ErbB-2 Activates Stat3α in a Src- and JAK2-dependent Manner*

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Stats (signal transducer and activator of transcription) are latent transcription factors that translocate from the cytoplasm to the nucleus. Constitutive activation of Stat3α by upstream oncoproteins and receptor tyrosine kinases has been found in many human tumors and tumor-derived cell lines. Constitutively activated Stat3α is often correlated with the activation of ErbB-2, a member of the EGFR family. To explore the involvement of ErbB-2 in the activation of Stat3 and the mechanism underlying this event, an ErbB-2 point mutant was used as a model of constitutively activated receptor. Phenylalanine mutations (Y→F) were made in the autophosphorylation sites of the receptor, and their ability to activate Stat3α was evaluated. Our results suggest that Stat3α and JAK2 associates with ErbB-2 prior to phosphorylation of the receptor and that full activation of Stat3α by ErbB-2 requires the participation of other non-receptor tyrosine kinases. Both Src and Jak2 kinases contribute to the activation of Stat3α but Src binds to ErbB-2 only when the receptor is phosphorylated. Our results also suggest that tyrosine 1139 may be important for Src homology 2 domain association because a mutant lacking this tyrosine reduces the ability of the Src homology 2 domain to bind to ErbB-2 and significantly decreases its ability to activate Stat3α.

Stats† (signal transducer and activator of transcription) are a family of latent signaling transcriptional factors that are activated by a variety of extracellular signals. To date, there have been seven mammalian Stat genes identified (1). When they were first discovered, Stat proteins and the mechanism of their activation were considered primarily in the context of normal cytokine signaling, and their function was believed to specifically regulate host defense. Upon further study, Stats were found to be not only activated by cytokines but also in response to activation of other membrane growth factor receptors with intrinsic tyrosine kinase activity, including EGFR (2), platelet-derived growth factor receptor (3), and vascular endothelial growth factor receptor (4). According to those findings, it is not surprising that Stats have highly diverse biological functions and are essential in regulating many critical biological responses such as development, differentiation, cell-cycle progression, and apoptosis. Consequently, precise regulation of Stat activity is necessary; aberrant activation of Stats may contribute to the imbalance between cell proliferation and cell death and eventually lead to cancer. Emerging evidence shows that Stats are also frequently activated by many oncoproteins, and constitutive activation of Stat proteins is found in transformed cell lines, tumors, and tumor-derived cell lines (5–8). Stat proteins contain several domains including a coiled-coil domain necessary for dimer oligomerization, as well as DNA binding, SH2, and transactivation domains. Upon the initiation of specific activation signals, a single tyrosine residue in the C terminus of the Stat protein is phosphorylated by receptor kinases, JAK family kinases associated with cytokine receptors, or cytoplasmic kinases (c-Src, c-Abl) (9). Once the tyrosine is phosphorylated, the Stat can form homo- or heterodimers via reciprocal SH2 domain-phosphotyrosine interactions and translocate into the nucleus. Through binding to specific DNA sequences, the Stat dimers can directly regulate gene expression. Despite intense research, relatively few Stat3-regulated genes have been clearly characterized. However, the proteins encoded by these genes play important roles in maintaining the balance between cell proliferation and cell death. While cyclin D (10) and p21 (11) are essential in regulating cell proliferation and cell cycle progression, Bel-7XL prevents apoptosis (10). Another target gene, c-myc (12), is important in both cell proliferation and survival. Hence it is believed that Stat3 can induce cell malignant transformation by promoting uncontrolled proliferation and inhibiting the regular response to apoptotic signals.

ErbB-2 is the second member of Erb family identified and is 85% homologous with EGFR (13). While the ErbB family members are essential during development (14, 15), abnormal activation (by overexpression or mutation) is often observed during tumorigenesis. ErbB-2 is a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity. Although no ligand that specifically binds to ErbB-2 has been identified, the ErbB-2 receptor is reported to form homodimers or heterodimers with other ErbB family members and may contribute to changes in downstream gene expression profiles (16). Overexpression or abnormal activation of ErbB-2 is found in tumors such as brain (17), head, and neck (18), breast cancer (19), non-small-cell lung cancer (20), bladder cancer (21), and melanoma (22). Furthermore, ErbB-2 expression is also correlated with poor prognosis due to the resistance to chemotherapy (23) and increased metastasis (24, 25). The correlation of ErbB-2 receptor and human cancers indicates that abnormal expression of this receptor is implicated in tumor development and progression. In vitro experiments have shown that overexpression of ErbB-2 receptor or constitutively activated ErbB-2 mutants can transform NIH3T3 fibroblasts (26, 27). The mechanism underlying this has been intensely studied and numerous signal transduction pathways are involved in this process (28).

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§ The abbreviations used are: Stats, signal transducer and activator of transcription; EGFR, epidermal growth factor receptor; SH, Src homology; JAK, Janus family kinase; GST, glutathione S-transferase; DM, double mutation; M1, mutant 1.
Though the mechanism of Stat3α activation by EGFR has been well investigated, only a few studies have indicated that its sibling ErbB-2 is also involved (29, 30). In this report we show that Stat3α can be activated by ErbB-2 and investigate the mechanism of this activation. We used an ErbB-2 mutant, V659E, as a model of constitutively activated ErbB-2 receptor because it has higher intrinsic kinase activity and increased ability to induce transformation compared with a wild-type receptor. The results presented show direct evidence that ErbB-2 can activate Stat3α through both JAK2- and SRC-dependent manners.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—NIH3T3, B104-1-1, and COS-7 cells were obtained from American Type Culture Collection (ATCC). NIH3T3 and B104-1-1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% calf serum. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The day before transfection, the cells were plated at a density of 3 x 10^6 per 6-cm dish and 1 x 10^6 per 10-cm dish. Transient transfection was performed using FuGENE-6 (Roche Molecular Biochemicals). Plasmids were transfected at a ratio of 1 μg of DNA to 3 μl of FuGENE-6 reagent according to the protocol supplied. Cells were then harvested 2 days following transfection.

Plasmid Construction—ErbB-2 wild-type, ErbB-2 V → E, and ErbB-2 inactive plasmids were kindly provided by Dr. Ming Tan (Anderson Cancer Center). All the mutations were made by a two-step PCR-directed mutagenesis method. The primers used to generate the mutations were: M1: 5’-CCCCAGCCTGAATTGTTAACCAGCCAGAT-3’; M2: 5’-GAGACCCGCCATCTTCTGACCCCATG-3’; M3: 5’-GCCCTTGACAACACTTTTGACCGACGAGC-3’; M4: 5’-GACAACCTTCTATTTGAGAACCCCGAGTTCTTGACACCCCAG-3’; M5: 5’-GGAGACCCGACCTCTCAGGACCACG-3’. The bases underlined indicate the point mutations introduced. One HpaI and one AvrII site were introduced into M1 and M5, respectively, while a TaqI site was abolished in M3 to facilitate screening of potential mutants. GST-Src SH2 domain plasmid was provided by Dr. John McMurray (M.D. Anderson Cancer Center). 3XIE luciferase plasmid was constructed by inserting three tandem copies of the hSIE element (GTGCAGTTGCCGCAACG-3’) into the pGL3 promoter plasmid (Promega). The consensus Stat site is underlined. The α2-macroglobulin luciferase reporter plasmid was kindly provided by Dr. Joanne Richards ( Baylor College of Medicine). The dominant-negative Src plasmid was kindly provide by Dr. Sara Courtneidge (Van Andel Research Institute), and the dominant-negative Jak2 plasmid by Dr. David Levy (New York University, School of Medicine).

Luciferase Assay—Luciferase assay reagents were purchased from Promega, and the assays performed according to their protocol. In brief, cells were washed twice with ice-old phosphate-buffered saline and then collected. After spinning at 4500 rpm for 1 min, the cell pellets were lysed in 1x lysis buffer at room temperature for 30 min. The lysate was cleared by centrifugation at 13,000 rpm for 10 min. Then 50 μl of luciferase substrate was added to 10 μl of supernatant, and the luciferase value was read by luminosimeter (Sirus Luminometer, Berthold Detection Systems). Each transfection was normalized to concomitant β-galactosidase expression from a control-transfected plasmid pYN3214-fuzC.

Western Blot and Immunoprecipitation—Cells were harvested using the method described above, and the cell pellets were lysed in lysis buffer (1% Triton X-100 in phosphate-buffered saline, 1 mM NaF, 1 mM sodium orthovanadate and 1× complete (Roche Molecular Biochemicals) protease inhibitor tablets) for 40 min in 4°C. The lysates were then cleared by centrifugation at 13,000 rpm for 10 min, and the protein concentration was determined by the Bradford method (BioRad). For Western blots, equivalent amounts of protein were heated to 95°C for 5 min in Laemmli buffer. Proteins were resolved on 7.5% SDS-polyacrylamide gels followed by electrophoretic transfer to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline supplemented with 5% nonfat milk and 0.1% Tween 20 and incubated with primary antibody for 1 h at room temperature. Proteins recognized by the antibody were detected by enhanced chemiluminescence using horseradish peroxidase–coupled secondary antibody and reagents from Pierce. For immunoprecipitation, equal amounts of protein were pre-cleared with 40 μl of protein A/G beads (Santa Cruz) for 1 h. After collecting the beads by centrifugation at 8000 rpm for 1 min, the supernatants were transferred to new tubes, and the appropriate antibody (0.8 μg) was then added. After binding in 4°C for another 1 h, 40 μl of protein A/G beads were added and allowed to bind for 1 h. The beads were then collected by centrifugation and washed three times with lysis buffer and once with phosphate-buffered saline. The immunopurified protein were boiled in Laemmli buffer and used in immunoblotting experiments.

GST Fusion Protein Purification and GST Pull-Down Assay—GST-Src SH2 domain plasmid was transformed into DH5α (Invitrogen). A single colony was picked, and a small-scale culture (5 ml) was grown overnight. The following day a large culture was inoculated with the saturated culture at a 1:50 dilution. Following a 2-h incubation with shaking at 37°C, 1 mM isopropyl-1-thio-β-D-galactopyranoside was added to induce the expression of the protein for an additional 2 h. The cells were harvested by resuspending them in 5000 rpm for 5 min. The cell pellets were then resuspended in 10 ml of lysis buffer (1% Triton X-100 in phosphate-buffered saline, 1 mM NaF, 1 mM sodium orthovanadate and 1× complete) and sonicated four times for 15 s at 50% power on ice. The cell lysates were clarified by centrifugation at 12,000 rpm for 30 min at 4°C. 1 ml of slurry glutathione-Sepharose resin equilibrated in lysis buffer was then added to recover the protein. After rotating at 4°C for 1 h, the resin was collected by centrifugation and washed three times with lysis buffer. GST pull-down assays were performed similar to immunoprecipitations; the primary antibody was replaced by 4 μg of GST-Src SH2 domain protein, and the protein A/G beads were replaced by 40 μl of GST beads (Amersham Biociences).

RESULTS

B104-1-1 Cells Have a Higher Ratio of Activated Stat3 than NIH3T3 Cells—As reported previously, Stat3α activation is frequently correlated with ErbB-2 activation (29–31). To begin understand this observation in greater detail, we assessed Stat3 activation in NIH3T3 and B104-1-1 cells. The latter line was established by Schechter et al. by transfecting NIH3T3 cells with EcoR1-digested DNA from the rat neuroblastoma cell line B-104 (32). The cells express the neu-tropic gene, which codes for an 185,000-Dalton antigen designated p185. It has higher intrinsic kinase activity than wild-type ErbB-2 and can transform normal cells (33, 34); it therefore serves as an ideal cell line to determine the activation status of Stat3α in cells expressing an activated ErbB-2 receptor. As shown in Fig. 1A, when total cell lysates representing equivalent amounts of Stat3α were assayed by immunoblotting there was significantly more phospho-Stat3α detected in the B104-1-1 cells compared with extracts from NIH3T3 cells (lower panel). Consistent with this observation, when transfected with a reporter plasmid containing three tandem copies of a Stat3α-responsive element (3xSIE) proximal to a minimal promoter element, B104-1-1 cells showed higher luciferase activity than NIH3T3 fibroblasts (Fig. 1B). To confirm that activation of the Stat3α-dependent reporter plasmid was due to transcriptionally functional Stat3α, we performed similar experiments using a Stat3α dominant-negative plasmid. As shown in Fig. 1C, in B104-1-1 cells, a dose-dependent decrease in transcription from the Stat3α-responsive reporter plasmid was seen when increasing amounts of a dominant-negative Stat3α (Stat3α Y705F) expression plasmid was transfected into the cells (35). This strongly indicated that the expression of the activated form of ErbB-2 in fibroblasts results in the specific tyrosine phosphorylation (on tyrosine 705) and transcriptional activation of Stat3α.

Mutations of the ErbB-2 Cytoplasmic Autophosphorylation Sites Result in Receptors with Different Capacity to Activate Stat3—The activation is ErbB-2 kinase-activity dependent. Although there is no known ligand that specifically binds to ErbB-2 receptor, it can be activated either by overexpression or by a point mutation (33, 36). One such mutation in the transmembrane domain of ErbB-2 receptor, valine 659 to glutamic acid, confers on the mutant increased kinase activity and ability to transform cells (33, 34). As shown in Fig. 2, although wild-type ErbB-2 does not appreciably activate Stat3 transcriptional activity, the V659E mutant results in nearly 7-fold in-
ErbB-2 activates Stat3α in a Src- and JAK2-dependent manner.

Production of exogenous Stat3 (V659E also mildly activated endogenous Stat3 [forth column from the left]). Because of this observation, we chose to use the ErbB-2 V→E mutant as a model of constitutively activated receptor. As previously reported, there are five autophosphorylation sites in ErbB-2 that can be tyrosine phosphorylated (tyrosines 1139, 1196, 1221, 1222, and 1249), and maximal autophosphorylation is required for optimal cell transformation (26, 27, 37). Among these five residues, tyrosines 1139 and 1222 are contained within a YXXQ motif (where X is any amino acid). Phosphotyrosines in this context have been found to be important for recruiting Stat3α to other receptor molecules due to pY/SH2 domain interactions (38–41). To examine the function of each site in ErbB-2 Stat3 activation, sensitive 3XSIE-luciferase was used, and the luciferase value was normalized by β-galactosidase from the lacZ plasmid. Experiments were performed in duplicate, and relative luciferase value is shown as average ± S.D.

Fig. 1. A, B104-1-1 cells have a higher ratio of phospho-Stat3 than NIH3T3 cells. Whole-cell extracts were prepared from NIH3T3 and B104-1-1 cells. Total proteins containing comparable levels of Stat3 protein were separated on 7.5% SDS–polyacrylamide gels and then analyzed by immunoblotting with anti-phospho-Stat3 Y705 antibody (Cell Signaling). The membrane was then stripped and blotted with anti-total Stat3 monoclonal antibody (Upstate Biotechnology). The membrane was then stripped and blotted with anti-total Stat3 monoclonal antibody (Upstate Biotechnology). B104-1-1 cells have higher Stat3 transcriptional activity than NIH3T3 cells. 3XSIE luciferase and lacZ plasmids were transiently transfected into B104-1-1 and NIH3T3 cells. 48 h after transfection, cells were harvested, and luciferase assays were performed. Results are shown as relative luciferase values ± S.D. of duplicate dishes. The results are representative of two experiments. B. NIH3T3 and B104-1-1 cells were transfected with increasing amount of Stat3 Y705F expression plasmid (0.25, 0.5, and 0.75 μg per dish). 48 h after transfection, cells were harvested, and luciferase assays were performed. Results are shown as representative of two experiments. C, endogenous Stat3 transcriptional activity was blocked by the expression of dominant-negative Stat3. B104-1-1 cells were transfected with increasing amount of Stat3 Y705F expression plasmid (0.25, 0.5, and 0.75 μg per dish). 48 h after transfection, cells were harvested, and luciferase assays were performed. To detect endogenous Stat3 activation, sensitive 3XSIE-luciferase was used, and the luciferase value was normalized by β-galactosidase activity from co-transfected lacZ plasmid. Experiments were performed in duplicate dishes, and the value is shown as fold induction ± S.D. The results are representative of two experiments.

Fig. 2. The ErbB2 V→E mutant activates Stat3 to higher levels than wild-type receptor. COS-7 cells were transfected with the ErbB-2 and Stat3 plasmids indicated in the figure. In addition, the Stat3-responsive α2-macroglobulin luciferase reporter and lacZ plasmid were also cotransfected. 48 h after transfection, cells were harvested, and luciferase assays performed according to the protocol provided by Promega. Luciferase values were equalized to the expression of β-galactosidase from the lacZ plasmid. Experiments were performed in duplicate, and relative luciferase value is shown as average ± S.D. The results are representative of two experiments.

Fig. 3. ErbB2 mutant constructs. The schematic representations of the ErbB2 receptor constructs are shown. TM indicates transmembrane domain; kinase indicates kinase domain; and autophosphorylation tyrosines (Y) and their changes to phenylalanine (F) are indicated. All domains containing mutations are marked with a shadow. The names of the mutants are indicated on the right side of the figure.

| ErbB2 mutants | Lysine | Y | Y | Y | Y | Y |
|---------------|--------|---|---|---|---|---|
| ErbB2 wt      |        |   |   |   |   |   |
| V→E M1       |        | F | F | F | F | F |
| V→E M2       |        | Y | Y | Y | Y | Y |
| V→E M3       |        | Y | F | Y | F | F |
| V→E M4       |        | F | Y | Y | Y | Y |
| V→E M5       |        | F | Y | Y | Y | Y |

Fig. 4. All of the mutants have fairly comparable expression levels when transiently transfected into COS-7 cells. Similar results were also obtained in NIH3T3 cells (data not shown).

To further analyze the properties of these mutants, immunoprecipitations were performed to assess the autophosphorylation status of the mutants. Whole-cell extracts from cells transfected individually with the ErbB2 constructs were im-

2 M. Tan, unpublished data.
munoprecipitated with ErbB-2 antibody followed by immunoblotting with 4G10, a pan-anti-phosphotyrosine antibody. As expected, all the mutants derived from the V→E mutant have similar autophosphorylation levels except the kinase-inactive mutant, and all are tyrosine-phosphorylated to a higher level than wild-type receptor (Fig. 5, top panel). Although a previous report showed that some combined Tyr-Phe mutations could reduce the overall level of autophosphorylation of ErbB-2 (27), the DM mutant did not show a dramatic change in the level of receptor phosphorylation. Furthermore, this result also indicated that the intrinsic kinase activity was required for receptor autophosphorylation.

We then tested the ability of these plasmids to activate Stat3a transcriptional activity. To achieve this, the plasmids were transiently transfected into COS-7 along with a Stat3a expression plasmid and a Stat3a-responsive reporter plasmid. Cell extracts were prepared and used to perform luciferase assays to determine the relative level of activated Stat3a. The results in Fig. 6 show that each of the mutants was capable of activating Stat3a transcriptional activity. Among the V→E mutants, expression of mutant 1 (M1) and DM consistently showed a decreased ability to activate Stat3a compared with the other mutants.

Because the two putative Stat3 SH2 domain binding motifs (Tyr-1139 and Tyr-1222) were abolished in mutant 1 and 4, one or both of them was expected to have lower Stat3 activation ability. To test whether the reduced capacity to activate Stat3a was due to the impaired ability of Stat3 to bind to the receptor, we performed immunoprecipitation experiments with an anti-ErbB-2 antibody to isolate ErbB-2 immune complexes. Extracts from cells transfected with Stat3a together with one of the mutant ErbB-2 constructs were used, and the amount of Stat3a associated with ErbB-2 was determined by immunoblotting with an antibody to Stat3. As seen in Fig. 5, Stat3a was detected in complexes with each of the mutants (middle panel).

Stat3a Activation by ErbB-2 Is Partially Src-dependent—Although the activation of Stat3a by ErbB-2 is strictly dependent on the kinase activity of the receptor, we also wanted to determine whether other kinases participate in the process. Other reports have shown that c-Src and JAK2 can mediate the activation of Stat3a by ErbB family members (30) and platelet-derived growth factor receptors (42, 43, 44). Although there is some controversy as to the involvement of each of these kinases in the transduction of signals through specific receptor molecules (3, 43–45), we considered c-Src and JAK2 to be likely candidates. To investigate the role of these proteins in Stat3a activation by ErbB-2 receptor, we used dominant-negative expression constructs of c-Src and JAK2 to block their function.

First, we investigated the role of c-Src in Stat3a activation by ErbB-2. Transfection of c-Src DN into cells resulted in an ~50% decrease in the Stat3a transcriptional activity by ErbB-2 V→E (Fig. 7). Reductions in Stat3a activation by M1 and DM were also decreased but to a lesser extent. To further investigate the mechanism underlying the involvement of Src in the ErbB-2 activation of Stat3a, we tested whether the c-Src SH2 domain associates with ErbB-2. A previous report showed that the Src SH2 domain alone can bind to ErbB-2 although the Src SH3 and SH2 together bound with a higher affinity (46). GST-Src-SH2 protein pull-down assays were performed to detect its ability to bind to different ErbB-2 mutant proteins. As demonstrated in Fig. 8, the ErbB-2 receptors showed different binding affinities to the Src SH2 domain; the ErbB-2 mutant bound with the highest affinity followed by M1 and DM, whereas the ErbB-2 wild type and ErbB-2 kinase-inactive receptor association to the SH2 domain was barely detectable.

Stat3a Activation by ErbB-2 Is Partially JAK2-dependent—JAK2 is known to be involved in the activation of Stat3a; specifically it was found to be necessary for Stat3a activation by IL-6 (47) and EGF (48). Because of these findings, we also investigated the role of JAK2 in Stat3a activation by ErbB-2 as assessed by the transcriptional activation experiment described above. Transfection of JAK2 DN reduces Stat3a-mediated transcription by ErbB-2 V→E by nearly 70% (Fig. 9). The dominant-negative also reduces Stat3a activation by the M1 and DM mutants by about 50%. To investigate the binding status of JAK2 and ErbB-2 receptor, we also performed co-immunoprecipitation assays. Because the ErbB-2 is known to form heterodimers with other ErbB family members, especially EGFR, we performed control experiments with EGFR antibody to prove that the activation observed was due to ErbB-2 and not to JAK association with endogenous EGFR. As seen in Fig. 10, JAK2 associated with kinase-active and kinase-inactive ErbB-2, whereas no JAK2 was detected in either control reactions, which were immunoprecipitated with anti-hemagglutinin and anti-EGFR antibody, respectively. These results suggest that JAK2 is associated with ErbB-2 and not with endogenous EGFR.
Because constitutively activated Stat3 is seen in many human tumors (7, 8, 48) and an activated Stat3 form (Stat3C) can transform NIH3T3 cells (49), Stat3 is believed to play an important role in tumorigenesis. The aberrant activation of the Stat3 signaling pathway together with the concomitant expression of downstream genes makes it an effective regulator of the balance between cell growth and death. Because no naturally occurring constitutively Stat3 mutants have been identified thus far, alternative mechanisms must be required to maintain Stat3 in the activated (i.e., tyrosine-phosphorylated) state observed in numerous neoplastic cells. Previous studies have suggested that Stat3 may participate in ErbB-mediated tumorigenesis (29, 31). Although Stat3 activation by EGFR has been intensely studied, less attention has focused on ErbB-2. The characteristic of maintaining constitutive activation when overexpressed renders ErbB-2 the ability to activate downstream molecules without extracellular signals. Not surprisingly, activated ErbB-2 has been reported in many human malignant neoplasia (17, 18, 21, 22, 50). Resistance to chemical therapy such as Taxol and increased ability of invasion of tumor cells have also been attributed to the expression of ErbB-2 receptors (25, 51–53). The studies reported here provide direct evidence that Stat3 can be activated by ErbB-2 and that full activation depends on the participation of both Src and JAK2 kinases.

To verify the potential relationship between Stat3 and ErbB-2 activation, we examined the tyrosine-705 phosphorylated form of Stat3α in B104-1-1 cells. These cells are derived from NIH3T3 fibroblasts that have been transformed by the...
neu-transforming gene (32). They were shown to have a higher ratio of tyrosine-705-phosphorylated Stat3/H9251 to total Stat3/H9251 protein compared with NIH3T3 cells. Transfection of B104-1-1 cells also resulted in a higher transcriptional activation of a Stat3-dependent reporter gene when compared with NIH3T3 cells. Introduction of Stat3/H9251Y705F, a dominant-negative form of Stat3/H9251, into B104-1-1 cells effectively blocked the endogenous Stat3/H9251 transcriptional activity in a dose-dependent manner, indicating that the Stat3/H9251 in these cells is transcriptionally active. These results strongly suggest that there is a correlation between expression of activated ErbB-2 and the activation of Stat3/H9251.

To further prove that Stat3/H9251 activation is induced by ErbB-2, we used an ErbB-2 mutant (V659E) to model constitutively activated receptor. Six mutant receptors were derived from this receptor in which individual or combinations of autophosphorylation mutations were introduced. The effect of these mutants on the Stat3/H9251 activity was then determined. We used two independent cell lines in our studies to eliminate any cell type-specific biases: COS-7 cells, which transfect with a high efficiency, and NIH3T3 cells, which are reported to lack expression of ErbB family members except for low levels of ErbB-2 (30). Comparable results were obtained with both lines. Compared with wild-type ErbB-2, the V659E mutant has a high capacity of activating Stat3/H9251, which may be the result of its elevated intrinsic kinase activity. Our results showed that the kinase activity of the ErbB-2 receptor is necessary for this activation because the kinase-inactive mutant of ErbB-2 is incapable of activating Stat3/H9251. Despite a comparable tyrosine phosphorylation level of all the mutants, the ability of M1 and DM to activate Stat3/H9251 was consistently decreased. Because the M1 and DM abolished one (1139) of two tyrosines located in the YXXQ amino acid context (1139 and 1222), we initially inferred this decrease to be due to the impaired ability of these mutants to recruit Stat3/H9251 to the receptor. However, our immunoprecipitation experiments showed that Stat3/H9251 forms complexes with all the mutants, even the one with an inactive kinase. Similar observations have been reported with EGFR and platelet-derived growth factor receptor (30, 42). These results contrast with the standard model of Stat3/H9251 being recruited to a receptor

**FIG. 9.** Stat3 activation by ErbB-2 receptor is partially JAK2-dependent. COS-7 cells were transfected with the plasmids indicated, along with α-2 macroglobulin luciferase and lacZ plasmids. 48 h after transfection, the cells were harvested, and luciferase assay was performed according to the protocol provided by Promega. Data shown were the relative luciferase values ± S.D of duplicate dishes. Similar results were also obtained in NIH3T3 cells. The results are representative of two experiments.

**FIG. 10.** JAK2 is preassembled with ErbB-2 receptors. COS-7 cells were transfected with Stat3/H9251, JAK2, and the ErbB-2 expression plasmids indicated. Cells were harvested 48 h after transfection, and whole-cell lysates containing 1 mg of protein were immunoprecipitated with 0.8 μg of anti-ErbB-2 antibody (Santa Cruz C-18), anti EGFR, and anti-hemagglutinin antibody as indicated (below B). Proteins bound to protein A/G beads (Santa Cruz) were analyzed by immunoblotting with JAK2 antibody (Santa Cruz) (A). The same membrane was stripped and blotted with ErbB-2 antibody (B). Expression of each of ErbB-2 mutants is shown by immunoblot analysis of whole-cell lysates using the ErbB-2 antibody (C).
tyrosine kinase via an interaction between its SH2 domain and a phosphorylated tyrosine on the receptor. If this is the case, other portion(s) of the Stat3 molecule may contribute to the association of Stat3 and ErbB-2. Alternatively, other proteins may serve as “bridging” molecules that participate in maintaining Stat3α in a complex with ErbB-2 (54). In either scenario, determining means by which Stat3α associates with ErbB-2 warrants additional study.

Despite being present in ErbB-2 immune complexes (Fig. 5) we were unable to detect tyrosine 705-phosphorylated Stat3α using a phosphotyrosine-specific antibody (data not shown). The cells do, however, contain activated Stat3α based on the results of the transcriptional activation experiments. We believe this apparent discrepancy to be due to the rapid disassociation of Stat3α from the receptor following phosphorylation on tyrosine 705 and subsequent translocation to the nucleus. Thus, the activated Stat3α is not detected in complexes with the membrane-bound receptor.

Previous studies have reported the involvement of the Src family and JAK2 kinase in the activation of Stat3α. Several models have been proposed for Stat3α activation by cell surface receptors, and thus far, there is no consensus as to the contribution of cytoplasmic tyrosine kinases (30, 42, 48). We used dominant-negative molecules to assess the involvement of Src and JAK in the activation of Stat3α by ErbB-2. We favored this method over the use of tyrosine kinase inhibitors due to the lack of specificity of many of these compounds. For example, the JAK2-specific inhibitor AG490 can block the kinase activity EGFR family members at concentrations as low as 100 nM (55). We interpret the 50–70% reduction of Stat3α transcriptional activation due to expression of activated ErbB-2 by both Src and JAK dominant-negative genes to indicate the involvement of both proteins in full activation of Stat3α. This dependence is quite similar to the manner by which Stat3α is activated by the EGF receptor (48). Dominant-negative JAK2 consistently showed a higher ability of blocking Stat3α activation than the dominant-negative Src kinase. Whether this difference indicates a more important role for JAK2 in the activation of Stat3α or simply reflects a higher amount of expression of this gene from the transfected plasmid or its greater effectiveness in acting as a dominant-negative is not known.

Immunoprecipitation and GST fusion pull-down assays were performed to investigate the protein/protein interactions involved the activation of Stat3α by ErbB-2. When immunoprecipitated with ErbB-2 receptor, both Stat3α and JAK2 kinase were found associated with all the mutants tested, including the one lacking kinase activity. This result suggests that ErbB-2 receptor, JAK2 kinase, and Stat3α may form a complex prior to Stat3α activation. To rule out the possibility that those molecules associated to ErbB-2 receptor through interactions with endogenous EGFR receptors, immunoprecipitation assays using anti-EGFR antibody were performed as well, and no association was detected (Fig. 10). As reported previously, the Src SH2 domain can bind to ErbB-2 (46). We used purified recombinant GST-Src SH2 domain fusion protein to examine its binding abilities to the different ErbB-2 mutants expressed in cells. Interestingly, we found different affinities among ErbB-2 constructs; the affinity to ErbB-2 V659E was the highest, binding to M1 and DM was reduced, and ErbB-2 wild-type was the lowest. The association between the kinase-inactive receptor and GST-Src SH2 domain was undetectable. This hierarchy of binding of the receptors to Src and is reminiscent of their respective capacity to activate Stat3α. This correlation suggests that Src plays a role in Stat3α activation by ErbB-2 receptors. Although no consensus Src binding motif is found in any of the ErbB-2 autophosphorylation sites, the decreased (compared with wild-type) association of Src and ErbB-2 when tyrosine 1139 suggests that it may be a potential Src binding site or may influence Src binding. A previous study has shown that Src can phosphorylate tyrosines other than autophosphorylation sites on EGFR and ErbB-2 and then itself use these residues as “docking” sites (56).

Although several signal transduction pathways have been shown to be involved in ErbB-2 activation (28), the direct evidence involving Stat3α activation provides greater insights into the mechanism of ErbB-2 signaling in tumorigenesis. Furthermore, it underscores the importance of other cytoplasmic tyrosine kinases in the signaling by receptors with intrinsic tyrosine kinase activity. Additional studies on Stat3α activation in the receptor kinase/Stat3α complex will provide greater understanding of these mechanisms. The knowledge that Stat3α is a direct downstream molecule of ErbB-2 receptor signaling also provides a new target for therapy for tumors expressing ErbB-2 receptors.

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