular domain of the EGF-R and the transmembrane and cytoplasmic domains of ErbB-2/neu. Two different EGF-R/ErbB-2/neu chimeric plasmids were used: the TEC construct which contains a point mutation (Val1664 → Glu) in the ErbB-2/neu transmembrane domain causing constitutive activation of the molecule, and the NEC construct that contains the wild-type sequence of the ErbB-2/neu cytoplasmic domain and therefore can be stimulated by EGF (19). NIH3T3 cells were stably transfected with either the NEC or TEC construct. The transfected cells were analyzed for construct expression by immunoprecipitation followed by Western blot analysis using anti-EGF-R antibodies. Upon EGF stimulation, CHKSH2 was associated with NEC (Fig. 2, B-II), whereas its association with TEC was constitutive and not dependent on EGF stimulation (Fig. 2, A-II). Therefore, the cytoplasmic domain of the ErbB-2/neu receptor appears to be sufficient for its interaction with the CHKSH2 domain.

**Inhibition of the CHKSH2-ErbB-2/neu Interaction by Tyrosine-phosphorylated Peptides—**To identify the binding site of CHKSH2 within the ErbB-2/neu receptor, we synthesized a series of tyrosine-phosphorylated peptides derived from the five autophosphorylated tyrosine residue sites of the cytoplasmic domain of the ErbB-2/neu receptor (Tyr1028, Tyr1144, Tyr12867, and Tyr12553). These tyrosine-phosphorylated peptides were used to inhibit the interaction between the CHKSH2 domain and the activated ErbB-2/neu receptor. COS cells were transiently transfected with the transformed ErbB-2/neu (neu*) plasmid that codes for the constitutively phosphorylated receptor (point mutation Val1664 → Glu), as described previously (19–20). Complexes of ErbB-2/neu and CHKSH2 were indicated by the presence of ErbB-2/neu in the washed CHKSH2 GST fusion protein precipitates (Fig. 3, A and B). Of the four peptides, peptide P1 (ENPEY*LGLDV, where * indicates the phosphophorylated tyrosine residue) most significantly inhibited complex formation (Fig. 3A). We also found inhibition by peptide P5 (AEYY*LVPQQ). To compare the relative abilities of the P1 and P5 peptides to inhibit the CHKSH2-ErbB-2/neu interaction, various concentrations of peptides from 5 to 100 μM were tested. The results indicate that inhibition by the P1 peptide is much more significant throughout all the tested concentrations as compared with other peptides (Fig. 3C) and suggest that binding of CHK is primarily at the P1 (ENPEY*LGLDV) site of the ErbB-2/neu receptor. Moreover, inhibition by the P1 peptide was phosphorylation-dependent, since the P5 non-phosphorylated peptide had no inhibitory effect (data not shown).

**Binding of the CHKSH2 Domain and Native CHK to Immobilized Peptides—**To test further the binding of CHK to the phosphorylated P1 site, we linked the tyrosine-phosphorylated P1 peptide or the non-phosphorylated P1 peptide to Affi-Gel 15 beads, and the association of either CHKSH2 GST fusion protein or native CHK to the beads was analyzed. As shown in Fig. 4A, CHKSH2 GST was associated in a phosphotyrosine-dependent manner to the phosphorylated P1 peptide. Similar specificity was observed when we tested the association of native CHK to the peptide beads. The (P1)7 peptide was able to associate with native CHK from extracts of CHK-Flag-MCF-7-transfected cells (Fig. 4B). No binding was observed by the MCF-7 or neo-1 lysates, which do not express CHK. Similar results were demonstrated using CHK obtained from the vaccinia expression system (Fig. 4C). This specificity was in agreement with the peptide inhibition experiments, indicating a direct association between ErbB-2/neu and CHKSH2 mediated by the P1 tyrosine-phosphorylated site.

**CHK Is Associated with the P1, Phosphotyrosine as Shown by Site-directed Mutagenesis—**To confirm the significance of the phosphorylated peptide studies, we analyzed whether the CHKSH2 GST fusion protein could bind to the ErbB-2/neu receptor bearing the P1 phosphotyrosine site alone (Tyr12553). Two constructs of the activated ErbB-2/neu receptor (neu*-mutated Val1664 → Glu) (19, 20) were used to transfect the COS cells as
FIG. 1. Confocal micrographs of breast tumors and CHK-Flag-MCF-7 cells. A, co-localization analysis of ErbB-2 and CHK in primary breast tumors. Confocal micrographs of breast cancer tissues immunolabeled with ErbB-2 staining (dilution 1:500), followed by rhodamine-labeled secondary antibody (red), CHK staining (dilution 1:100), followed by fluorescein isothiocyanate-labeled secondary antibody (green) are shown. For overlay images, yellow represents co-localized staining. Using co-localization analysis, the co-localized pixels were depicted (blue) and overlaid on the red-green image. A graphic representation of the distribution of green (y axis) and red (x axis) fluorescence for each pixel is shown. The number of pixels is color coded where dark blue indicates one to few, yellow is high, and dark red is the highest co-localization. Similar results were obtained with an additional five breast cancer specimens. B, CHK-Flag-MCF-7 transfected cells. CHK-Flag-MCF-7 cells were stimulated with HRG (10 nM...
were added to CHKSH2 which was preincubated individually with each of four different ErbB-2/neu phosphopeptides. Extracts from neu* transiently transfected COS cells were added to CHKSH2 GST fusion protein which was preincubated individually with each of four different ErbB-2/neu phosphopeptides as indicated below. The final concentration of peptide was 100 μM for each incubation. The samples were washed and separated on 7% SDS-PAGE and immunoblotted with monoclonal anti-phosphotyrosine antibody (4G10) or with polyclonal anti-EGF-R antibodies. The other half of the lysates was immunoprecipitated using monoclonal antibodies for EGF-R (A-I and A-II) or with the chimeric plasmid EGF-NEC-ErbB-2/neu (TEC) (B). TEC and NEC cells (5 × 10⁶ cells/plate) were serum-starved and then stimulated with 100 ng of EGF at room temperature for 5 min. The lysates were divided into two parts; one-half of the lysates was precipitated with the CHKSH2 GST fusion protein (10 μg) for 90 min at 4 °C (A-II and B-II). After washing, the precipitates were separated by 7% SDS-PAGE and immunoblotted with monoclonal anti-phosphotyrosine antibody (4G10) or with polyclonal anti-EGF-R antibodies. The other half of the lysates was immunoprecipitated using monoclonal antibodies for EGF-R (A-I and B-I) for 16 h at 4 °C. The washed precipitates were run on 7% SDS-PAGE and blotted with 4G10 or with anti-EGF-R antibodies.

follows: 1) the P1 construct that contains the extracellular and transmembrane domains of ErbB-2/neu and the P1 binding site, and 2) the Y1253F construct that contains the full sequence of the constitutively activated ErbB-2/neu, including a point mutation at the P1 site (Tyr1253 → Phe). Both ErbB-2/neu constructs were tyrosine-phosphorylated (Fig. 5A). Cell extracts from the same experiment were incubated with the CHKSH2 GST fusion protein, separated by SDS-PAGE, and analyzed by Western blotting using ErbB-2 antibody. The CHKSH2 GST precipitated the ErbB-2/neu in the COS cells transfected with the P1 construct (Fig. 5B), whereas no association was found with the ErbB-2/neu carrying the point mutation on the P1 site (Y1253F). Therefore, the CHKSH2 exclusively bound to the P1-Tyr1253 site of ErbB-2/neu.

To examine for the in vivo association of the CHK molecule with the P1-Tyr1253 site, we used the T7 polymerase vaccinia system to overexpress CHK. COS cells were first transiently transfected with the P1 plasmid, and then 2 days post-transfection the cells were co-infected with the T7 polymerase virus alone or with the T7 polymerase and CHK recombinant viruses. CHK was expressed in cells infected with the CHK recombinant virus but not in cells that were infected with the T7 polymerase virus alone (Fig. 5C). Lysates from the same experiment were tested for the expression of the P1-ErbB-2/neu molecule using anti-ErbB-2/neu antibody (Fig. 5D).

To demonstrate the in vivo association between P1-ErbB-2/neu and CHK, the same cell extracts were immunoprecipitated using CHK antibody and then immunoblotted with ErbB-2/neu antibody. P1-ErbB-2/neu was present only in precipitates from the CHK-expressing cells and not in those of the T7-infected cells (Fig. 5D). Taken together, these results indicate an in vivo association between CHK and the P1 binding site (Tyr1253) of ErbB-2/neu.

Investigation of ErbB-2/neu Signaling Mechanisms by Vaccinia-driven Overexpression of CHK in MCF-7 Cells—To elucidate the involvement of CHK in the regulation of Src kinase activity, we again overexpressed CHK using the T7 polymerase-vaccinia expression system. MCF-7 cells were co-infected either with a CHK vaccinia recombinant virus (CHK-vacc) and T7 polymerase virus (T7) or with the T7 virus alone as a control. One day after co-infection, the cells were starved for 4 h and then stimulated with heregulin (10 nM). Cell extracts were immunoprecipitated using Src antibody, and the enzymatic activity of Src was determined using poly(Glu/Tyr) (4:1) as a substrate. In CHK-expressing cells, poly(Glu/Tyr) phosphorylation was decreased about 4-fold compared with the control T7-infected cells upon stimulation with HRG (Fig. 6). Therefore, CHK expression resulted in a significant reduction in Src activity of Src.
Suppression of Cell Growth by CHK—To elucidate whether CHK might affect the growth of MCF-7 cells, the proliferation rate of CHK-MCF-7 clones was analyzed using the 3-(4,5-di-

methyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide method (Fig. 7). The proliferation rate of CHK-expressing cells, compared with the control untransfected MCF-7 cells or cells transfected with vector alone (neo-1) (Fig. 7A). Furthermore, when the cells expressing CHK were stimulated with HRG, we observed a significant reduction in their proliferative response to HRG (Fig. 7A). These data suggest that CHK can reduce the proliferative activity of breast cancer cells and cause desensitization to the growth-promoting effects of HRG.

The anti-transforming potential of CHK was evaluated in MCF-7 clones by examining the ability of CHK-transfected cells to escape contact inhibition when grown on tissue culture plastic and to support anchorage-independent growth in soft agar. MCF-7 cells, as well as neo-1 cells, grew in culture plates to a higher density, displayed a tendency to pile up (data not shown), and acquired the ability to form colonies in soft agar (Fig. 7B). CHK-expressing cells did not show anchorage-independent growth, and the number of colonies formed in soft agar decreased approximately 4-fold compared with control MCF-7 or neo-1 cells (Fig. 7B). Taken together, these results demonstrate that CHK expression is associated with anti-proliferative activity and can reduce the transformation ability of breast cancer cells.
to demonstrate that the interaction of CHKSH2 at known to confer oncogenicity and transforming abilities to the chimeric EGFR-Erbb-2/neu receptor constructs, NEC and TEC (Fig. 2). Moreover, we identified the binding site of CHKSH2 to the nuclear CREB-binding protein (CBP) (14–15, 25). However, the identification of CHK as a signaling molecule that directly interacts with the P1-Tyr1253 site implies that CHK might modulate ErbB-2/neu activity and oncogenicity, since this C-terminal sequence enables coupling of ErbB-2/neu signaling to downstream pathways that include Ras, mitogen-activated protein kinase, and transactivation of c-Jun (19). The results presented here further suggest that CHK exerts anti-mitogenic activity, since its overexpression in MCF-7 cells decreased their proliferative response to HRG stimulation. In addition, MCF-7 cells overexpressing CHK showed a reduction in colony size and number when assayed for growth in soft agar (Fig. 7).

Activation of not only ErbB-2/neu but downstream Src tyrosine kinases plays an important role in mammary tumorigenesis (44–47). Human breast cancer specimens possess more than a 4-fold increase in c-Src tyrosine kinase activity when compared with normal breast tissues (16), and neu-induced mouse mammary tumors possess a 6–7-fold increase in c-Src tyrosine kinase activity as compared with their normal counterparts (14, 15). Transgenic mice expressing the middle T antigen in the mammary epithelium (44) have 4–5-fold increases in the tyrosine kinase activities of both c-Src and the Src family kinase c-Yes (45). Furthermore, expression in transgenic mice of a constitutively active form of c-Src (V527F) under the control of the murine mammary tumor virus promoter/enhancer results in epithelial hyperplasia and mammary tumors (23). The increased Src kinase activity observed in neu-induced tumors also results from the ability of the SrcSH2 domain to directly interact with ErbB-2/neu in a phosphotyrosine-independent manner. Both Src and Yes were found to bind to the same site on ErbB-2/neu (14–15, 25, 43). However, the site of interaction between Src family members and ErbB-2/neu is not yet known. In the present study, we demonstrate that upon HRG stimulation of breast cancer cells, there is extensive elevation of Src kinase activity and that expression of CHK completely inhibits this activity. CHK down-regulates Src kinase activity probably by phosphorylation of its C-terminal regulatory tyrosine. The results presented here further suggest that CHK can exert anti-proliferative activity. Overexpression of CHK in MCF-7 cells resulted in decreased cell proliferation and a significant decline in the number and size of the colonies formed in soft agar (Fig. 7).

The observations that activation of Src in ErbB-2/neu-expressing cells occurs through tyrosine-phosphorylated ErbB-2/neu and that CHK can down-regulate ErbB-2/neu-activated Src kinases have important implications in understanding the biological properties of the molecules. It is conceivable that CHK association to ErbB-2/neu upon HRG stimulation is followed by the down-regulation of ErbB-2/neu-activated Src kinases. We suggest a model for this regulation of ErbB-2/neu-activated Src kinases by CHK based on our data (Fig. 8). In this model, stimulation of ErbB-2/neu by HRG, presumably via
FIG. 8. Model for the regulation of ErbB-2-activated Src kinases by CHK.

ligand-driven heterodimerization and cross-phosphorylation, leads to autophosphorylation of ErbB-2/neu and an initial association of Src kinase to one of the phosphorylated tyrosine sites, thereby resulting in the activation of the Src kinase (Fig. 8A). Association of CHK with P_1-Tyr^{1253} on activated ErbB-2/neu is likely to facilitate phosphorylation of Src at its C-terminal phosphotyrosine (Fig. 8B). These interactions lead to the inactivation and dissociation of the Src kinase due to the self-association of the C-terminal phosphotyrosine to its own SH2 domain (Fig. 8C). Further studies will address this model, particularly whether Src kinase bound to ErbB-2/neu is the primary substrate for CHK. Investigation of the role of CHK in breast cancer may contribute to an understanding of the mechanisms of oncoprotein signal transduction and provide a basis for utilizing this oncogenic kinase to oppose the malignant process.

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Sheila Zrihan-Licht, Bijia Deng, Yosef Yarden, Gina McShan, Iafa Keydar and Hava Avraham

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