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Abstract

Activation of Src kinase plays important roles in the development of many neoplasias. Most of the previous Src studies focused on the deregulation of Src kinase activity. The deregulated Src protein synthesis and stability in mediating malignant phenotypes of cancer cells, however, have been neglected. While investigating the signal transduction pathways contributing to ErbB2-mediated metastasis, we found that ErbB2-activated breast cancer cells that had higher metastatic potentials also had increased Src activity compared with ErbB2 low-expressing cells. The increased Src activity in ErbB2-activated cells paralleled higher Src protein levels, whereas Src RNA levels were not significantly altered. Our studies revealed two novel mechanisms that are involved in Src protein up-regulation and activation by ErbB2: (a) ErbB2 increased Src translation through activation of the Akt/mammalian target of rapamycin/4E-BP1 pathway and (b) ErbB2 increased Src stability most likely through the inhibition of the calpain protease. Furthermore, inhibition of Src activity by a Src-specific inhibitor, PP2, or a Src dominant-negative mutant dramatically reduced ErbB2-mediated cancer cell invasion in vitro and metastasis in an experimental metastasis animal model. Together, activation of ErbB2 and downstream signaling pathways can lead to increased Src protein synthesis and decreased Src protein degradation resulting in Src up-regulation and activation, which play critical roles in ErbB2-mediated breast cancer invasion and metastasis.

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Introduction

ErbB2 is a member of the epidermal growth factor receptor family called class I receptor tyrosine kinases (RTK; refs. 1, 2). The erbB2 gene has been found amplified or overexpressed in ~30% of human breast cancers (3) and in many other cancer types (4). Patients with ErbB2-overexpressing breast cancer have substantially lower survival rates and shorter times to relapse than patients without the overexpression. Moreover, many clinical and laboratory investigations have shown that overexpression of ErbB2 leads to increased breast cancer metastasis (5, 6). Results from our laboratory also showed that ErbB2 overexpression enhances the intrinsic metastatic potential of human breast cancer cells (7).

The ErbB2 RTK has intrinsic tyrosine kinase activity (1, 6). Activation of the ErbB2 tyrosine kinase leads to autophosphorylation of tyrosine residues in its carboxyl-terminal domain. The phosphorylated tyrosine residues then function as docking sites for ErbB2 downstream signaling molecules (6, 8). Previous studies showed that kinase-activated ErbB2 physically associates with Shc (9), Grb-7 (10), Ras-GTPase-activating protein (11), phospholipase Cγ (11, 12), the 85-kDa regulatory subunit (p85) of phosphatidylinositol 3-kinase (12, 13), Src (14, 15), β-catenin (16), Csk-homologous kinase (17), Erbin (18), Ralt (19), c-Cbl (20), and p34cdc2 (21). However, the overall picture of how ErbB2 exerts its function through these signaling molecules is unclear, and the signaling events responsible for ErbB2-mediated cancer metastasis remain elusive. Because most patients with ErbB2-overexpressing breast cancer die from metastasis, unraveling the molecular mechanisms and signaling events that underlie ErbB2-mediated metastasis is extremely important and may facilitate the development of therapies or prevention strategies for patients with ErbB2-overexpressing tumors.

The human c-Src proto-oncogene encodes an intracellular tyrosine kinase, pp60-src (22, 23). c-Src was originally identified as a homologue of the transforming gene of the Rous sarcoma virus (24) and is the prototype of a family of highly conserved genes that includes yes, fyn, fgr, lyn, lck, hck, bbl, and yrk. Src family proteins have been implicated in many signal transduction pathways and a wide variety of cellular functions (25). Activation of Src kinase has been linked to the development of many human neoplasias, especially those of the colon, breast, lung, and pancreas (26). Most of the previous Src studies focused on the deregulation of Src kinase activity but not the altered expression levels of Src protein. The deregulated Src protein synthesis and stability in mediating malignant phenotypes of cancer cells have been largely neglected.

Tumors and cell lines that overexpress ErbB2 have been reported to contain activated Src family kinases, especially pp60-src (14, 15, 27). Although these previous studies suggest that Src activation might play a role in the malignant phenotypes induced by ErbB2, whether Src is important in ErbB2-mediated breast cancer metastasis has not been investigated, and the mechanism by which ErbB2 activates Src has not been well established.

To elucidate the signaling pathways downstream of ErbB2 that contribute to ErbB2-mediated breast cancer metastasis, we expressed wild-type (wt) or mutant ErbB2 proteins with altered signaling capacity in the MDA-MB-435 human breast cancer cell line that expresses low levels of ErbB2. We then examined the metastatic potentials of these cells and their relationship with the activation of ErbB2 downstream signals that might be involved in metastasis. We found that ErbB2 activated Src kinase through the
up-regulation of the Src protein level via increasing Src protein synthesis and stability. Moreover, we showed that increased Src activities were required for ErbB2-mediated breast cancer metastasis.

Materials and Methods

Antibodies and Reagents. Antibodies and reagents were purchased from commercial sources: antibodies against Src and ErbB2 (Ab3) were from Oncogene Science (Cambridge, MA); antibodies against Akt, phospho-Akt (Ser473), 4E-BP1, phospho-4E-BP1 (Ser65), phosphospecific ErbB2 (Tyr1248), and rapamycin were purchased from Cell Signaling Technology (Beverley, MA); antibodies against phosphotyrosine (PY20) were from BD Transduction Laboratories (San Diego, CA); polyclonal antibodies against [35S]methionine were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-hemagglutinin (HA) monoclonal antibodies (HA.11) were from Babco (Richmond, CA); and the Src-specific inhibitor PP2 and its control chemical PP3, calpain-specific inhibitor N-acetyl-leucyl-leucyl-norleucinal, calpain-specific inhibitor N-acetyl-leucyl-leucyl-methional, and MG-132 were purchased from Calbiochem (San Diego, CA). The Src kinase substrate elonase, purified calpain, and cycloheximide were from Sigma (St. Louis, MO).

Design and Construction of Small Interfering RNA. Small interfering RNAs (siRNA) were designed to target ErbB2-specific sequences. We synthesized two oligonucleotides: 5'-AAGTACACGATGGCGGAGACGTCTGTCTC-3' and 5'-AACAGCTCCGCCATGTACCGTCTC-3'. The Silencer siRNA Construction kit (Ambion, Austin, TX) was used to obtain the double-stranded siRNA according to the manufacturer's instruction.

Cell Lines and Cell Cultures. The MDA-MB-435 human breast cancer cell line, 435.eB cells (which are MDA-MB-435 cells stably expressing wt ErbB2), and 435.neo cells were obtained, established, and cultured as reported previously (28). The ErbB2 mutants were subcloned from the pSV2 vectors into pcDNA3 expression vectors (Invitrogen, Carlsbad, CA) in which transcription is driven by a cytomegalovirus promoter. The resulting plasmids were transfected into MDA-MB-435 cells as described (29), and the stable transfectants were selected by G418 (geneticin, Life Technologies, Rockville, MD). Western blot was done to identify transfectants that expressed the ErbB2 protein. Stable wt ErbB2 transfectants of the human breast cancer cell line MDA-MB-231 were established using the same method. The dominant-negative mutant sequence of Src (DNsrc) was subcloned from the expression vector pcR295 into the pRUESpuro2 vector (Clontech, Palo Alto, CA). The resulting plasmid (DNsrc) was transfected into 435.neo or 435.eB cells, and the transfectants were selected using puromycin (Clontech). HA-tagged ubiquitin expression vector (pCGB-Ub) was constructed and expressed as described previously (30). All breast cancer cells used in this study were grown in DMEM and Ham's F-12 (DMEM/F-12, Life Technologies). Osteosarcoma cell line Saos-2 was cultured in RPMI 1640 (Life Technologies).

Western Blot and Immunoprecipitation Analyses. Western blot and immunoprecipitation analyses were done as described previously (31). The protein inputs in the Western blot and immunoprecipitation analyses were normalized by loading the equal amount of total protein lysates onto the SDS-PAGE gels. The nonradioactive signals were quantified by Alpha Imager 2000 (Alpha Innotech, San Leandro, CA). The radioactive signals were quantified by Phospholmager (Molecular Dynamics, Sunnyvale, CA).

In vitro Cell Invasion Assay. Cell invasion was assayed as described previously (7).

In vivo Metastatic Survival Rates. Single-cell suspensions of each cell line were injected into the lateral tail veins of 8-week-old female severe combined immunodeficient (ICR-SCID) mice (1 × 10^6 cells per mouse, Harlan, Madison, WI). The injected mice were maintained under identical conditions and were monitored regularly. Once a mouse became moribund, euthanasia and necropsy were done, and a veterinary pathologist was consulted to determine the cause of the animal's death. The times of death for the mice that died of metastasis were plotted. Survival curves were drawn and analyzed using Prism software (GraphPad Software, San Diego, CA). The survival curves were compared by the Mantel-Haenszel log-rank test. All animal experiments in this study were done under the animal use guidelines of the NIH (Bethesda, MD) and the Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center (Houston, TX).

Src Kinase Assay. Cells were lysed and immunoprecipitated with anti-Src antibodies. The immunoprecipitates were washed thrice with immunoprecipitation buffer and twice with a kinase buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10 mmol/L MgCl2, 1 mmol/L DTT, and 0.5 mmol/L orthovanadate. Kinase activity was assayed by incubating the immunoprecipitates with 40 μL kinase buffer containing 250 μg/mL enolase, 2 μCi [γ-32P]ATP, and 3 μL of 100 mmol/L ATP. After 30 minutes at 37°C, the reaction was terminated by adding 15 μL of 6× SDS sample buffer and boiling for 5 minutes. The samples were subjected to SDS-PAGE. The [γ-32P]ATP-labeled enolase was visualized by autoradiography.

RNA Interference. BT474 breast cancer cells were cultured to ~30% confluence and then transfected with various concentrations of ErbB2 siRNA. After 3 hours of transfection, cells were incubated with serum-containing medium and cell lysates were collected for Western blot analysis 48 hours after transfection. A siRNA concentration of 66.7 μmol/L was able to significantly decrease ErbB2 protein levels compared with that in the control GFP siRNA- or mock-transfected cells.

Assay of Protein Half-life by Pulse-Chase Experiment. The cells were washed thrice with [35S]methionine-free DMEM (Life Technologies) supplemented with 3% fetal bovine serum. The cells were incubated for 1 hour with the wash medium containing 500 μCi/mL [35S]methionine (Amersham Pharmacia, Piscataway, NJ). The cells were washed thrice and incubated in normal growth medium. At the different chase time points, the cells were lysed in immunoprecipitation buffer, and Src protein was immunoprecipitated by the Src antibody. The immunoprecipitates were subjected to SDS-PAGE, and radiolabeled Src was detected by autoradiography.

Protein Synthesis Assay. The cells were washed thrice with [35S]methionine-free DMEM (Life Technologies) supplemented with 3% fetal bovine serum and then incubated for 1 or 4 hours with the wash medium containing 500 μCi/mL [35S]methionine. Cell lysates were extracted and equal amounts of protein lysates were subjected to immunoprecipitation with the antibody against Src or β-actin, and the immunoprecipitates were analyzed by SDS-PAGE and radiolabeled proteins were detected by autoradiography.

Experimental Metastasis Assays. We injected 1 × 10^6 cancer cells into the tail veins of female ICR-SCID mice. On the same day of the injections, the mice were injected i.p. with 1 mg/kg body weight of Src kinase inhibitor PP2 or its solvent, 10% DMSO-PBS. Three days later, the mice were injected with a second dose of PP2 or 10% DMSO-PBS. The injection schedules were then changed to once a week, and the injections continued for 12 weeks. The mice were euthanized 90 days later, and their lungs were instilled with India ink. Metastatic lung nodules >0.5 mm in diameter were counted. Statistical analyses were done by Student's t test.

The experimental metastasis assay by i.v. injection of the 435.eB or eBDNsrc was done and analyzed similar to the above described, except that 2 × 10^6 cancer cells were injected, mice were not treated with PP2, and mice were euthanized 60 days after injection.

Results

Breast Cancer Cells with Increased ErbB2 Kinase Activity Have Increased Metastatic Potential. To investigate the ErbB2 downstream signals that contribute to ErbB2-mediated breast cancer metastasis, we chose to determine the metastatic potential of isogenic breast cancer cells that overexpress wt or mutant forms of ErbB2 and exhibit impaired or enhanced ErbB2 RTK activities (32). The V659E mutant has a Val->Glu mutation at the transmembrane domain that leads to constitutive activation of the ErbB2 tyrosine kinase. The K753M mutant, in addition to
ErbB2 enhances human breast cancer cell invasion and metastasis. A, ErbB2 protein expression and tyrosine phosphorylation in MDA-MB-435 cells, 435.neo cells, and ErbB2 transfectants were detected by using an ErbB2 antibody and a phosphospecific ErbB2 antibody (top). Levels of tyrosine phosphorylation on ErbB2 and other proteins were detected by whole cell lysate Western blotting with PY20 antibody (bottom). B, ErbB2 increased the invasiveness of ErbB2-activated transfectants. Transwells with an 8 μm pore polycarbonate filter were coated with Matrigel. The lower compartment of the transwells contained 0.6 mL of 40 μg/mL laminin in DMEM/F-12 as a chemoattractant. Cells (1 × 10^5) in 0.1 mL DMEM/F-12 containing 0.1% bovine serum albumin were placed in the upper compartment and incubated for 24 hours at 37°C. After incubation, the filters were fixed with 3% glutaraldehyde in PBS and stained with Giemsa. Cells on the upper surface of the filter were removed with a cotton swab, and the invasive ability of the cells was determined by counting the number of cells per high-power field (200×) that had migrated to the lower side of the filter. Matrigel-invaded cells were counted by at least three high-power fields per filter. Each sample was assayed in triplicate, and the results confirm that ErbB2 kinase activities are increased in 435.eB and V659E cells but not in K753M cells.

To determine the impact of ErbB2 activation on breast cancer metastasis, we examined MDA-MB-435 cells, 435.neo cells, and wt or mutant ErbB2 transfectants for their metastatic potential in vitro and in vivo. An invasion assay showed that 435.eB and V659E cells were highly invasive compared with MDA-MB-435, 435.neo, and K753M cells (Fig. 1B), which correlated with increased ErbB2 kinase activities (Fig. 1A). To test the metastatic potential of these cells in vivo, we injected the cells into the lateral tail veins of immunodeficient ICR-SCID mice. The mice later died of metastases, and the metastatic survival rates of the mice were recorded. The mice injected with 435.eB or V659E cells had significantly lower metastatic survival rates than those injected with MDA-MB-435 and 435.neo cells (435.eB versus 435.neo, P = 0.0420; V659E versus 435.neo, P = 0.0252; Fig. 1C). The metastatic survival rates of mice injected with K753M cells were similar to those of mice injected with MDA-MB-435 and 435.neo cells (Fig. 1C). In summary, 435.eB and V659E cells with high ErbB2 kinase activities were highly metastatic, whereas MDA-MB-435, 435.neo, and K753M cells with low kinase activities were not. These results show that ErbB2 kinase activity plays a critical role in ErbB2-mediated metastasis in these breast cancer cells.

Up-regulation of Src Protein by ErbB2. We next investigated multiple ErbB2 downstream signaling pathways that may contribute to ErbB2-mediated metastasis, and we focused on Src because it was tightly regulated by altered ErbB2 RTK activities and closely correlated with the metastatic potential of our panel of breast cancer cells (see below). When we compared Src activity in MDA-MB-435 cells, 435.neo cells, and wt and mutant ErbB2 transfectants by immunocomplex kinase assay (Fig. 2A), we found that the Src kinase was activated at dramatically higher levels in 435.eB and V659E cells than in the control MDA-MB-435, 435.neo, and K753M cells. Thus, activation having the Val659Glu mutation, has a Lys753Met mutation at the ATP binding site of the ErbB2 kinase domain, resulting in the loss of its tyrosine kinase activity. We stably transfected these two mutants, the wt ErbB2 or the pcDNA3 vector, into the ErbB2 low-expressing MDA-MB-435 human breast cancer cell line. ErbB2 protein expression levels were detected by immunoblotting on protein lysates from the parental MDA-MB-435 cells, vector-transfected control 435.neo cells, and wt or mutant ErbB2 transfectants. ErbB2 transfectants that expressed the ErbB2 protein at comparable levels were selected for further studies (Fig. 1A, top).

ErbB2 tyrosine phosphorylation corresponds to the kinase activity of ErbB2 (33–35). Therefore, we determined the changes in ErbB2 tyrosine autophosphorylation in wt and mutant ErbB2-expressing MDA-MB-435 cells by immunoblotting using an ErbB2 phosphotyrosine-specific antibody (Fig. 1A, top). As expected, little ErbB2 tyrosine phosphorylation was detected in parental MDA-MB-435, 435.neo, or kinase-defective K753M cells, indicating very low levels of ErbB2 kinase activation in these cells. However, 435.eB and V659E cells overexpressing wt or activated ErbB2 had high levels of ErbB2 phosphorylation, indicating that the ErbB2 kinase was activated in these cells. Transphosphorylation of other cellular proteins was detected on whole cell lysate by immunoblotting with antibody against phosphorytine (Fig. 1A, bottom). The 435.neo and K753M cells showed low levels of tyrosine transphosphorylation (Fig. 1A, bottom). In contrast, 435.eB and V659E cells, which had higher levels of ErbB2 autophosphorylation (Fig. 1A, top), had higher levels of transphosphorylation (Fig. 1A, bottom).
of ErbB2 RTK by overexpression or activating mutation can lead to Src activation in these breast cancer cells.

To explore the mechanism by which ErbB2 activates Src, we examined the Src protein levels in these cells by Western blot (Fig. 2A) and quantified and correlated the Src protein level and kinase activity in each cell line (Fig. 2A and B). Surprisingly, Src protein levels dramatically increased in ErbB2-overexpressing 435.eB cells and ErbB2-activated V659E cells, which correlated very well with their higher Src kinase activities. This indicates that the higher Src kinase activities in 435.eB and V659E cells were most likely due to the increased Src protein level rather than the increased kinase activity per Src molecule. We next did Northern blotting to determine whether the increased Src protein level resulted from an increased Src RNA level (Fig. 2C). Src RNA levels were similar among all of the cell lines, indicating that the up-regulation of Src expression by ErbB2 did not occur at the RNA level.

To further confirm that ErbB2 regulates Src protein expression levels, the ErbB2 high-expressing breast cancer cell line BT474 was transfected with siRNA to ErbB2 (Fig. 2D, left). Immunoblotting results showed that ErbB2 siRNA down-regulated ErbB2 expression in BT474 cells and subsequently reduced Src protein levels. In addition, compared with 435.eB, the Src protein level was reduced in the 433eBR spontaneous revertant, a 435.eB derivative that partially lost the transfected erbb2 gene and expresses lower levels of ErbB2 (Fig. 2D, middle). Moreover, elevated Src protein expression could also be observed in the wt ErbB2-transfected breast cancer cell line MDA-MB-231 (231.eB) compared with their parental cells (MDA-MB-231; Fig. 2D, right). Furthermore, by immunohistochemistry analysis, we studied Src protein expression levels in breast cancer patient samples, which were strongly correlated with ErbB2 expression levels (data not shown) as reported previously (36). These data show that Src protein expression is regulated by ErbB2 in breast cancer cells. Taken together, we conclude that ErbB2 activation can up-regulate Src at the post-transcriptional level and thereby increase Src protein and kinase activity in ErbB2-activated human breast cancer cells.

### ErbB2 Increases Src Protein Synthesis by Activating the Akt/Mammalian Target of Rapamycin/4E-BP1 Protein Translation Pathway

To investigate the mechanism of Src protein up-regulation by ErbB2, we first tested whether the rate of Src protein synthesis was greater in ErbB2-overexpressing cells than in ErbB2 low-expressing cells. MDA-MB-435 and 435.eB cells were cultured with medium containing [35S]methionine to metabolically label the newly synthesized proteins for 1 or 4 hours. Src proteins from the cell lysates were immunoprecipitated with a Src-specific antibody, analyzed by SDS-PAGE, and visualized by autoradiography. The results showed that ErbB2-overexpressing 435.eB cells had higher levels of [35S]methionine-labeled Src protein than MDA-MB-435 cells at both 1- and 4-hour [35S]methionine labeling (Fig. 3A, left and middle), although the overall rate of incorporation of [35S]methionine was similar between the two cell lines (data not shown). Meanwhile, immunoprecipitation on the same [35S]methionine-labeled Src protein levels were similar among all of the cell lines, indicating that the increased Src protein level resulted from an increased Src RNA level (Fig. 2C). Src RNA levels were similar among all of the cell lines, indicating that the up-regulation of Src expression by ErbB2 did not occur at the RNA level.

To further confirm that ErbB2 regulates Src protein expression levels, the ErbB2 high-expressing breast cancer cell line BT474 was transfected with siRNA to ErbB2 (Fig. 2D, left). Immunoblotting results showed that ErbB2 siRNA down-regulated ErbB2 expression in BT474 cells and subsequently reduced Src protein levels. In addition, compared with 435.eB, the Src protein level was reduced in the 433eBR spontaneous revertant, a 435.eB derivative that partially lost the transfected erbb2 gene and expresses lower levels of ErbB2 (Fig. 2D, middle). Moreover, elevated Src protein expression could also be observed in the wt ErbB2-transfected breast cancer cell line MDA-MB-231 (231.eB) compared with their parental cells (MDA-MB-231; Fig. 2D, right). Furthermore, by immunohistochemistry analysis, we studied Src protein expression levels in breast cancer patient samples, which were strongly correlated with ErbB2 expression levels (data not shown) as reported previously (36). These data show that Src protein expression is regulated by ErbB2 in breast cancer cells. Taken together, we conclude that ErbB2 activation can up-regulate Src at the post-transcriptional level and thereby increase Src protein and kinase activity in ErbB2-activated human breast cancer cells.

An important regulator of protein synthesis and translation is the mammalian target of rapamycin (mTOR), which is a phosphatidylinositol 3-kinase family protein that functions downstream of Akt (37, 38). We therefore examined whether the Akt/mTOR pathway was involved in ErbB2-mediated increase of Src protein synthesis. We detected higher levels of Akt phosphorylation in ErbB2-activated 435.eB and V659E cells but not in MDA-MB-435, 435.neo, or K753M cells (Fig. 3B), whereas the total amount of Akt remained similar. Because activated Akt

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**Figure 2.** Up-regulation of Src protein by ErbB2. A, ErbB2 overexpression or activation led to increased Src protein levels and Src activity in MDA-MB-435 human breast cancer cells. Cell lysates were immunoprecipitated with Src antibodies. Immunocomplexes were subjected to Src kinase assays using enolase as the substrate (top). Src (middle) and β-actin (bottom) protein levels were determined by Western blot. B, quantification of the Src protein level and kinase activity in MDA-MB-435 cells and ErbB2 transfecants. Results from A were quantified by Alpha Imager 2000. Relative Src protein levels and kinase activities were calculated using the protein level and kinase activity of 435.neo as 1.0. C, similar RNA levels among MDA-MB-435 cells and ErbB2 transfecants. Src RNA level was detected by Northern blot using a Src-specific sequence as the probe. D, ErbB2 regulates Src protein levels in human breast cancer cell lines. Cell lysates from BT474 cells treated with ErbB2 siRNA or control siRNA (left), 435.eB and its revertant 435eBR (middle), and MDA-MB-231 and its wt ErbB2-transfectant 231.eB cells (right) were immunoblotted with anti-ErbB2 (top), anti-Src (middle), and anti-β-actin antibodies (bottom).
is known to activate mTOR, and mTOR in turn can phosphorylate its downstream substrate 4E-BP1, a translation repressor, on the inhibitory phosphorylation site at Ser65 (37), we tested whether ErbB2, which activates Akt, may further increase 4E-BP1 phosphorylation and lead to its inhibition. We examined the phosphorylation of 4E-BP1 by Western blot with a 4E-BP1-Ser65 phosphorylation-specific antibody. Compared with MDA-MB-435 cells, 435.eB cells had increased 4E-BP1 phosphorylation (Fig. 3C), indicating that there were higher levels of mTOR kinase activity in 435.eB cells than in MDA-MB-435 cells. The increased phosphorylation of 4E-BP1 on Ser65 could also be observed in wt ErbB2-transfected MDA-MB-231 human breast cancer cells but not in their parental cells (data not shown).

To test whether activation of the mTOR pathway was responsible for increasing 4E-BP1-Ser65 phosphorylation and ErbB2-mediated Src protein synthesis, we treated MDA-MB-435 and 435.eB cells with rapamycin, which is a mTOR-specific inhibitor (39). Inhibition of mTOR by rapamycin effectively reduced 4E-BP1-Ser65 phosphorylation (Fig. 3C). Notably, MDA-MB-435 cells had a minimal reduction in Src protein levels after rapamycin treatment, whereas ErbB2-overexpressing 435.eB cells had a evident reduction in Src protein levels (Fig. 3D), indicating that activation of the Akt/mTOR/4E-BP1 pathway by ErbB2 in 435.eB cells is involved in the enhanced Src protein synthesis. To further test this hypothesis, we treated the cells with or without rapamycin and labeled the cells with $[^{35}S]$methionine for 4 hours. Immunoprecipitation of Src protein was done, and the immunoprecipitates were analyzed by SDS-PAGE. The results showed that without rapamycin more $[^{35}S]$methionine-labeled Src protein was detected in 435.eB cells than that of in MDA-MB-435 cells (Fig. 3A, left and middle). However, in rapamycin-treated cells, the $[^{35}S]$methionine-labeled Src protein in 435.eB cells was reduced to a level similar to that in MDA-MB-435 cells (Fig. 3A, left), indicating that ErbB2-dependent mTOR activation is required for ErbB2-promoted Src protein translation.

**ErbB2 Stabilizes Src by Inhibiting Calpain-Mediated Src Degradation.** To test whether the elevated Src protein level detected in ErbB2-activated cells may also result from reduced Src protein degradation, pulse-chase analyses were done to test whether ErbB2 increases the stability of Src protein. MDA-MB-435 and 435.eB cells were incubated with $[^{35}S]$methionine for 1 or 4 hours. Immunoprecipitations with Src-specific antibodies (left and middle) and $\beta$-actin specific antibodies (right). Immunoprecipitates were washed, analyzed by SDS-PAGE, and visualized by autoradiography. IgG was used as a control for immunoprecipitation. Rel. intensity, relative protein band intensity. Experiments were repeated thrice. SD < 0.21. B, Akt activation in MDA-MB-435 cells and ErbB2 transfectants. Western blot was done with phosphorylated Akt-specific antibodies (top). Total Akt proteins were immunoblated with antibodies specifically against Akt (bottom). C, rapamycin inhibited a higher 4E-BP1-Ser65 phosphorylation level in ErbB2-overexpressing 435.eB cells. MDA-MB-435 and 435.eB cells were treated with or without 25 mmol/L rapamycin for 4 hours, protein lysates were extracted, and Western blot was done with 4E-BP1-Ser65 phosphorylation-specific antibodies (top) and 4E-BP1-specific antibodies (bottom). D, Src protein levels were more obviously reduced by rapamycin treatment in ErbB2-overexpressing 435.eB cells than in the parental MDA-MB-435 cells. Cells were treated with or without rapamycin for the indicated concentrations for 24 hours, protein lysates were extracted, and Western blot was done with Src-specific antibodies (top) and antibodies against $\beta$-actin (bottom). Experiments were repeated thrice. SD < 0.19.

**Figure 3.** ErbB2 increases Src protein synthesis by activating a mTOR-mediated translational mechanism. A, Src protein synthesis increased in ErbB2-overexpressing 435.eB cells. MDA-MB-435 and 435.eB cells were treated without or with 25 mmol/L rapamycin and labeled with $[^{35}S]$methionine for 1 or 4 hours. Immunoprecipitations with Src-specific antibodies (left and middle) and $\beta$-actin specific antibodies (right). Immunoprecipitates were washed, analyzed by SDS-PAGE, and visualized by autoradiography. IgG was used as a control for immunoprecipitation. Rel. intensity, relative protein band intensity. Experiments were repeated thrice. SD < 0.21. B, Akt activation in MDA-MB-435 cells and ErbB2 transfectants. Western blot was done with phosphorylated Akt-specific antibodies (top). Total Akt proteins were immunoblated with antibodies specifically against Akt (bottom). C, rapamycin inhibited a higher 4E-BP1-Ser65 phosphorylation level in ErbB2-overexpressing 435.eB cells. MDA-MB-435 and 435.eB cells were treated with or without 25 mmol/L rapamycin for 4 hours, protein lysates were extracted, and Western blot was done with 4E-BP1-Ser65 phosphorylation-specific antibodies (top) and 4E-BP1-specific antibodies (bottom). D, Src protein levels were more obviously reduced by rapamycin treatment in ErbB2-overexpressing 435.eB cells than in the parental MDA-MB-435 cells. Cells were treated with or without rapamycin for the indicated concentrations for 24 hours, protein lysates were extracted, and Western blot was done with Src-specific antibodies (top) and antibodies against $\beta$-actin (bottom). Experiments were repeated thrice. SD < 0.19.
did not lead to Src protein accumulation in either of the cell lines (Fig. 4C). This, in conjunction with the failure of detecting Src protein ubiquitination, suggests that the ubiquitin-proteasome pathway may not be a significant pathway for Src degradation in these cells.

Calpains belong to a family of Ca^{2+}-dependent proteases and have been implicated in a wide range of cellular functions, including apoptosis, proliferation, and migration (42). Previous reports suggested that calpain could degrade Src and lead to decreased Src kinase activity in human platelets (43, 44). Therefore, we hypothesized that ErbB2-overexpressing breast cancer cells might inhibit calpain-mediated Src protein degradation, which could be involved in stabilizing Src. We first examined whether Src was a substrate of calpain in our cell system.
lyastes from 435.eB cells were incubated with or without 5 or 50 μU purified calpain under various conditions, and the Src protein level was determined by Western blot analysis (Fig. 4D, top). In the presence of 5 mmol/L EGTA, a chelator of Ca2+, there was no obvious calpain activity toward Src. In the absence of added Ca2+, calpain activity toward Src was minimal. However, in the presence of 5 mmol/L CaCl2, calpain caused a dramatic Src protein cleavage into smaller fragments in a concentration-dependent manner. In contrast, immunoblotting with antibody against 14-3-3, a control protein, did not detect 14-3-3 degradation by calpain (Fig. 4D, top), indicating that the proteolytic cleavage is Src specific. To examine the time course of calpain-mediated Src degradation, protein lysates from 435.eB cells were treated with 50 μU calpain in the presence of 5 mmol/L CaCl2 for different times (Fig. 4D, bottom). Immunoblotting with Src antibody showed that the total Src protein detected by the antibody was reduced with prolonged incubation, suggesting that calpain can trigger complete degradation of Src. These data indicate that Src could be a substrate of calpain in these cells.

To test whether an inhibition of calpain-mediated Src degradation was involved in ErbB2-mediated Src stabilization, we incubated MDA-MB-435 and 435.eB cells in the presence or absence of cycloheximide to block protein synthesis or cycloheximide plus calpain-specific inhibitor N-acetyl-leucyl-leucyl-norleucinal. At different time points, protein lysates were collected and the Src protein level was determined by Western blot analysis (Fig. 4E). In the presence of the calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal, degradation of Src protein was delayed in both MDA-MB-435 and 435.eB cells compared with cells treated with cycloheximide alone (Fig. 4E, left). Notably, the delay of Src protein degradation was more evident in MDA-MB-435 cells than in 435.eB cells, suggesting that Src degradation by the calpain pathway was less active in 435.eB cells than in MDA-MB-435 cells most likely as a result of ErbB2 activation. These findings were supported by using another calpain-specific inhibitor N-acetyl-leucyl-leucyl-methioninal, which has been shown to have little inhibition of proteasome-mediated protein degradation (ref. 41; Fig. 4E, right). Thus, in addition to up-regulating the Src protein level by increasing Src protein synthesis via activation of the Akt/mTOR/4E-BP1 pathway, ErbB2 may also stabilize Src protein via inhibition of calpain-mediated Src degradation.

Src Plays a Crucial Role in ErbB2-Mediated Breast Cancer Metastasis. To assess the role of Src activation in ErbB2-induced breast cancer metastasis, we tested whether PP2, a Src-specific inhibitor (45), can inhibit the invasiveness of the ErbB2 transfectants that contain activated Src. The cells were treated with PP2 or its control chemical PP3 and transferred into transwell units for chemoinvasion assays. PP2 dramatically inhibited the basement membrane invasion abilities of ErbB2-overexpressing, Src-activated 435.eB and V659E cells and to a much lesser extent the invasion abilities of 435.neo control cells (Fig. 5A). Moreover, expression of a DNSrc in 435.neo and 435.eB cells reduced their invasive abilities in the chemoinvasion assay (Fig. 5B). Similar to the treatment with PP2, the inhibition of invasiveness by the DNSrc was more dramatic in 435.eB cells compared with 435.neo cells. These results show that Src kinase plays a critical role in ErbB2-induced cancer cell invasion. We then did experimental metastasis assays to determine the role of Src in ErbB2-mediated metastasis in vivo. The 435.neo cells, which had lower ErbB2 expression and Src kinase activity, and the V659E cells, which overexpressed activated ErbB2 and had a higher Src kinase activity, were injected into the tail veins of female ICR-SCID mice. The mice were sacrificed 90 days after the injection. Metastatic lung nodules were counted and statistically analyzed by Student’s t test (Table 1). Consistent with the results from the in vitro invasion assay (Fig. 5A and B), PP2 dramatically inhibited the metastatic potential of ErbB2-activated V659E cells (P < 0.05) but did not significantly inhibit metastasis of 435.neo control cells (P > 0.05). To further investigate the role of Src in ErbB2-mediated cancer cell metastasis, another experimental metastasis assay was done to compare the metastatic potentials of 435.eB cells and 435.eB cells expressing a DNSrc (eB.DNSrc). The result showed that compared with the mice injected with 435.eB cells mice injected with eB.DNSrc cells formed fewer pulmonary nodules (P < 0.05; Fig. 5C), and their lungs weighed less (P < 0.01; Fig. 5D). These in vitro and in vivo results firmly show that Src plays a critical role in ErbB2-mediated human breast cancer cell invasion and metastasis.

Discussion

In this study, we found that ErbB2 activation could up-regulate Src protein levels and thereby increase Src kinase activity in ErbB2-activated human breast cancer cells. The increased Src protein levels in ErbB2-activated cells resulted from at least two mechanisms: (a) ErbB2 promoted Src protein synthesis by activating the Akt/mTOR/4E-BP1 protein translation pathway and (b) ErbB2 stabilized the Src protein most likely by inhibiting calpain-mediated Src protein degradation. Moreover, we showed that Src played a critical role in ErbB2-mediated breast cancer invasion and metastasis (Fig. 5F).

Understanding the molecular mechanisms of ErbB2-mediated metastasis is important for the development of new therapies to treat metastases derived from ErbB2-overexpressing breast cancer. ErbB2 overexpression can be detected in 50% to 60% of high-grade ductal carcinoma in situ breast cancers (46). Interestingly, activation of ErbB2 receptor in preformed mammary acini grown in three-dimensional cell cultures has led to altered mammary acini structures that share properties with ductal carcinoma in situ. No invasive phenotypes were found in these altered acini structures (47). The findings suggest that activation of ErbB2 can induce early stages of mammary carcinogenesis but is not sufficient to induce invasive phenotypes (47). It has been suggested that additional genetic or epigenetic events are needed for mammary epithelial cells to acquire invasive properties (47). The MDA-MB-435 is a highly aggressive metastatic breast cancer cell line and it harbors multiple genetic or epigenetic alterations that may facilitate the development of metastasis (48).

Little was known on the role of Src in ErbB2-induced breast cancer metastasis and on the mechanisms of Src regulation by ErbB2. In this study, we showed that Src plays a critical role in ErbB2-mediated breast cancer invasion and metastasis. To our knowledge, this study reports the first experimental evidence that the higher metastatic potential of ErbB2-overexpressing cancer cells is mediated at least in part through the activation of Src. Therefore, this study provides a rationale for targeting Src as a therapeutic strategy for the treatment or prevention of ErbB2-mediated breast cancer metastasis. It may also have important implications in treating other RTK-mediated malignant cancer behaviors.

Tumor samples from a substantial portion of colon cancers (49, 50) and other cancer types (50), including ~48% of breast cancers (51), were shown to have elevated Src protein levels in
cancer cells compared with normal cells. However, few studies were devoted to investigating the role and mechanisms of deregulated Src protein synthesis and stability in mediating malignant phenotypes. Our study revealed that the up-regulation of Src protein is an important mechanism for Src activation in ErbB2-overexpressing breast cancer cells. This mechanism may also be exploited by other growth factor receptors or other types of cancer cells in addition to ErbB2 and breast cancer cells. Based on our findings, the overexpressed Src protein in the cancer patient samples may conceivably be due at least in part to growth factor receptor–induced protein up-regulation. The new mechanisms revealed in this study may also have important clinical implications for designing Src-targeting therapies. For example, drug designers may enhance Src blocking efficiency by inhibiting Src protein synthesis and/or inducing Src protein degradation in addition to inhibiting Src kinase activity.

In cancer research, studies on how signaling pathways regulate the transcriptional control of gene expression have been pursued extensively. In contrast, fewer studies have focused on how these pathways control other fundamental processes (52, 53). Recently, modulating protein translational control by signaling pathway activation has emerged as another important regulation mecha-

Table 1. PP2 inhibited metastatic potential of ErbB2 transfectants

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Pulmonary metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mice with metastasis/mice injected (%)</td>
</tr>
<tr>
<td>435.neo + solvent</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>435.neo + PP2</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>V659E + solvent</td>
<td>8/9 (89)</td>
</tr>
<tr>
<td>V659E + PP2</td>
<td>4/7 (57)</td>
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</tbody>
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*Lung metastasis nodules >0.5 mm in diameter were counted in the assay.
mTOR/4E-BP1 translation pathway and thereby increases Src protein synthesis. Notably, ErbB2 preferentially promotes the translation of Src, which is a typical growth-regulated protein, but not the translation of β-actin, which is a typical constitutive protein in most cells (Fig. 3A). This is consistent with previous reports that the activation of mTOR preferentially up-regulates the translation of mRNAs of growth-regulated proteins (56).

Previous reports suggested that Src might be a substrate of the Ca²⁺-dependent protease calpain in human platelets (43, 44). However, calpain inhibitors or activators were used to modulate Src protein in these previous studies, and it was not clear whether Src was a direct substrate of the calpain protease. By using purified calpain, we provided another evidence to support that Src might be a substrate of calpain (Fig. 4D). Furthermore, in our study, we discovered a biological function for calpain-mediated Src degradation as a way of regulating Src signaling by RTK, which might play an important role in ErbB2 (or other RTKs)–enhanced cancer metastasis. These were not addressed by previous studies. However, the following questions remain to be answered: whether ErbB2 directly or indirectly modulates calpain-associated protease activity, what the mechanism of this modulation is, and what other biological functions this modulation may exert. Given the fact that both ErbB2 and Src play important roles in cancer development and progression and the ubiquitous expression and important functions of calpain protease in the human body, future investigations of these questions will lead to a better understanding of how cancer metastasis signaling is regulated as well as to better strategies for metastasis intervention.

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