Association of Csk-homologous Kinase (CHK) (formerly MATK) with HER-2/ErbB-2 in Breast Cancer Cells*

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Protein-tyrosine kinases, such as HER-2/ErbB-2, have been specifically linked to breast cancer. The Csk-homologous kinase (CHK), formerly MATK, is a tyrosine kinase that contains the Src homology 2 and 3 (SH2 and SH3) domains and demonstrates homology (~50%) to the Csk tyrosine kinase. Like Csk, CHK is able to phosphorylate and inactivate Src family kinases. In this report, we investigated whether CHK is expressed in breast cancer tissues and whether it participates in the ErbB-2 signaling pathway in T47D and MCF-7 breast cancer cell lines. Immunostaining of the CHK protein in breast tissues demonstrated that primary invasive ductal carcinomas, stage II (13 of 15 cases) and stage I (8 of 15 cases), expressed the CHK protein, while this protein was not detected in the adjacent normal tissues from the same patients. To study the role of CHK in the ErbB-2 signaling pathway, glutathione S-transferase fusion proteins containing the SH2 and SH3 domains of CHK were generated. CHK-SH2 and CHK-SH2-SH3, but not CHK-SH3 or CHK-NH2-SH3, precipitated the tyrosine-phosphorylated ErbB-2 upon stimulation with heregulin. EGF or interleukin-6 stimulation of T47D cells failed to induce CHK-SH2 association with ErbB-2, the EGF-receptor, or the interleukin-6 receptor. In vivo association of the tyrosine-phosphorylated ErbB-2 with CHK was observed in co-immunoprecipitation studies with anti-CHK antibodies.

EGF-R, ErbB-3, and ErbB-4 were not detected in the CHK immunoprecipitates or in the precipitates of the GST-SH2 fusion proteins of CHK, suggesting that the association of CHK with ErbB-2 upon heregulin stimulation is receptor-specific (ErbB-2) and ligand-specific (heregulin). These results indicate that CHK might participate in signaling in breast cancer cells by associating, via its SH2 domain, with ErbB-2 following heregulin stimulation.

Protein-tyrosine kinases are involved in the regulation of cell growth and differentiation. The binding of a ligand to the extracellular domain of a cognate receptor protein-tyrosine kinase induces receptor dimerization, stimulation of intrinsic kinase activity, and autophosphorylation. Protein-tyrosine kinases elicit their function by binding and/or phosphorylating intracellular substrate proteins (1–3). The tyrosine-phosphorylated sites in the activated receptors function as high affinity binding sites for proteins containing SH2 domains (1–2, 4–6).

Constitutive activation of these signaling pathways is apparent in many malignancies. A variety of human tumors overexpress the ErbB (HER) family of type I receptor protein-tyrosine kinases (7, 8). Four members of this family are presently known: p170ErbB-1 (epidermal growth factor receptor; EGF-R), p185ErbB-2, p180ErbB-3, and p185ErbB-4 (9–13). In particular, the overexpression of p185ErbB-2 correlates with a poor clinical prognosis of breast cancer (8–9, 14). The overall amino acid homology within this receptor family ranges from 40 to 50%. All of the family members are characterized by two cysteine-rich regions in the extracellular domain, a single transmembrane region and a large cytoplasmic domain that exhibits tyrosine kinase activity (14).

Several ligands that bind to and stimulate the kinase activity of the ErbB family members have been identified and are classified as EGF-like ligands. EGF, HB-EGF, amphiregulin, betacellulin, epiregulin, and transforming growth factor-α (TGF-α) are the ligands for the EGFR (ErbB-1) (15–17). Heregulin (HRG) and its rat homologue neu differentiation factor (NDF) are a subfamily of neueregulins, which are EGF-like ligands that bind to and activate both ErbB-3 and ErbB-4 (15, 18–24). Recently, betacellulin has been shown to bind ErbB-4 as well as EGFR (25). Although none of these factors bind directly to ErbB-2, both EGF and HRG induce its tyrosine phosphorylation, presumably by ligand-driven heterodimerization and cross-phosphorylation (15, 24, 26–29). Interestingly, ErbB-2, by heterodimerizing with the EGFR and ErbB-3, confers high affinity binding sites for EGF and HRG, respectively (9, 28).

Upon ligand binding, the activated ErbB receptor family members interact with different signaling molecules. EGF-R has been shown to associate with phospholipase C-y1, Shc, and Grb-2 (15, 30–32), and ErbB-2 can associate with phospho-

1 The abbreviations used are: SH2 and SH3, Src homology domains 2 and 3, respectively; CHK, Csk-homologous kinase (previously referred to as MATK, or megakaryocyte-associated tyrosine kinase); EGF, epidermal growth factor; EGF-R, EGF receptor; HRG, heregulin; HER-2/Erbb-2, protein-tyrosine kinase ErbB-2 related to the epidermal growth factor receptor; GST, glutathione S-transferase; CHK-SH2, GST fusion protein containing the SH2 domain of CHK; CHK-SH3, GST fusion protein containing the SH3 domain of CHK; SH3-SH2, GST fusion protein containing SH3 and SH2 domains of CHK; NH2-SH3, GST fusion protein containing the N terminus and the SH3 domain of CHK; IL, interleukin; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation; WB, Western blot.
Enhanced chemiluminescence (ECL) reagents were purchased from model 394. Reagents for electrophoresis were obtained from Bio-Rad. Primers for the polymerase chain reaction (PCR) were synthesized by an automated DNA synthesizer (Applied Biosystems). The primers for the polymerase chain reaction (PCR) were designed to amplify specific regions of CHK cDNA from nucleotides 127–150 as the sense primer and from nucleotides 343–321 as the antisense primer. The DNA fragments obtained from PCR were restriction-digested with BanHI and EcoRI and ligated into the pGEX-2T vector (Pharmacia). The sequence and orientation were confirmed by sequencing both strands. Construction of the GST fusion proteins of CHK-SH2 and CHK-SH3 was described previously (45).

GST fusion proteins were produced by the induction of transformed bacteria using 10 mM isopropyl-β-D-thiogalactopyranoside and purified on a large scale by affinity chromatography on glutathione-Sepharose beads according to the manufacturer’s protocol (Pharmacia).

RESULTS

Expression of CHK in Breast Cancer Tissues—Analyses of CHK expression in human breast cancer tissues were performed on paraffin-embedded 5-μm-thick tissue sections of human breast cancer. Sections were deparaffinized in xylene and then incubated in decreasing concentrations of ethyl alcohol. After several rinses in water, the slides were incubated in 1% hydrogen peroxide (1:1) and briefly rinsed in water and then in phosphate-buffered saline (pH 7.6). Subsequent immunohistochemical staining was performed using a 1:100 dilution in phosphate-buffered saline of rabbit anti-CHK antisera (1-h incubation) followed by the addition of the secondary antibodies, peroxidase-conjugated rabbit anti-mouse IgG (Sigma) at 50 μg/ml in phosphate-buffered saline.

Cell Lines—The T47D and MCF-7 human breast cancer cell lines were obtained from ATCC (American Type Culture Collection, Rockville, MD). T47D cells were grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 3.5 μg/ml insulin (Sigma). The MCF-7 cells were grown in minimal essential medium (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, EGF, or IL-6, cells were starved overnight in media containing 1% fetal bovine serum and then for 4 h in serum-free medium.

Generation of Flag-CHK Construct in pCDNA3 Vector—The CHK cDNA (1.6-kilobase pairs) was cloned into EcoRI sites in the pCDNA3-neo vector. The nucleotide sequence for the Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) was introduced into the 5′ end of the open reading frame of the CHK cDNA sequence by PCR, using a 1.6-kilobase pair CHK cDNA as a template. The 5′ sense primer included a BamHI restriction site, ATG initiation codon, the Flag sequence, and CHK sequences from nucleotides 269–295 (43). The 3′ antisense primer was composed of CHK sequences from nucleotides 510–481 (43). The PCR product was double digested with BamHI and BstEII (New England Biolabs, Beverly, MA), gel-purified, and then cloned into BamHI and BstEII sites in the pCDNA3-neo.Flag-CHK. The construct was analyzed by restriction mapping and nucleotide sequencing.

Transfection—Transfection of MCF-7 cells was performed using the Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s protocol. The transfected cells were selected in 1.2 μg/ml G418 (Sigma). Positive transfectants were chosen based on their immunoreactivity on Western blots probed with polyclonal anti-CHK and monoclonal anti-Flag (M5) antibodies (Eastman Kodak Co.).

Construction and Purification of GST Fusion Proteins of CHK—To express the NH2-SH3 and SH3-SH2 domains of CHK as GST fusion proteins, the corresponding DNA sequences were amplified by PCR with sense and antisense primers of CHK cDNA, which contained BamHI and EcoRI restriction sites. For the NH2-SH3 construct, we used the sense primer from nucleotides 4–27 and the antisense primer from nucleotides 343–321 (43). For the SH3-SH2 construct, we used the sequences from nucleotides 127–150 as the sense primer and from nucleotides 657–634 as the antisense primer (43). The DNA fragments obtained from PCR were restriction-digested with BamHI and EcoRI and ligated into the pGEX-2T vector (Pharmacia). The sequence and orientation were confirmed by sequencing both strands. Construction of the GST fusion proteins of CHK-SH2 and CHK-SH3 was described previously (45).

GST fusion proteins were produced by the induction of transformed bacteria using 10 mM isopropyl-β-D-thiogalactopyranoside and purified on a large scale by affinity chromatography on glutathione-Sepharose beads according to the manufacturer’s protocol (Pharmacia).

Purification of GST Fusion Proteins and Immunoprecipitations—In order to detect the binding of other proteins to CHK GST fusion proteins, approximately 5 × 109 cells/plate were starved overnight in media containing 1% fetal bovine serum, followed by additional starvation in serum-free medium for 4 h at 37°C. The starved cells were then stimulated with 10 ng HRG for 8 min or with 100 ng/ml EGF or 100 ng/ml IL-6 for 5 min at room temperature. The stimulation was terminated by the addition of an ice-cold lysis buffer (0.1% Triton X-100, 1% sodium deoxycholate, 0.5% sodium EDTA, 0.5% sodium NαVO3, 0.1 mg/ml leupeptin, 10 mM NaF, and 10 μM pepstatin A). Lysates were cleared by centrifugation (14,000 rpm, 15 min) and then incubated for 90 min at 4°C with 5 μg of GST fusion proteins coupled to glutathione-Sepharose beads. The beads were washed three times with the lysis buffer. For the immunoprecipitation experiments, polyclonal anti-CHK antibody (10 μl), monoclonal anti-ERB-2 antibody, 3E8 (10 μg/ml), polyclonal anti-ERB-3 antibody (10 μg/ml), or polyclonal anti-ERB-4 antibody (10 μg/ml) was used. SDS-sample buffer was added to the samples and analyzed on % polyacrylamide SDS-PAGE. Proteins were transferred onto nitrocellulose or Immobilon-P (Millipore Corp., Bedford, MA) membranes. Bound proteins were immunoblotted with anti-phosphotyrosine antibody (PY20), polyclonal anti-ERB-2 antibody, or polyclonal anti-CHK, EGF-R, ErbB-3, or ErbB-4 antibodies. The blots were developed using the ECL system (Amersham). Blots were stripped for 20 min at 55°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7), according to the manufacturer’s protocol (Amersham).

RESULTS

Expression of CHK in Breast Cancer Tissues—Analyses of CHK expression in human breast cancer tissues were performed on paraffin sections derived from 12 breast cancer patients using immunohistochemistry. CHK protein was found in 13 of 15 invasive ductal carcinomas, stage II, and in 8 of 15 stage I carcinomas (Fig. 1), while no CHK was detected in the adjacent normal tissues from the same patients.

CHK Is Associated with Activated ErbB-2 upon Stimulation with HRG—Following our observation that CHK was expressed in human breast cancer tissues, we investigated whether CHK might be involved in one of the major signaling pathways in breast cancer mediated by the ErbB family receptors. Experiments were performed using the T47D breast cancer cell line and the GST fusion protein containing the SH2 domain of CHK (CHK-SH2). T47D cells express the ErbB family receptors and the CHK protein as observed by immunohistochemistry (data not shown). T47D cells were starved overnight in 1% fetal calf serum in RPMI 1640 and then incubated...
in serum-free medium for 4 h. The starved cells were then stimulated with HRG (10 nM) for the indicated times (Fig. 2). Cells were lysed, and the supernatants were incubated with the purified CHK-SH2 fusion protein (Fig. 2, A and B) or with the 3E8 monoclonal antibody to ErbB-2 (Fig. 2, C and D). The co-precipitated proteins were analyzed on 7% SDS-PAGE, and immunoblotted with PY20 (Fig. 2, A and C). As shown in Fig. 2A, a tyrosine-phosphorylated 185-kDa protein was associated with CHK-SH2 within 2 min of the HRG stimulation. The association of the 185-kDa protein with CHK-SH2 was maximal at 2–8 min after HRG stimulation and then gradually decreased. In order to determine whether the 185-kDa protein was ErbB-2, the blot was deprobed and rebotted with polyclonal anti-ErbB-2 antibody. As shown in Fig. 2B, the 185-kDa protein was confirmed to be the ErbB-2 protein. These results indicated that the CHK protein can interact with the HRG-activated ErbB-2 receptor.

When lysates from HRG-treated cells were immunoprecipitated with the 3E8 monoclonal anti-ErbB-2 antibody, the pattern of the phosphorylated ErbB-2 was different from that of the ErbB-2 precipitated with the SH2 domain of CHK (compare Fig. 2C with Fig. 2A). Blotting of the same samples with the polyclonal anti-ErbB-2 antibody (Fig. 2D) confirmed these observations. This difference might indicate that tyrosine phosphatase(s) are involved in the association of CHK with the activated ErbB-2.

CHK-SH2 fusion proteins also precipitated another as yet unidentified tyrosine-phosphorylated proteins, as shown in Fig. 2A. However, these phosphorylated proteins were also precipitated from the unstimulated cells, and their phosphorylation pattern did not appear to change over the time course of these studies.

The Association of CHK with ErbB-2 Is Specific for HRG Stimulation—in order to determine whether the observed association of CHK with ErbB-2 was receptor-specific and stimulus-specific, we analyzed whether CHK could associate with either the EGF-R or IL-6 receptors, which are both known to be expressed in T47D cells (1, 59). We compared the association of CHK-SH2 with ErbB-2 in lysates from HRG, EGF, and IL-6-stimulated cells (Fig. 3). T47D cells were serum-starved as described above and then activated either with HRG (10 nM) for 8 min or with EGF (100 ng/ml) or IL-6 (100 ng/ml) for 5 min. The experimental time points and the concentrations of EGF and IL-6 were optimized in initial kinetic studies (data not shown). The stimulated cells were lysed and precipitated with the CHK-SH2 fusion protein as described above. The precipitates were then analyzed on SDS-PAGE and immunoblotted with PY20 antibodies or with polyclonal anti-ErbB-2 antibodies. Only HRG stimulation induced the association of ErbB-2 with the purified CHK-SH2 fusion protein. EGF or IL-6 stimulation failed to induce CHK-SH2 association to ErbB-2 (Fig. 3), to the EGF receptor, or to the IL-6 receptor (data not shown).

The association of ErbB-2 with other SH2 domain-containing signaling molecules such as p85 of PI 3-kinase, phospholipase C-γ1 (Fig. 3A), or She was also examined. The SH2-SH2-SH3 domain of phospholipase C-γ1 was found to be associated with the HRG-activated ErbB-2 (Fig. 3A) as well as with She (data not shown). The SH2 domain of PI 3-kinase precipitated ErbB-2, probably as a result of the ErbB-2 heterodimerization with ErbB-3 (60). Taken together, these results indicate that ErbB-2 associates with all three signaling molecules in HRG-activated T47D cells.

SH3 Domain of CHK Is Not Involved in the Interaction between CHK and ErbB-2—The potential involvement of other domains of CHK in the interaction with ErbB-2 was examined. GST fusion proteins containing the SH3 domain of CHK (CHK-SH3), the N-terminal domain plus SH3 domain (NH2-SH3), the SH3 and SH2 domains of CHK (SH3-SH2), and the SH2 domain of CHK as well as the GST protein alone were prepared as described under “Experimental Procedures.” HRG-stimulated T47D cell lysates were incubated with the different GST fusion proteins, analyzed by SDS-PAGE, and immunoblotted with PY20, rabbit anti-ErbB-2 antibody, or with anti-GST antibody (Fig. 4). Neither the SH3 domain of the CHK protein nor the NH2-SH3 domain precipitated ErbB-2 (Fig. 4A). Binding to ErbB-2 was detected only in the presence of the CHK-SH2 (data not shown) and CHK-SH3-SH2 fusion proteins (Fig. 4B). As expected, no binding was detected when the same lysates were incubated with the GST protein alone. The amounts of the different fusion proteins loaded on the gel were comparable (as shown in Fig. 4C). Therefore, we conclude that CHK can interact with the HRG-stimulated ErbB-2 in a specific manner via its SH2 domain.

In Vivo Association of Intact CHK with ErbB-2—To further confirm the association of ErbB-2 with CHK, we overexpressed the CHK protein in MCF-7 breast cancer cells. CHK expression in MCF-7 cells was detected only by PCR analysis (data not shown). Expression of the ErbB receptor family in MCF-7 cells was similar to that observed in T47D cells. Stable transfections were performed using the Flag-CHK pcDNA3-neo construct as described under “Experimental Procedures.” The transfected cells were analyzed for CHK expression by Western blot using anti-Flag and anti-CHK antibodies and also by immunofluorescence using confocal microscopy (data not shown). MCF-7 cells transfected with Flag-CHK pcDNA3-neo (Flag-CHK), MCF-7 cells transfected with the pcDNA3-neo vector alone, or untransfected MCF-7 control cells were stimulated with HRG and then lysed. The lysates were immunoprecipitated with anti-Flag antibodies (data not shown) and anti-CHK antibodies, and the associated proteins were analyzed by SDS-PAGE and immunoblotted with PY20 or polyclonal anti-ErbB-2 antibody (Fig. 5). The 185-kDa tyrosine-phosphorylated protein was immunoprecipitated with anti-Flag antibodies (data not shown) or anti-CHK antibodies only in HRG-stimulated Flag-CHK-transfected cell lysates (Fig. 5A) but not in the untransfected MCF-7 cell lysates (Fig. 5A) or the MCF-7 cell lysates transfected with the pcDNA3-neo Flag vector alone (data not shown). Blotting with the anti-ErbB-2 antibody confirmed that the co-precipitated 185-kDa protein was indeed the ErbB-2...
Analysis of the total lysates from the same experiment revealed that the ErbB-2 was tyrosine-phosphorylated as a result of the HRG stimulation in the Flag-CHK cells as well as in the MCF-7 untransfected cells (Fig. 5C). The expression of ErbB-2 appeared to be equal in both the Flag-CHK and MCF-7 cells (Fig. 5D). Taken together, these in vitro and in vivo data indicate that the HRG-stimulated ErbB-2 associates with CHK through the SH2 domain.

Involvement of Other ErbB Family Members in the Interaction with CHK—To further investigate the possible involvement of other members of the ErbB family in the observed interaction between CHK and ErbB-2, we performed co-immunoprecipitation experiments using MCF-7 cells transfected with Flag-CHK. Flag-CHK-transfected cells were stimulated with HRG and then lysed and immunoprecipitated with anti-CHK antibody. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-ErbB-2 antibody (Fig. 6A) or with anti-ErbB-3 antibody (Fig. 6B). The results indicated that anti-CHK antibody immunoprecipitated the HRG-activated ErbB-2. In contrast, no detectable ErbB-3 was found. However, the possibility that very low amounts of ErbB-3 were present in the precipitates as a result of the heterodimerization with the ErbB-2 receptor upon HRG stimulation cannot be excluded. We also investigated whether ErbB-4 interacted with CHK under these conditions; however, our findings indicated that ErbB-4 was not involved in the ErbB-2-CHK association (data not shown).

In order to confirm the presence and phosphorylation of the ErbB-3 as well as the heterodimerization of ErbB-3 with ErbB-2 in the Flag-CHK-transfected cells, lysates from HRG-stimulated Flag-CHK cells were immunoprecipitated with anti-ErbB-3 antibodies or with anti-ErbB-2 antibodies (B). In parallel, starved T47D cells were stimulated for 5 min at room temperature with 100 ng/ml of EGF (C) or with IL-6 (D). The lysed cells were then precipitated with GST fusion proteins of CHK-SH2 as described above. The precipitates were separated on 7% SDS-PAGE and immunoblotted with PY20 (lanes 1 and 2) or with anti-ErbB-2 antibodies (lanes 3 and 4).
precipitated with anti-CHK-antibodies in the HRG-stimulated lysates (Fig. 7, A and B). No tyrosine-phosphorylated proteins were detected in the immunoprecipitates with anti-CHK antibodies from the EGF-stimulated cells (Fig. 7C). Reprobing of this blot in Fig. 7C with anti-ErbB-2 (data not shown) or with anti-EGF-R (Fig. 7D) antibodies confirmed that neither of these receptors was present in the CHK immunoprecipitates. As a control, we performed immunoprecipitations with anti-EGF-R antibodies of the EGF-stimulated Flag-CHK cell lysates as well as of lysates from untransfected MCF-7 cells. The EGF-R and the ErbB-2 proteins were present in the immunoprecipitates from the EGF-stimulated cells (not shown) as a result of the EGF-ErbB-2 heterodimerization. Probing of the same blot with anti-ErbB-2 or anti-EGF-R antibodies confirmed this observation (data not shown).

These analyses indicate that CHK associates via its SH2 domain with HRG-stimulated ErbB-2 and does not appear to prominently involve other ErbB family members.

**DISCUSSION**

In this report, we have shown the interaction of CHK, a recently identified cytoplasmic protein-tyrosine kinase, with ErbB-2 upon the activation of breast cancer cells by HRG. This interaction occurred via the SH2 domain of CHK and was specific to the activated ErbB-2 receptor upon HRG stimulation.

CHK was recently cloned, and its expression pattern was characterized by us and by others (43-52). CHK is highly restricted in expression in normal tissues of brain and hematopoietic cells. Recently, we have observed that CHK interacts in a specific and SH2-dependent manner with the c-Kit recep-

tor and participates in the c-Kit signaling pathway in human megakaryocytes (45). Our observation of CHK expression in breast cancer tissues suggests that CHK might be involved in signaling in some cases of breast cancer.

In this report, we demonstrated the association of the SH2 domain of CHK with the HRG-activated ErbB-2 receptor using GST fusion proteins containing different domains of the CHK molecule. Other domains, such as the SH3 domain or the NH2-terminal region of CHK did not appear to be required for the association of CHK with ErbB-2 (Fig. 4). The CHK-ErbB-2 interaction occurred upon the HRG-induced phosphorylation of ErbB-2 (Fig. 2). After 10 min of HRG stimulation, binding of ErbB-2 to the CHK-SH2 domain decreased gradually, while the immunoprecipitated ErbB-2 with anti-ErbB-2 antibodies from the same lysates seemed to remain phosphorylated. This difference in the binding pattern of ErbB-2 to CHK-SH2 as compared with anti-ErbB-2 antibodies could stem from the higher accessibility of the CHK recognition site in the ErbB-2 receptor to tyrosine phosphatases, compared with other tyrosine residues in the ErbB-2 receptor. These observations might indicate a possible regulation and involvement of tyrosine phosphatase(s) in the association of CHK and activated ErbB-2.

In this study, we also addressed the receptor ligand specificity of the observed association between CHK and ErbB-2. Neither EGF nor IL-6 induced the association of CHK with ErbB-2 (Fig. 3) in T47D cells, although treatment of the same cells with EGF did cause the tyrosine phosphorylation of ErbB-2 by heterodimerization as previously reported (1, 24, 26, 28, 61). It is possible that the binding site of CHK on the ErbB-2 receptor is not trans-phosphorylated by the EGF receptor. This might also explain the difference of cell response to binding of EGF versus HRG to heterodimers that contain ErbB-2 and EGF-R, ErbB-2 and ErbB-3 or ErbB-4. For example, it has been shown that ErbB-2 alone or in combination with EGF-R resulted in the malignant transformation of murine fibroblasts (34, 62, 63), while HRG affected both mitogenesis and differentiation in different mammary tumor cells (62, 64-67).

The SH2 domain of CHK was found to interact specifically with ErbB-2, while no interaction was observed between CHK and either IL-6 receptor or EGF-R (Fig. 3). In addition, neither ErbB-3 nor ErbB-4 was detected in the precipitates of the GST-SH2 fusion proteins of CHK (data not shown).

The possible involvement of other ErbB family members in the CHK-ErbB-2 association was also analyzed by co-immunoprecipitation studies. Upon HRG stimulation, no detectable amounts of either ErbB-3 or ErbB-4 were found in transfected cells immunoprecipitated with anti-CHK antibodies (Fig. 5). The co-immunoprecipitation of ErbB-2 with CHK occurred upon HRG stimulation in the presence of CHK in Flag-CHK MCF-7 transfected cells, but not in untransfected MCF-7 cells or cells transfected with the vector alone. These observations suggest that the association of ErbB-2 with CHK occurs in vivo in those breast cancer cells that express CHK.

The possible involvement of other ErbB family members in the CHK-ErbB-2 association was also analyzed by co-immunoprecipitation studies. Upon HRG stimulation, no detectable amounts of either ErbB-3 or ErbB-4 were found in transfected cells immunoprecipitated with anti-CHK antibodies (Fig. 6). We also could not detect any EGF-R molecules in the EGF-stimulated Flag-CHK-transfected cell lysates that were immunoprecipitated with the anti-CHK antibodies (Fig. 7).
to interact with phospholipase C-γ1 and GTPase-activating protein, while ErbB-3 does not interact with those two molecules (60, 39). However, ErbB-3 is the main ErbB family member that has been shown to interact with PI 3-kinase (39). ErbB-3 may thus play a role in coupling PI 3-kinase to other ErbB family receptor molecules (68, 69). Similar relationships have been demonstrated for the insulin receptor substrate-1, the insulin receptor, and the insulin-like growth factor I-receptor (68, 70, 71). Insulin receptor substrate-1 was shown to function as an accessory protein to the insulin receptor involved in the recruitment and activation of PI 3-kinase (68). Similarly, CD19, a membrane protein found in B cells, also has a PI 3-kinase binding site to which membrane IgM can be coupled (68, 72).

To our knowledge, no similar interactions between CHK or Csk and the ErbB family receptors have been reported to date. Recently, the sites where CHK binds to the c-Kit receptor have been identified in our laboratory (73). Comparison of these sites to the five autophosphorylated sites in the ErbB-2 receptor did not reveal any shared identities. Interestingly, one of the three proposed binding sites of CHK to the c-Kit receptor is known to

**FIG. 5. In vivo association of ErbB-2 with CHK.** MCF-7 cells transfected with a pcDNA3-neo Flag-CHK construct (Flag-CHK) (A–D, lanes 1 and 2) or untransfected MCF-7 cells (Panels A–D, lanes 3 and 4) were starved and HRG-stimulated as described in the legend to Fig. 2. The lysates were immunoprecipitated with anti-CHK antibodies for 16 h at 4 °C. The precipitates were analyzed on 7% SDS-PAGE and immunoblotted with PY20 (A) or with anti-ErbB-2 antibodies (B). A fraction of the total lysates used for immunoprecipitation was separated on 7% SDS-PAGE and immunoblotted with PY20 (C) or with anti-ErbB-2 antibodies (D).

**FIG. 6. ErbB-3 is not involved in the CHK-ErbB-2 association.** Flag-CHK-transfected cells were HRG-stimulated (A and B, lane 1, and C and D, lanes 1 and 3). The lysates were immunoprecipitated with anti-CHK antibodies and then analyzed by 7% SDS-PAGE. Immunoblotting was performed using anti-ErbB-2 antibodies (A) or anti-ErbB-3 antibodies (B). The same lysates were also immunoprecipitated with anti-ErbB-3 antibodies (C, D, and E, lane 1) or with 3E8 monoclonal anti-ErbB-2 antibodies (C, D, and E, lanes 2 and 3) for 16 h at 4 °C. The associated proteins were analyzed as described above, and immunoblotted with PY20 (C), anti-ErbB-2 antibodies (D), or anti-ErbB-3 antibodies (E).

**FIG. 7. EGF-R is not involved in the CHK-ErbB-2 association.** CHK-Flag-transfected cells were stimulated with either HRG (10 nM for 8 min) (A and B, lane 1) or with EGF (100 ng/ml for 5 min) (C and D, lane 1). The lysates were immunoprecipitated with anti-CHK antibodies. The separated proteins were immunoblotted with PY20 (A and C). The blot in A was reblotted with anti-ErbB-2 antibodies (B). The blot in C was reblotted with anti-EGF-R antibodies (D).
bind the pS8 regulatory subunit of PI 3-kinase (73). Similar sites exist within EGF-R and ErbB-2, but they do not appear to be autophosphorylated. Others have recently reported that EGF-R as well as ErbB-2 are phosphorylated by c-Src at non-autophosphorylation sites and that these novel sites can act as docking sites for Src, pS8 of PI 3-kinase, and potentially other SH2-containing proteins (74).

In addition to sequence homology, CHK and Csk also share functional properties. Like Csk, CHK was also shown to phospho-rylate purified Src protein in vitro (44, 45, 53–57). Therefore, CHK might act as a negative regulator of Src kinase activity. Interestingly, Src kinase has been reported to be di-phorylated in vitro (37–38, 75). In light of these findings, future studies will aim to characterize the involvement of CHK in Src-ErbB receptor family signaling.

Recently, ErbB-2 has been proposed to act as a modulating subunit of the ErbB family receptors by serving as an essential common subunit of the receptors for HRG and EGF (62). This function is similar to the non-ligand binding components of the interleukin receptors; e.g. gp130 is shared by the receptors for IL-6, leukemia inhibitory factor, oncostatin M, IL-11, and ciliary neurotrophic factor (76). Overexpression of ErbB-2 has been shown to enhance the binding affinity to both EGF and neu differentiation factor through deceleration of ligand dissociation rates (58, 59). Likewise, the removal of ErbB-2 from the cell surface almost completely abolished ligand binding by accelerating the dissociation of both growth factors (62), resulting in an impairment of both HRG and EGF signaling (1, 9, 62). Here we propose an additional modulating role for the ErbB-2 receptor; by interacting with CHK, ErbB-2 recruits CHK within the proximity of its potential substrate, Src, which can be associated with the cytoplasmic domain of another ErbB family member present in a given heterodimer. CHK-ErbB-2 association may lead to the down-regulation of Src activity through its phosphorylation by CHK and hence the attenuation of the receptor signal. Future studies will seek to experimentally assess this model in the context of breast cancer proliferation.

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