STAT5b, a Mediator of Synergism between c-Src and the Epidermal Growth Factor Receptor

Overexpression of the epidermal growth factor receptor (EGFR) and its association with the tyrosine kinase, c-Src, is correlated with increased cellular proliferation and tumorigenesis. Previous studies have shown that EGFR and c-Src co-overexpression and association leads to the c-Src-mediated phosphorylation of tyrosine 845 of the EGFR and that mutation of Tyr845 ablates epidermal growth factor (EGF)-induced DNA synthesis. Here, we investigate the contribution of the signal transducers and activators of transcription (STAT5b) in the signaling pathways regulated by EGFR and c-Src overexpression in human breast tumor cell lines as well as in a mouse fibroblast model (C3H10T1/2). We demonstrate that 1) activation of STAT5b by EGF requires overexpression of the EGFR, 2) co-overexpression of c-Src alone does not result in EGF-induced activation of STAT5b but enhances that seen in EGFR-overexpressing cells, and 3) EGF-induced tyrosine phosphorylation of STAT5b requires Tyr845 of the EGFR. Furthermore, the stable overexpression of a kinase-defective c-Src in the context of EGFR overexpression results in a decrease in the tyrosine phosphorylation of STAT5b in response to EGF and a more dramatic decrease in EGF-induced transcriptional activation of STAT5b, suggesting an integral role for c-Src in the physiological actions of STAT5b. Using a dominant negative STAT5b, we provide evidence that one such physiological action is to mediate EGF-induced DNA-synthesis. Finally, the use of site-specific tyrosine mutants demonstrates that EGF-induced phosphorylation of STAT5b involves not only tyrosine 699 of STAT5b, which is required for its transcriptional activation, but also three previously identified tyrosines in the C terminus of STAT5b (Tyr725/Tyr740/Tyr743).

Tumorigenesis frequently occurs as a result of the overexpression of proteins that are otherwise involved in normal cellular processes. Examples of such proteins include the human epidermal growth factor receptor (HER) family and cellular c-Src tyrosine kinase (1, 2). It has been estimated that overexpression of HER family members (HER1/EGFR, HER2/neu, HER3, and HER4) occurs in ~67% of human breast cancers (3–5). Elevated levels of the EGFR are associated with the loss of estrogen-dependent growth of tumors and are found predominantly in metastatic sites rather than in primary tumors, suggesting a role for the EGFR in later stages of tumor progression (3, 4, 6). Overexpression of c-Src is found in greater than 70% of breast cancers and thus frequently accompanies EGFR overexpression (2, 7, 8). Not only has c-Src been shown to be involved in the normal process of EGF-induced mitogenesis (9–11), but evidence is also accumulating to suggest that c-Src potentiates the action of EGFR family members during tumorigenesis (1, 12–14).

Model cell lines have been instrumental in characterizing the role of EGFR and c-Src co-overexpression and association in the process of tumor formation. In the mouse fibroblast cell model (C3H10T1/2), overexpression of both the EGFR receptor and c-Src leads to synergistic increases in mitogenesis, anchorage-independent growth, and tumorigenesis in nude mice as compared with overexpression of the EGFR or c-Src alone (14). This increased tumorigenesis correlates with the EGF-induced physical association of the EGFR receptor with c-Src, tyrosine phosphorylation of EGFR receptor at two novel sites, and an increased tyrosine phosphorylation of two EGFR receptor substrates: the Src homology 2-containing adapter protein (Shc) and phospholipase Cγ. These studies provide evidence for the cooperativity between c-Src and EGFR receptor in the process of tumor formation. Importantly, this association between EGF receptor and c-Src has also been described in a number of human breast cancer cell lines (13). A panel of 14 human breast tumor cell lines was characterized with regard to the presence or absence of the estrogen receptor, the levels of EGFR and c-Src, and the presence or absence of EGFR/c-Src complexes. Five of the 14 cell lines were found to overexpress both the EGF receptor and c-Src, and such co-overexpression correlated with high association between these two proteins. Phosphorylation of the EGFR on the same two novel sites as was found in the mouse fibroblast system. These findings suggested a mechanistic cooperation between the two tyrosine kinases. In fact, as compared with breast tumor cell lines expressing normal levels of either protein, the EGFR/c-Src double overexpressors generally exhibited increased EGF-stimulated mitogen-activated protein kinase activity and formation of aggressive tumors in nude mice.
Tyr845 is dependent on the kinase activity of c-Src (16). Furthermore, the kinase activity of c-Src is required for the biological synergy between c-Src and the EGFR that results in increased mitogenesis, growth in soft agar, and tumour formation.

The mechanisms responsible for the cooperation between the EGFR and c-Src tyrosine kinases that lead to tumorigenesis are beginning to be elucidated. The two novel tyrosine phosphorylations on the EGFR that are dependent upon c-Src have been identified as Tyr845 and Tyr1101 (15). Tyr1101 on the EGFR, which itself is not an autophosphorylation site, is located in the C-terminal tail of the EGFR among the known autophosphorylation sites. The function of Tyr1101 is unknown. Tyr845 is located on the activation loop of the catalytic domain. In other tyrosine kinase receptors, such as platelet-derived growth factor, colony-stimulating factor, insulin, hepatocyte growth factor, and fibroblast growth factor, the homologous site is an autophosphorylation site required for full activity of the enzyme (12). However, EGFR-stimulated phosphorylation of Tyr845 is dependent on the kinase activity of c-Src (16). Furthermore, the kinase activity of c-Src is required for the biological synergy between c-Src and the EGFR that results in increased mitogenesis, growth in soft agar, and tumour formation.

Most importantly, mutation of Tyr845 to phenylalanine ablates EGF-induced increases in DNA synthesis. This ablation occurs despite the presence of intact EGFR kinase activity, increases in Shc tyrosine phosphorylation, and activation of mitogen-activated protein kinase (15, 16). This finding suggests that other, as yet unidentified, biochemical signaling pathways that are activated by the c-Src mediated phosphorylation of Tyr845 of the EGFR must be operative in triggering cell proliferation and tumorigenesis.

The signal transducers and activators of transcription (STATs), originally identified in interferon signaling, can be activated by a number of cytokines and growth factors, including EGF (17, 18). Activation of STATs involves phosphorylation of a single tyrosine at the C terminus, hetero- or homodimerization, and translocation to the nucleus, where STATs bind to consensus elements in the promoter of genes that are regulated (19, 20). STAT proteins are normally involved in a variety of cellular processes including mitogenesis, differentiation, and apoptosis (21), but accumulating evidence supports a role for STAT proteins in oncogenesis. STAT1, -3, and -5 are all activated in cells transformed by the v-Src oncogene (22–24), and studies have demonstrated that STAT3 is required for v-Src-induced transformation (25, 26). Our previous studies have shown that the stable overexpression of the EGFR in 293HEK cells leads to the EGFR-induced tyrosine phosphorylation, DNA binding, and transcriptional activation of STAT1b (27). Given the precedence for the role of STAT proteins in oncogenesis, we have investigated the potential role of these transcription factors in the signaling pathways activated by EGFR and c-Src co-overexpression in the C3H10T1/2 mouse fibroblast model and in human breast tumor cell lines. Here, we demonstrate that overexpression of the EGFR, the kinase activities of the EGFR and c-Src, and the c-Src-mediated phosphorylation of Tyr845 of the EGFR are required for EGFR-induced STAT1b activation in breast cancer cell models. Finally, evidence is provided that STAT1b is required for EGFR-induced DNA synthesis. Together, these studies support the potentially important role of STAT1b in the process of tumorigenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines—**C3H10T1/2 mouse fibroblast cell lines, engineered to stably overexpress the human EGFR, wild type c-Src (K+1), or kinase-inactive (A430V) c-Src (K-), have been described previously (14, 16). These cell lines were grown under selection in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 400 μg/ml G418. Mouse embryonic fibroblasts (MEFs) from STAT5a/5b knockout mice were kindly provided by Dr. J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN) (28). These cells were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. Human breast cancer cell lines (MDA-MB468, MDA-MB231, MCF-7, SK-BR-3, BT-20, and BT-549) were obtained from ATCC (Manassas, VA). Cells were passaged twice per week and maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum.

**Reagents—**Recombinant human growth hormone was from Genentech (San Francisco, CA); recombinant human EGF was from Invitrogen; and the c-Src inhibitor, PP2, was from Calbiochem. Monoclonal anti-phosphotyrosine antibody (sc-7020) and polyclonal anti-EGFR (sc-03) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-STAT5A/B (Tyr694/Tyr699) is a mouse monoclonal from Upstate biotechnology (Lake Placid, NY). Monoclonal anti-HA antibody (16B12) was purchased from Babco (Richmond, CA). Polyclonal STAT5a- and STAT5b-specific antibodies were developed in our laboratory (29). Construction of the tyrosine mutants of human STAT5b has been described previously (27). Prestained protein markers (Bio-Rad) and all tissue culture reagents were from Invitrogen. Except where noted, other reagents were either reagent or molecular biological grade from Sigma.

**Preparation of Protein Lyssates—**Cells were preincubated in Dulbecco’s modified Eagle’s medium containing 0.1% bovine serum albumin overnight. In some experiments, cells were pretreated for 15 min at 37 °C with 10 μM PP2. After preincubation, cells were treated either with medium alone (control), 200 ng/ml recombinant human growth hormone, or 100 ng/ml rhEGF at 37 °C for 15 min. At the end of this incubation, cells were washed once in phosphate-buffered saline containing 0.4 M sodium orthovandenate (PBS-Vo43–), scraped into 2 ml of PBS, and then pelleted by centrifugation. Cell pellets were lysed in radioimmunoprecipitation buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 50 mM Tris, pH 7.4). All lysis buffers contained protease and phosphatase inhibitors (25 μg/ml leupeptin, 0.071 trypsin inhibitory units/ml aprotinin, 1 mM vanadate, 200 μM phenylmethylsulfonyl fluoride). Lysates were stored at −70 °C until use. Upon thawing, lysates were subjected to ultracentrifugation (82,000 × g, 30 min, 4 °C), and resulting supernatants were analyzed as described below. The protein amount was determined using the BCA™ protein assay from Pierce.

**Western Blotting and Immunoprecipitation—**For immunoprecipitation, 500 ng of protein lysate was incubated with antibody overnight at 4 °C. Rabbit anti-HA-protein G-Plus agarose (Santa Cruz Biotechnology) was added for an additional 1 h at 4 °C. Agarose pellets were washed three times in radioimmunoprecipitation buffer, and bound proteins were removed by heating to 100 °C in 1× Laemmli buffer (30). For direct analysis of extracts, detergent lysates, prepared as described above, were mixed 1:1 with 2× Laemmli buffer. Both total lysates and immunoprecipitates were fractionated through a 7.5% polyacrylamide gel, electrophoretically transferred to nitrocellulose, and blotted as described previously (31). Blocking buffers contained TBS-T (0.15 M NaCl, 0.1% Tween 20, 50 mM Tris, pH 8.0) and either 3% bovine serum albumin for the anti-phosphotyrosine antibody or 5% nonfat dry milk for all other antibodies. Donkey anti-rabbit or sheep anti-mouse antibodies and radiolabeled secondary antibodies were used to detect antibodies and detected by ECL™ (Amersham Biosciences). In some cases, blots were reprobed after stripping in buffer (2% SDS, 0.1 M 2-mercaptoethanol, 62.5 mM Tris, pH 6.8) at 70 °C for 80 min.

**Transient Transfection and Luciferase Assays—**C3H10T1/2 mouse fibroblasts were transfected according to the manufacturer’s protocol with Effectene Reagent™ from Qiagen (Valencia, CA) and a reporter plasmid containing the lactogenic hormone response region linked to firefly luciferase (LHRR-luciferase) as well as a plasmid encoding Renilla luciferase under the control of the thymidine kinase promoter commercially available from Promega Biotech and kindly provided by Dr. Theodorescu (Department of Urology, University of Virginia). The plasmid contains two copies of the STAT5 consensus sequence from the bovine β-casein gene (AGATTTCAGGAACTTTCAACTC) as described (32). Renilla luciferase expression was used as a normalization control for transfection. Transfected cells (5 × 104/well) were plated in 96-well plates and treated for 24 h with control (serum free) medium or 100 ng/ml rhEGF. Lysates were prepared by means of a Dual-Luciferase™ reporter assay system from Promega (Madison, WI). Firefly and Renilla luciferases were measured sequentially from each sample using a Packard Topcount. Firefly luciferase values were normalized to Renilla luciferase values in each cell sample.

**The pCDNA3 expression vectors (Invitrogen) for wild type (WT) and the mutant Y845F EGFR have been described previously (15, 16). The kinase inactive EGF, which contains a lysine to alanine mutation in the ATP binding site (K721A) was a gift from L. Beguinot (Laboratory of Molecular Oncology, Milan, Italy). A vector containing a cytomegalo- virus promoter and the hemagglutinin (HA) epitope upstream of a unique Nofl site was used to construct the HA-tagged STAT5b expres-
EGF induces tyrosine phosphorylation of STAT5b and demonstrates that EGF induces the phosphorylation of STAT5 in the absence of EGFR overexpression. EGF activates STAT5b in EGFR-overexpressing breast cancer cells. Furthermore, overexpression of c-Src in the absence of EGFR overexpression (as in MCF-7 cells) is not sufficient for EGF-induced tyrosine phosphorylation of STAT5b. In Fig. 1C, Western blot analysis with the commercially available anti-phospho-STAT5b (Tyr694) antibody demonstrates the results seen in Fig. 1B and demonstrates that EGF induces the phosphorylation of STAT5b at Tyr694 in this panel of cell lines.

STAT5b Activation Requires EGFR Kinase Activity and Tyr694 of the EGFR—Studies in other cell systems have shown that the activation of STAT1, STAT3, and STAT5 by EGF requires the kinase activity of the EGFR receptor (19, 34, 35). In addition, studies by Biscardi et al. (15, 16) have demonstrated that co-overexpression and association of the EGFR and c-Src tyrosine kinase leads to the c-Src-mediated phosphorylation of Tyr694 on the EGFR. Thus, we investigated the role of the kinase activity and Tyr694 of the EGFR in the EGF-induced activation of STAT5b in the breast tumor model. MCF-7 cells were transiently transfected with plasmids encoding HA-tagged STAT5b and either wild type EGF, a K721A mutation that renders the EGFR kinase inactive, or an Y845F mutant of the EGFR. Transfection of empty vector (pcDNA3.1) does not result in an EGF-induced tyrosine phosphorylation of STAT5b (Fig. 2A), supporting the results of Fig. 1 (MCF-7 cells). However, the exogenous expression of the WT EGFR in MCF-7 cells was transiently transfected with plasmid encoding HA-tagged STAT5b and either wild type EGF, a K721A mutation that renders the EGFR kinase inactive, or an Y845F mutant of the EGFR. Transfection of empty vector (pcDNA3.1) does not result in an EGF-induced tyrosine phosphorylation of STAT5b (Fig. 2A), supporting the results of Fig. 1 (MCF-7 cells). However, the exogenous expression of the WT EGFR in MCF-7 cells was transiently transfected with plasmid encoding HA-tagged STAT5b and either wild type EGF, a K721A mutation that renders the EGFR kinase inactive, or an Y845F mutant of the EGFR. Transfection of empty vector (pcDNA3.1) does not result in an EGF-induced tyrosine phosphorylation of STAT5b (Fig. 2A), supporting the results of Fig. 1 (MCF-7 cells). 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as well as its kinase activity is required for EGF-induced activation of STAT5b in MCF-7 breast tumor cells. Using the same model, either the WT EGFR or the Y845F mutant were transiently transfected into MCF-7 cells along with HA-tagged STAT5b. Whereas expression of the WT EGFR resulted in EGF-induced STAT5b tyrosine phosphorylation, transfection of the same amount of the Y845F mutant plasmid resulted in little, if any, EGF-induced STAT5b tyrosine phosphorylation (Fig. 2B). Thus, both the EGFR kinase activity, as well as tyrosine 845 of the EGFR, is required for the EGF-induced tyrosine phosphorylation of STAT5b.

To investigate the effect of the EGFR on the transcriptional activity of STAT5b, MCF-7 cells were transiently transfected with HA-STAT5b, WT EGFR, K721A, or Y845F and the STAT5 response element (LHRR-luciferase). Fig. 2C demonstrates that expression of WT EGFR results in a ~3-fold increase in LHRR-luciferase by EGF (see legend to Fig. 2). This activation is completely ablated when the kinase-defective EGFR (K721A) or the tyrosine mutant of the EGFR (Y845F) are transfected, thus demonstrating that both the kinase activity and tyrosine 845 are required for EGF-induced transcriptional activation of STAT5b.

**STAT Activation in the C3H10T1/2 Mouse Fibroblast Model System**—To further investigate the mechanism of EGF-induced activation of STAT5b, mouse C3H10T1/2 cell lines that stably overexpress the EGFR (NeoR) or wild type c-Src (K’c-Src) were used (14). To compare STAT5b activation by EGF with that of a known activator of STAT5b signaling in fibroblasts, these cells were treated with EGF as well as with growth hormone (GH). Immunoblots of STAT5b immunoprecipitates from Neo, NeoR, and K’c-Src cells demonstrate that STAT5b is tyrosine-phosphorylated in response to the cytokine GH in all three cell types, regardless of EGFR or c-Src levels (Fig. 3A). In contrast, EGF was able to activate STAT5b only in the cell line that overexpresses the EGFR (NeoR), confirming results in the breast cancer panel (Fig. 1). Importantly, Fig. 3A also demonstrates that increased levels of c-Src alone do not result in EGF activation of STAT5b (K’c-Src). However, as shown in Fig. 3B, overexpression of kinase active c-Src plus the EGFR (K’c-Src/R) (16) resulted not only in an increase in basal levels of STAT5b tyrosine phosphorylation but also a reproducible increase in EGF-induced tyrosine phosphorylation of STAT5b (as compared with the NeoR cells overexpressing EGFR alone). In contrast, overexpression of catalytically inactive c-Src along with the EGFR (K’c-Src/R) led to a reduction in the level of EGF-induced STAT5b tyrosine phosphorylation. The results of this and three additional experiments were quantified by densitometry (taking into account levels of STAT5b protein in the immunoprecipitates) and depicted graphically in Fig. 3C. The Tyr(P)/STAT5b ratios were as follows: NeoR (1.03 ± 0.10), K’c-Src/R (1.94 ± 0.21), and K’c-Src/R (1.27 ± 0.11). Similar results were obtained when cells were stimulated with EGF for 5, 30, and 60 min (data not shown). These data suggest that c-Src kinase activity can regulate EGF-induced tyrosine phosphorylation of STAT5b but is not required for it.

The role of c-Src in the transcriptional activity of STAT5b was investigated by using the LHRR-luciferase reporter plasmid in the C3H10T1/2 cells. Fig. 3D demonstrates that EGF treatment results in an ~5-fold increase in LHRR-luciferase activity over the unstimulated levels in NeoR cells and an ~12-fold increase in K’c-Src/R cells. In contrast, EGF treatment results in less than 2-fold increase in the K’c-Src/R cells. Thus, the LHRR-luciferase data demonstrate a dramatic effect of c-Src activity on the EGF-induced transcriptional activation of STAT5b. Together, the results of Fig. 3 demonstrate that
c-Src influences not only the tyrosine phosphorylation of STAT5b but, more dramatically, the transcriptional activation of STAT5b in response to EGF.

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**Fig. 3. STAT activation in 10T1/2 mouse fibroblasts.** A, Neo, NeoR, and K+c-Src cells were treated for 15 min with serum-free medium (control), 200 ng/ml recombinant human GH, or 100 ng/ml rhEGF (EGF). Detergent lysates were prepared and incubated with a STAT5b-specific antibody. Immunoprecipitated proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose. Duplicate filters were probed with anti-phosphotyrosine antibody (top panels) or anti-STAT5b antibody (bottom panels). These results are representative of four independent experiments for STAT5b. B, NeoR cells that overexpress the EGFR and EGFR-overexpressing cells that also co-overexpress either the kinase-active c-Src (K+c-Src/R) or kinase-defective c-Src (K+c-Src/R) were treated for 15 min at 37°C with serum-free medium (Cont) or 100 ng/ml rhEGF. Lysates were prepared and incubated with anti-STAT5b antibody. Immunoprecipitates (Immunoppt) were analyzed as described above. C, densitometric analysis of Tyr(P) (pTyr) and protein levels of STAT5b from B and two additional experiments was carried out, and the Tyr/P/STAT5b ratios were determined for each cell line. The mean + S.E. ratio from NeoR cells was arbitrarily set at 1, and the ratios from K+c-Src/R and K+c-Src/R cells were determined relative to 1. The Tyr(P)/STAT5b ratios in EGF-treated were as follows: NeoR (1.03 ± 0.10), K+c-Src/R (1.94 ± 0.21), and K+c-Src/R (1.27 ± 0.11). D, cells were transfected with plasmids encoding LHRR-luciferase (firefly) and thymidine kinase-luciferase (Renilla), seeded in 96-well plates, and incubated with serum-free medium alone (Cont) or with 100 ng/ml EGF for 24 h. Cells were lysed, and firefly luciferase values were normalized to Renilla luciferase values in each treatment group. Shown is the mean-fold induction ± S.E. from four separate experiments, each performed in quadruplicate per treatment group. Fold EGF-stimulated values over control are as follows: NeoR (4.8 ± 2.7); K+c-Src/R (11.7 ± 3.7); and K+c-Src/R (1.6 ± 0.5).

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**Fig. 4. Signaling in the MEF model system.** A, MEFs from STAT5a/b knockout mice were transiently transfected with the EGF receptor as well as expression vectors for either wild type STAT5b (wt), the tyrosine 699 mutant (Y699F), or the mutant of the other three identified tyrosine phosphorylation sites (tyrosines 725/740/743). Forty-eight hours after transfection, cells were treated for 15 min either with medium alone (Cont) or 100 ng/ml EGF. Anti-STAT5b immunoprecipitates were analyzed by immunoblotting with either anti-phosphotyrosine (α-pTyr) or α-STAT5b. B, MEFs were transiently transfected with WT STAT5b and either WT EGFR or the Y845F mutant of the EGFR, or the kinase-defective K721A EGFR. Cells were treated with EGF (100 ng/ml) or not (Cont), and STAT5b immunoprecipitates were analyzed by immunoblotting with anti-phosphotyrosine or anti-STAT5b antibody. Analysis of three independent experiments resulted in an EGF-induced increase in STAT5b tyrosine phosphorylation of 26.9 ± 8.1-fold for WT EGF and 8.9 ± 4.6-fold for the Y845F form of the EGFR. No tyrosine signal was detectable with the K721A EGFR.

**Role of c-Src and EGFR Kinases in EGF-induced Tyrosine Phosphorylation**—The disparity between the level of EGF-induced tyrosine phosphorylation of STAT5b and its transcriptional activity in cells expressing kinase-defective c-Src (Fig. 3) suggests that there may be tyrosine phosphorylation sites on STAT5b that are insensitive to c-Src kinase activity. We have recently identified three EGF-induced tyrosine phosphorylation sites in the C-terminal transactivation domain of STAT5b (Tyr725, Tyr740, and Tyr743), and these tyrosines apparently exert a negative regulatory role in STAT5b transcriptional activation (27). We sought to determine the relative contribution of these three sites as well as the well described site of cytokine-induced phosphorylation (Tyr699) in model systems overexpressing the EGFR and c-Src tyrosine kinases. Tyrosine mutants of STAT5b along with the EGFR were transiently transfected into MEFs from STAT5a/ba knockout mice, and lysates from control and EGF-treated cells were isolated. Immunoblotting of STAT5b immunoprecipitates demonstrates that the STAT5b protein, wild type, or tyrosine mutants are expressed (Fig. 4A, lower panels). Anti-phosphotyrosine blotting (Fig. 4A, top panels) demonstrates that mutation of tyrosine 699 of STAT5b alone (Y699F) does not eliminate EGF-induced tyrosine phosphorylation. However, when tyrosines 725, 740, and 743 are mutated in addition to tyrosine 699, no EGF-induced tyrosine phosphorylation is observed, demonstrating that these four sites account for all of the detectable EGF-induced tyrosine phosphorylation of STAT5b in the MEF model.

We next sought to determine whether the kinase activity and Tyr699 of the EGFR were required for EGF-induced tyrosine phosphorylation of STAT5b but, more dramatically, the transcriptional activation of STAT5b in response to EGF.
phosphorylation of STAT5b in the MEF system, as previously seen in MCF-7 cells (Fig. 2). Fig. 4B demonstrates that whereas transient transfection of the WT EGFR results in an EGF-induced tyrosine phosphorylation of WT STAT5b in MEF cells, the kinase defective EGFR (K721A) does not. Finally, although transfection of the Y845F mutant results in a detectable level of EGF-induced tyrosine phosphorylation, it is at least 3-fold lower than that seen with the WT EGFR. This decrease is not as dramatic as that seen with the Y845F EGFR in the MCF-7 model system (Fig. 2); however, this result clearly demonstrates the importance of Tyr845 in signaling to STAT5b.

The Role of c-Src in the Phosphorylation of the Novel Tyrosine Sites—Thus, using the MEF transfection system characterized in Fig. 4, we investigated the role of the c-Src tyrosine kinase in EGF-induced tyrosine phosphorylations of STAT5b. Specifically, we sought to determine whether Tyr725/Tyr740/Tyr743 contributed to the EGF-induced phosphorylation detected in the absence of c-Src kinase activity (seen in Fig. 3B, K-c-Src/ R). MEF cells were transiently transfected with plasmids encoding EGFR plus the various STAT5b mutants and then either pretreated or not with the c-Src inhibitor PP2 before treatment with EGF. Fig. 5A demonstrates that pretreatment with PP2 results in a decreased basal and EGF-induced tyrosine phosphorylation of WT STAT5b. When Tyr699 is mutated (Fig. 5B), there is not as dramatic a decrease in the EGF-induced tyrosine phosphorylation (with some still remaining), indicating that the three sites of phosphorylation (Tyr725/ Tyr740/Tyr743) are not completely inhibited upon inhibition of c-Src kinase activity. In contrast, when Tyr725/Tyr740/Tyr743 are mutated (Fig. 5C), no basal or EGF-induced tyrosine phosphorylation is detected, indicating that most, if not all, of the phosphorylation of Tyr699 is dependent on c-Src kinase activity. Quantitation of four individual experiments is shown in Fig. 5D and supports the conclusion that PP2 has a more dramatic effect on the EGF-induced phosphorylation of tyrosine 699 compared with that of tyrosines 725/740/743.

To further investigate the effect of c-Src on EGF-induced tyrosine phosphorylation of STAT5b, a second approach was used. The EGFR, STAT5b (WT or tyrosine mutants), and c-Src (kinase-active (K) or kinase-defective (K D)) were transiently transfected into MEF cells, and the EGF-induced tyrosine phosphorylation of STAT5b was analyzed. Fig. 6A shows that overexpression of K-c-Src, along with WT EGFR and WT STAT5b, resulted in the constitutive tyrosine phosphorylation of the WT STAT5b protein. This result supports that seen in Fig. 3B with K-c-Src/R cells, demonstrating an increase in basal tyrosine phosphorylation upon wild type c-Src overexpression. In contrast, when a kinase-defective form of c-Src (K D) is expressed along with the EGFR and WT STAT5b, no tyrosine phosphorylation (constitutive or EGF-induced) of STAT5b is readily detected. These results confirm that c-Src plays a critical role in EGF-induced STAT5b tyrosine phosphorylation. Nevertheless, with longer exposure of the anti-phosphotyrosine blot, one can detect an EGF-induced tyrosine phosphorylation of WT STAT5b (Fig. 6A, longer exposure). These results indicate that EGF can induce STAT5b tyrosine phosphorylation even in the context of kinase-defective c-Src, but to a greatly reduced extent. To determine which tyrosine(s) was being phosphorylated on WT STAT5b, the EGF-induced phosphorylation of the tyrosine site mutants was investigated. Fig. 6B demonstrates that, as with WT STAT5b, the Y699F mutant can be tyrosine-phosphorylated in response to EGF even in the presence of K-c-Src (see longer exposure) but that catalytically active c-Src is required for full phosphorylation. In Fig. 6C, the Y725F/Y740F/Y743F mutant was analyzed in the context of K + or K D c-Src. As seen in Fig. 6, A and B, overexpression of K-c-Src results in a high level of constitutive tyrosine phosphorylation of STAT5b. However, the expression of K-c-Src inhibits the tyrosine phosphorylation of Y725F/Y740F/Y743F, even upon longer exposure. This result suggests that any amount of EGF-induced phosphorylation of Tyr699 is dependent on c-Src kinase activity. Thus, the results of these experiments are consistent with those shown in Fig. 5, demonstrating that c-Src plays a role in regulating the phosphorylation of all of the described tyrosines in the C terminus of STAT5b (699, 725, 740, and 743) but affects the EGF-induced phosphorylation of Tyr699 to a greater extent than the other three tyrosines. Since Tyr699 is known to be required for the transcriptional activation of STAT5b, these results offer an explanation for the disparate effects of kinase-defective c-Src on STAT5b tyrosine phosphorylation and transcriptional activation seen in the C3H10T1/2 cell model in Fig. 3.
以前的研究已在工程化的小鼠成纤维细胞系（15，16）和数据中提出，证明EGF诱导的磷酸化和转录活性STAT5b依赖于Tyr645。我们随后研究了STAT5b在EGF诱导的DNA合成中的作用。因此，K-c-Src转染的成纤维细胞被转染具有完全活性（K-wt）c-Src和野生型STAT5b（A），Y699F突变的STAT5b（B）或STAT5b三重突变（Y725F/Y740F/Y743F）（C）。48小时后，将100 ng/ml EGF或不含（Cont）细胞洗涤，制备细胞裂解物，然后用抗-Tyr(P)（α-pTyr）或抗-STAT5b免疫沉淀。免疫沉淀物用抗c-Src（K-wt）c-Src和野生型STAT5b

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**DISCUSSION**

先前的研究在具有c-Src和EGF诱导的DNA合成的两个鼠成纤维细胞系中均显示了这种协同效应。在EGF诱导的细胞中，DNA合成的总体水平较高。在不同浓度的EGF条件下，随着EGF浓度的增加，DNA合成的百分比也随之增加。然而，在不含EGF的对照组中，DNA合成的百分比保持在较低水平，未显示出显著的差异。这些结果表明，EGF诱导的DNA合成显著依赖于STAT5b的活性。

尽管STAT5b在EGF诱导的细胞中表现得非常活跃，但进一步的研究表明，STAT5b在EGF诱导的DNA合成中的作用可能不是直接的。STAT5b可能通过与其他蛋白质的相互作用来调节DNA合成。在这些研究中，我们还发现STAT5b的磷酸化水平在EGF诱导的细胞中显著提高，这进一步支持了STAT5b在EGF诱导的DNA合成中的调节作用。

总的来说，这些结果表明，STAT5b在EGF诱导的DNA合成中起着重要的作用。STAT5b的活性可以通过EGF或c-Src的共表达来上调，从而促进DNA合成。这些发现为理解EGF诱导的DNA合成的分子机制提供了新的见解。
growth of the cells in soft agar (transformation), and, most dramatically, in the ability of the cells to form tumors in vivo (1, 14, 16). Biochemical characterization demonstrated that simultaneous overexpression of the EGFR and c-Src tyrosine kinases led to their heterocomplex formation and the resulting c-Src-dependent phosphorylation of the EGFR on Tyr^{699} and Tyr^{1031} (15). Furthermore, mutation of Tyr^{694} (Y845F) resulted in a dominant negative EGFR, which reduced not only EGFR, but also serum- and lysophosphatidic acid-induced DNA synthesis, although it did not inhibit the tyrosine kinase activity of the EGFR or the ability of EGFR to activate the mitogen-activated protein kinase pathway (15, 16). In the studies presented here, we demonstrate in both models not only that STAT5b is tyrosine-phosphorylated in response to EGFR-overexpressing cells but that this tyrosine phosphorylation is dependent, in part, on c-Src kinase activity and the presence of the c-Src-mediated phosphorylation site on the EGFR, Tyr^{694} (Fig. 2). Thus, the activation of STAT5b is an integral signal in the pathway emanating from EGFR and c-Src overexpression, association, and the c-Src-mediated phosphorylation of Tyr^{694}. Although the precise role of tyrosine 845 phosphorylation is not known, it may function directly to serve as a docking site for STAT5b, or it may act more indirectly to promote the phosphorylation of another site on the EGFR or even other signaling molecules in the complex. Since the phosphorylation of Tyr^{845} is so labile (15), we propose that it serves an indirect rather than direct role.

Our results support a mechanistically relevant interaction between STAT5b and the EGFR that is mediated by c-Src tyrosine kinase activity. C3H10T1/2 cells stably expressing a kinase-inactive c-Src showed a decrease in EGF-induced STAT5b tyrosine phosphorylation. However, more dramatic than the detectable decrease in tyrosine phosphorylation was the observed decrease in transcriptional activation of STAT5b in cells expressing kinase-inactive c-Src. These results suggest that the mechanisms responsible for the effect of c-Src on the EGF-mediated transcriptional activation of STAT5b are complex. Until recently, tyrosine phosphorylation of STAT5b was reported to occur on a single tyrosine residue (tyrosine 699). Tyrosine phosphorylation at this site has been shown to be induced by cytokine treatment and to result in the dimerization, translocation of STAT5b to the nucleus, DNA binding, and transcriptional activation (17). However, our recently published studies have shown that a Y699F mutant form of STAT5b that is not tyrosine-phosphorylated in response to cytokine (GH) is tyrosine-phosphorylated in response to EGF (27). Interestingly, an EGF-induced, but not prolactin-induced, tyrosine phosphorylation of a similar mutant of STAT5a (Y694F) has been described, although the sites have not been identified (37). We have identified three tyrosine residues in the C-terminal transactivation domain of STAT5b that contribute to the non-Tyr^{699} EGF-induced tyrosine phosphorylation in our model systems. Fig. 4, using STAT5b/5a knockout MEFs, supports our previous studies in the 293HEK cell model and demonstrates that tyrosines 725, 740, and 743 contribute to the EGF-induced tyrosine phosphorylation of STAT5b. Furthermore, this tyrosine phosphorylation is dependent on the kinase activity as well as Tyr^{694} of the EGFR.

Having identified these additional sites of tyrosine phosphorylation, the potential role of the c-Src tyrosine kinase in the EGF-induced phosphorylation not only of Tyr^{699} but also of the newly identified sites of phosphorylation, Tyr^{725}/Tyr^{740}/Tyr^{743}, was investigated. Results of inhibitor studies as well as expression of dominant negative c-Src indicated that the kinase activity of c-Src is absolutely required for the EGF-induced tyrosine phosphorylation of Tyr^{699}. In contrast, c-Src kinase activity is not essential for the phosphorylation of tyrosines 725/740/743, although it does either directly or indirectly modulate their phosphorylation (Figs. 5 and 6). We propose that these results could explain the discrepancy between the complete lack of STAT5b transcriptional activity and the EGF-induced tyrosine phosphorylation in the K_c-Src/R cells (Fig. 3, B versus D), since the observed EGF-induced tyrosine phosphorylation may be due to phosphorylation of the other tyrosine sites (725/740/743). Previous studies demonstrate that the tyrosine 725/740/743 sites negatively regulate the transcriptional activation of STAT5b (27). Studies are currently under way to determine the role of each individual tyrosine in c-Src mediated, EGF-induced tyrosine phosphorylation of STAT5b, since a mechanistic understanding of the role of these sites may lead to the development of novel therapeutics to modulate STAT5b action in breast tumor cells.

An increasing number of studies support a role for the c-Src tyrosine kinase in the phosphorylation of STAT5b as well as other STAT proteins. Olayioye et al. (37) have demonstrated that c-Src kinase activity is required for the EGF-induced tyrosine phosphorylation of STAT1, -3, -5a, and -5b in both NIH3T3 cells engineered to overexpress the EGFR and in A431 cells, which endogenously express high levels of EGFR. This activation is most sustained for STAT5b and is independent of the activity of the JAK tyrosine kinases. A role for Src family members in the tyrosine phosphorylation of STAT5a and STAT5b in a COS cell transfection model has also been described by Kazansky et al. (38). In these studies, the c-Src-mediated tyrosine phosphorylation of STAT5b resulted in the nuclear translocation of STAT5b, although this STAT5b was unable to activate a well described STAT5 response element. Our studies provide evidence not only that c-Src plays an important role in the EGF-induced transcriptional activation of STAT5b but also that STAT5b tyrosine phosphorylation occurs through the c-Src-mediated phosphorylation of Tyr^{845} of the EGFR. Thus, a complex picture of the role of c-Src in STAT activation is emerging. Not only does c-Src potentially play a direct role in the phosphorylation of STAT5b, but it also plays an indirect role in the EGF-induced phosphorylation of STAT5b. Our studies demonstrate that one such role is through the c-Src-mediated phosphorylation of Tyr^{845} of the EGFR (Fig. 2). Furthermore, we now know that the c-Src-mediated phosphorylation of STAT5b occurs not only at Tyr^{699} but also at three other tyrosines 725/740/743 (Figs. 5 and 6).

In addition to our studies with STAT5b, there is now evidence that STAT3 is activated in both the C3H10T1/2 mouse fibroblast model as well as the human breast cancer cell lines (39). In their work, Garcia et al. (39) demonstrate that the c-Src and JAK family kinases mediate the constitutive activation of STAT3 in EGFR- and c-Src-overexpressing cells, whereas the EGFR mediates the EGF-induced STAT3 activation. We also find evidence of the constitutive activation of STAT5b in the K_c-Src/R fibroblasts (Fig. 2B) but not in the human breast cancer cell lines (Fig. 1). The role of the c-Src-mediated phosphorylation of Tyr^{845} of the EGFR in STAT3 activation has not yet been addressed. Nevertheless, the already apparent differences in the role of STATs in the fibroblast versus epithelial cell model or the subtle differences in STAT3 versus STAT5b signaling are not surprising given that these signaling molecules could be contributing different functions to the process of tumorigenesis. Our studies (not shown) in the EGFR/c-Src-overexpressing cell line, MDA-MB468, demonstrated that, in addition to STAT5b, STAT1, STAT3, and STAT5a were also tyrosine-phosphorylated in response to EGF treatment. Together, all of these studies provide supportive evidence for a potential role of STAT proteins in both the initiation and the
Finally, there is evidence that STAT3 and STAT5b are required for the basal and EGF-induced proliferation in these model systems. Garcia et al. (39) showed that the c-Src and JAK kinases are required not only for STAT3 activation but also for growth regulation in human breast cancer cells (39). Furthermore, a dominant negative STAT3 not only inhibited the growth of these cells but also resulted in cell death (apoptosis). Here, we provide evidence that a dominant negative STAT5b inhibits the EGF-induced stimulation of DNA synthesis (Fig. 7). Since previous studies showed that mutation of tyrosine 845 of the EGFR (Y845F) also leads to an inhibition of EGF-induced DNA synthesis, we propose that STAT5b provides a direct link between the increased EGF-induced proliferation in EGFR- and c-Src-overexpressing breast cancer cells.

Although proliferation is only the first step on the pathway toward eventual transformation and tumorogenesis, there is now increasing evidence that STAT proteins are involved in these processes also. Kazansky et al. (40) have shown that STAT5b can enhance the v-Src transformation of NIH 3T3 cells, whereas a dominant negative STAT5b decreases cell growth and transformation of these cells. Studies with STAT5a knockout mouse have shown that these mice have a delay in the onset of tumor growth factor-a-induced hyperplasia (which occurs through the EGFR) (41). Furthermore, STAT5a/−/− mice show a decreased level of STAT5a, and the involvement of this process in the progression of tumorogenesis. Future studies will be aimed at identifying the mechanisms involved in this progression, and we propose that STAT5b, as well as STAT5a and STAT3, will provide good therapeutic targets to inhibit the process of tumorogenesis.

Acknowledgments—We thank the Parsons-Weber-Parsons research group for thoughtful comments.

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