Novel Activation of STAT5b in Response to Epidermal Growth Factor*

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Of the seven signal transducers and activators of transcription that have been identified, STATs 1, 3, and 5a/5b can be activated not only by a multitude of cytokines but also by some growth factors. The data presented here demonstrate that, in contrast to activation by the cytokine, growth hormone (GH), the activation of STAT5b by the growth factor, epidermal growth factor (EGF), requires overexpression of the EGF receptor (EGFR). We have shown that EGF activates STAT5b not only in a HEK293 cell model in which the EGFR is stably overexpressed but also in the MDA-MB468 breast cancer cell line. Furthermore, EGF (but not GH) is able to activate tyrosine phosphorylation of a Tyr-699 mutant of STAT5b. Using metabolic labeling studies as well as site-directed mutagenesis, we have identified three novel EGF-induced tyrosine phosphorylation sites, Tyr-725, Tyr-740, and Tyr-743. Luciferase assays using a STAT5-specific DNA sequence demonstrate that, although Tyr-699 is absolutely required for transcriptional activation, tyrosines 725, 740, and 743 may be involved in a negative regulation of transcription. Because overexpression of the EGFR is common in many cancers, including advanced breast cancer, characterization of EGF-induced STAT5b may have direct implications in therapeutic applications.

The signal transducers and activators of transcription (STATs) transmit signals from cytokine receptors at the membrane of the cell to the nucleus. Seven STAT proteins have been cloned and four of these (STATs 1, 3, 5a, and 5b) can be activated by a number of different cytokines (1–3). Similar to growth factor receptors, cytokine receptors are single transmembrane receptors that are activated by ligand-induced dimerization. However, the cytokine receptors do not contain a tyrosine kinase activity; rather they recruit one or more of a family of intracellular tyrosine kinases known as JAK kinases (JAKs 1, 2, 3, and tyk2) to the cytokine receptor complex. Activation of these kinases results in the tyrosine phosphorylation of one or more of the recruited STAT proteins (2). Some growth factor receptors have also been shown to activate the tyrosine phosphorylation of STAT proteins (4). Platelet-derived growth factor treatment results in the activation of STAT1 and STAT3, and EGF can activate STATs 1, 3, and 5 (5–8). Although JAK kinases are activated after growth factor stimulation, they are not required for growth factor-stimulated tyrosine phosphorylation of STAT1 or STAT3 (4). In response to cytokine treatment, STAT proteins are activated by the phosphorylation of a single tyrosine residue at the C terminus of the molecule. Tyrosine phosphorylation of the STATs results in STAT dimerization through phosphotyrosine-SH2 domain interactions. Dimerization leads to nuclear translocation and binding of STAT dimers to consensus elements upstream of regulated genes. There is evidence that STATs 1, 3, and 5 are also serine-phosphorylated in their C-terminal transactivation domain resulting in maximal transcriptional activation (9). The importance of this C-terminal domain is evidenced by the fact that its truncation results in dominant negative STAT proteins that are able to inhibit the transcriptional activity of the wild type STAT proteins (2). Therefore, these C-terminal truncated proteins provide a direct means of inhibiting the biological function of wild type STATs at the cellular level. The biological actions of STAT proteins are diverse and involve a number of cellular processes, including proliferation, differentiation, and apoptosis (10).

In our previous work on growth hormone (GH) receptor signaling, a member of the cytokine receptor superfamily, we identified and cloned the human forms of STAT5a and STAT5b, encoded by two separate genes that result in two highly homologous proteins (94% homology at the amino acid level) (11). Cytokine activation results in the phosphorylation of tyrosine 694 of STAT5a and tyrosine 699 of STAT5b (6). Others have also reported the serine phosphorylation of these proteins. Rui et al. (12) demonstrated that serine 725 of STAT5a is constitutively phosphorylated and that the corresponding serine 730 of STAT5b is phosphorylated in response to prolactin. Although they found that neither of these serines needed to be phosphorylated for maximal transcriptional activation, serine 715 of STAT5b was required for maximal transcriptional activation. Hynes et al. (13) subsequently found that serine 779 was also constitutively phosphorylated but not required for transcriptional activation of STAT5a.

We have continued our investigation of STAT5b activation not only in response to GH but also in response to the growth factor, EGF. We have found that EGF-induced activation of STAT5b requires overexpression of the EGF receptor (EGFR)
and that this activation involves the phosphorylation of three novel tyrosines. These results provide insight into potential mechanisms of STAT5b activation in states of EGFR overexpression, found in many cancer types.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Recombinant human growth hormone (rhGH) was obtained from Genentech (San Francisco, CA). Recombinant, human EGFR was purchased from Invitrogen (Gaithersburg, MD). The enhanced chemiluminescence kit (ECL) was purchased from Amersham Biosciences, Inc. (Arlington Heights, IL). The polyclonal STAT5b-specific antibody was developed in our laboratory by using a peptide corresponding to the unique C terminus of STAT5b and does not cross-react with STAT5a (14). Monoclonal anti-phosphorysine antibody (p99) was from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Shc antibody was from Transduction Laboratories (Lexington, KY), and monoclonal anti-phospho-STAT5a/b (Y705) (Tyr-695/Tyr-699) was from Upstate Biotechnology Inc. (Lake Placid, NY). Monoclonal anti-EGFR antibody (F4) was from Sigma Biochemicals (St. Louis, MO). Acrylamide and bisacrylamide were from Bio-Rad (Hercules, CA), and prestained molecular weight standards and all tissue culture reagents were from Invitrogen (Rockville, MD). Except where noted, other reagents were of either reagent or molecular biological grade from Sigma.

**Cell Culture—**Human breast cancer cell lines (MDA-MB-468 and MCF-7) were obtained from ATCC (Rockville, MD). They were grown in DMEM/10% fetal calf serum/1 mM sodium pyruvate and passaged twice per week. They were preincubated in serum-free media containing 0.1% BSA overnight at 4 °C until use. Upon thawing, lysates were centrifuged at 100,000 × g and the supernatant was used for EMSA.

**Site-directed Mutagenesis—**The Tyr-695 and Tyr-699 residues were mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Tyr-695F mutant was done by the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Construction of the Y699F mutant was done by the Molecular Biology Core Laboratory at the University of Virginia. All constructs of STAT5b were sequenced by the Biomolecular Research Facility (University of Virginia).

**Metabolic Labeling—**Cells were incubated in phosphate-free media for 1 h and then for 4 h at 37 °C in 10% conditioned media plus 50 μCi [35S]methionine (50 μCi/mM, PerkinElmer Life Sciences, Boston, MA) followed by stimulation with 100 ng/ml EGF or 200 ng/ml rhGH for 15 min. Anti-HA immunoprecipitates were prepared as above except that protein G-agarose pellets were washed eight times with buffer before isolation of immunoprecipitates. Immunoprecipitates were analyzed by SDS-PAGE and transferred to nitrocellulose. After transfer to nitrocellulose, tryptic phosphopeptide mapping and phosphoamino acid analysis were performed as described previously (19, 20).

**Transient Transfections and Luciferase Assays—**For Western blotting analysis, GHR293 cells were transfected with the plasmid encoding the human EGFR and epitope-tagged forms of wtSTAT5b or STAT5b mutants. Empty expression vector (CMV-His or CMV-VA) was used as a negative control. At 36 h after transfection, cells were treated for 15 min with 100 ng/ml rhGH, and detergent lysates were prepared (as described above).

**Reporter Assays**—For reporter assays, 293 cell stable clones were transiently transfected with a plasmid containing the lactogenic hormone response region linked to luciferase (LHRR-luciferase). The LHRR sequence contains two copies of the consensus sequence from the bovine β-casein gene (AGATTTCAGGATCTCAAATC) and has been previously used in electrophoretic mobility shift assays (11). It was labeled using T4 polynucleotide kinase (New England BioLabs) and γ-32P]ATP (PerkinElmer Life Sciences). 1–10 μg of protein was incubated with the labeled probe (10K to 20K cpn) in EMSA buffer (10 μL HEPS, pH 7.9; 75 mM KCl; 2.5 mM MgCl2; 0.1 mM EDTA; 1 mM dithiothreitol; 3% Ficoll; 0.5 μg/ml BSA; 0.05 mg/ml poly(dI/dC)) for 15 min at room temperature. For supershift, overnight, and Upjohn, Sweden). Cells were transfected with 1.4 μg Spi-luc, 1.4 μg of CMV-VA, and 0.042 μg of Spi-luc, 1.4 μg of CMV-VA, and transfected the plasmid encoding the human EGFR and epitope-tagged forms of wtSTAT5b or STAT5b mutants. Empty expression vector (CMV-His or CMV-VA) was used as a negative control. At 36 h after transfection, cells were treated for 15 min with 100 ng/ml rhGH, and detergent lysates were prepared (as described above).

**RESULTS**

To begin our investigation of EGFR signaling to the STAT pathway, we transfected the coding sequence for the human EGFR into HEK293 cells and selected for stable colonies. After selection, three clones were analyzed for expression of the

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buffer containing 1% Triton X-100, 10 mM imidazole, 50 mM NaHPO4/ 300 mM NaCl, pH 8.0. The wash buffer contained 20 mM imidazole, and the elution buffer, 250 mM imidazole. Elution buffer containing the extracted proteins was mixed 1:1 with 2x Laemmli and analyzed as described above for immunoblotting.

Constitution of Epitope HA- or His-tagged Expression Vectors of STAT5b—STAT5b expression vectors were constructed using a vector with a CMV promoter and NolI site for cloning (19). The vectors contained either the HA tag (YPYDVPDYA) or a polyhistidine (HHHHHH) tag (His). Expressed proteins were isolated using either a monoclonal anti-HA antibody (Covance) or nickel-NTA-agarose magnetic beads (Qiagen). Isolation of the His-tagged proteins required lysis of cells in a

Electrophoretic Mobility Shift Assay—Forty-eight hours after transfection, MEF cells were treated with media alone or 100 ng/ml rhEGF for 15 min, at which time nuclear extracts were prepared. Cells were lysed in buffer A (10 mM HEPS, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.4% Nonidet P-40) plus 1 mM dithiothreitol and protease and phosphatase inhibitors (as above). After 10 min, lysates were centrifuged at 100,000 × g. The resulting pellets were extracted by vigorous shaking at 4 °C for 2 h in buffer B (20 mM HEPS, pH 7.9; 0.4 mM NaCl). After centrifugation, the resulting extracts were assayed for protein amount, aliquoted, quick frozen, and stored at −80 °C until use. The oligonucleotide used in EMSA is the LHRR sequence that contains two copies of the consensus sequence from the bovine β-casein gene (AGATTTCAGGATCTCAAATC) and has been previously used in electrophoretic mobility shift assays (11). It was labeled using T4 polynucleotide kinase (New England BioLabs) and γ-32P]ATP (PerkinElmer Life Sciences). 1–10 μg of protein was incubated with the labeled probe (10K to 20K cpn) in EMSA buffer (10 μL HEPS, pH 7.9; 75 mM KCl; 2.5 mM MgCl2; 0.1 mM EDTA; 1 mM dithiothreitol; 3% Ficoll; 0.5 μg/ml BSA; 0.05 mg/ml poly(dI/dC)) for 15 min at room temperature. For supershift, overnight, and Upjohn, Sweden). Cells were transfected with 1.4 μg Spi-luc, 1.4 μg of CMV-VA, and 0.042 μg of Spi-luc, 1.4 μg of CMV-VA, and 0.042 μg of Spi-luc, 1.4 μg of CMV-VA, and transfected the plasmid encoding the human EGFR and epitope-tagged forms of wtSTAT5b or STAT5b mutants. Empty expression vector (CMV-His or CMV-VA) was used as a negative control. At 36 h after transfection, cells were treated for 15 min with 100 ng/ml rhGH, and detergent lysates were prepared (as described above).

**RESULTS**

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**Fig. 1.** EGF receptor expression and signaling in 293 clones. HEK293 cells were transfected with the human EGFR. After stable selection, three of the clones were treated with media alone (Cont), 200 ng/ml rhGH, or 100 ng/ml EGF for 15 min, and lysates were analyzed for: A, expression of the EGFR by Western blotting with a monoclonal anti-EGFR antibody (F4); B, STAT5b tyrosine phosphorylation by immunoprecipitation with our STAT5b-specific antibody followed by Western blotting with anti-phosphotyrosine (top) or anti-STAT5b (bottom); and C, activation of Shc by immunoprecipitation with an Shc-specific antibody followed by anti-phosphotyrosine blotting. D, a plasmid containing the STAT5 response element (LHRR) linked to the luciferase reporter was transiently transfected into the 293EGFR clones. The ability of EGF to induce transcription of this STAT5 reporter construct was analyzed as described under “Experimental Procedures.” The data shown represent three individual experiments with each point done in triplicate in each experiment. Luciferase values are normalized to protein amount. All are compared with untreated controls transfected with the same plasmid and are -fold ± S.E.; Empty Vector (1.6 ± 0.09); Clone #3 (3.9 ± 0.16); Clone #5 (52.7 ± 0.12).

EGFR. Total lysates were analyzed by Western blotting with an anti-EGFR antibody (Fig. 1A). Clone #5 showed detectable and increased levels of the EGFR, whereas clone #3 and the negative control (empty vector) did not. We then investigated the ability of EGF to activate signaling in these three clones. As seen in Fig. 1B, EGF-induced STAT5b tyrosine phosphorylation is seen only in the clone that overexpresses the EGFR (clone #5). In contrast, growth hormone (GH) activates STAT5b in all three clones (293 cells endogenously express low levels of the GH receptor). For comparison, we also analyzed another EGF-induced signaling molecule, the adapter protein Shc. Fig. 1C demonstrates that Shc is tyrosine-phosphorylated in response to EGF in all three clones, indicating that, although endogenous levels of the EGFR do not result in STAT5b activation, they are sufficient to activate the Shc signaling pathway. We also investigated whether the EGF-induced tyrosine phosphorylation of STAT5b in the EGFR-overexpressing cells had an effect on the transcriptional activation of STAT5b. The stable clones were transiently transfected with an LHRR-luciferase reporter construct, and EGF-induced luciferase activity was analyzed as detailed under “Experimental Procedures.” As shown in Fig. 1D, the clone expressing the empty vector has only a very low level of EGF-induced luciferase activity, which is due to endogenous levels of the EGFR (1.6-fold ± 0.09). In clone #3, which expresses undetectable levels of EGFR and of EGF-induced STAT5b tyrosine phosphorylation, the EGF-induced luciferase activity is only slightly higher than that seen in cells expressing endogenous levels of EGFR (3.9-fold ± 0.16), indicating that some exogenous EGFR (not detectable by blotting) is expressed. In contrast, clone #5, which expresses high levels of EGFR and EGF-induced STAT5b tyrosine phosphorylation, shows a greater than 50-fold increase in EGF-induced luciferase (52.7-fold ± 0.12). These results demonstrate that EGF overexpression and EGF-induced STAT5b tyrosine phosphorylation lead to a significant increase in the transcriptional activity of STAT5b.

To determine whether the EGF-induced activation of STAT5b is biologically relevant, we investigated the activation of STAT5b in a panel of breast cancer cell lines. Overexpression of the EGFR is known to occur in advanced, estrogen-independent breast cancer (22–24). The details of our work on this model have been submitted for publication separately and reviewed in Biscardi et al. (25). Results from two of these breast cancer cell lines are shown in Fig. 2. As described previously, MDA-MB468 cells do not express the estrogen receptor, overexpress the EGFR (40-fold normal levels), and form large, aggressive tumors in nude mice. In contrast, MCF-7 cells express the estrogen receptor, normal levels of EGFR, and do not form tumors in nude mice in the absence of estrogen (26). Fig. 2A (top panels) confirms the overexpression of the EGFR in MDA-MB468 cells but not in MCF-7 cells. Fig. 2A (bottom panels) demonstrates that EGF-induced phosphorylation of tyrosine 699 (the well-described site of STAT5b phosphorylation) is detected only in the EGFR-overexpressing MDA-MB468 cells and not the MCF-7 cells. Specific immunoprecipitation of STAT5b (Fig. 2B) confirms that STAT5b is tyrosine-phosphorylated in response to EGF in MDA-MB468 cells but not in MCF-7 cells. These studies support the findings that the EGFR activates STAT5b in breast cancer cells. Fig. 2C shows a representative EMSA analysis using the STAT5 consensus sequence oligonucleotide (LHRR) and lysates from control- and EGF-treated cells (MCF-7 or MDA-MB468). The lower filled arrow points to the EGF-induced band in MDA-MB468 cells (lane 8), which is completely supershifted by anti-STAT5b antibody (lane 7, upper filled arrow). These shifted bands demonstrate that STAT5b binds to the LHRR sequence in an EGF-induced manner. The open arrow indicates a band that appears in control and EGF-treated samples and appears to be stabilized but not shifted by anti-STAT5b antibody. Therefore, this shifted band does not contain STAT5b but may represent other STATs or related proteins. Most importantly, these shifted complexes are not seen in the MCF-7 cells (lanes 1–4), demonstrating that STAT5b DNA binding is not induced in the MCF-7 cells; thus confirming the results seen with STAT5b immunoprecipitation.

We continued our characterization of EGF-induced STAT5b activation by investigating the role of the tyrosine 699 site, which has been shown previously to be tyrosine-phosphorylated in response to cytokine treatment and is required for dimerization, translocation, DNA binding, and transcriptional activation (6). HA-epitope-tagged forms of the wild type and Y699F mutants of human STAT5b were transiently transfected along with the human EGFR receptor (EGFR) into GHR293 cells. Analysis of anti-HA immunoprecipitates by anti-STAT5b blotting (Fig. 3, bottom panel) showed that both forms of STAT5b were expressed to similar levels in these cells. Anti-
phosphotyrosine blotting of anti-HA immunoprecipitates from GH- and EGF-treated cells showed that wtSTAT5b was tyrosine-phosphorylated in response to both cytokine and growth factor treatment (Fig. 3, top left panel). In contrast, anti-phosphotyrosine blotting of anti-HA immunoprecipitates from Y699F-expressing cells treated with GH showed no tyrosine phosphorylation. However, anti-phosphotyrosine blotting of anti-HA immunoprecipitates from Y699F-expressing cells treated with EGF showed a major tyrosine-phosphorylated band (Fig. 3, top right panel). These results suggest that EGF, but not GH, induces tyrosine phosphorylation of STAT5b on sites other than Tyr-699. This novel tyrosine phosphorylation was confirmed with other anti-phosphotyrosine antibodies (data not shown). Furthermore, blotting with the anti-phospho-699-specific STAT5 antibody confirmed that this signal was not due to phosphorylation of tyrosine 699 (Fig. 3, middle panels). Also, close inspection of the anti-STAT5b blotting (bottom panel) shows that, although there is a shift in wtSTAT5b with GH and EGF treatment, there is no shift in EGF-treated Y699F despite the presence of a tyrosine phosphorylation. Together, these results demonstrate that the growth factor, EGF, can induce tyrosine phosphorylation of tyrosine 699 of STAT5b as well as one or more other tyrosines.

As a means toward identifying the novel EGF-induced tyrosine phosphorylation site(s), GHR293 cells were metabolically labeled with [32P]orthophosphate and treated with media alone (Cont), 200 ng/ml GH, or 100 ng/ml EGF. Anti-HA immunoprecipitates were analyzed by SDS-PAGE, and the labeled STAT5b band was excised from the blot and digested with trypsin. Tryptic peptides were then analyzed by two-dimensional electrophoresis and chromatography. As shown in Fig. 4, the large white-circled spot represents the peptide containing Tyr-699 (as determined by phosphoamino acid analysis and Edman degradation). As expected, phosphorylation of Tyr-699 is seen in both GH- and EGF-treated wtSTAT5b but not in the Y699F mutant (compare top versus bottom panels). To characterize the novel EGF-induced tyrosine phosphorylation, we focused on those spots that remained in the EGF-treated Y699F mutant (bottom, right panel). The largest of the spots remaining (circled in black) was seen not only in EGF-treated Y699F but also in the GH-treated sample (and to some extent, the untreated Cont). Amino acid analysis allowed us to determine that this spot corresponded to phosphoserine. The other two series of five and three spots that were seen only in the EGF-treated Y699F mutant (bracketed) were found to contain phosphothreonine and phosphoserine (as labeled). The fact that these two series of spots are not seen in the GH-treated Y699F sample (bottom, middle panel) suggests that they represent one or more of the novel EGF-induced tyrosine phosphorylations. Importantly, these spots are also seen in the EGF-treated wtSTAT5b (top, right panel), indicating that these phosphorylations may also be present under physiological conditions.

The pattern of spots seen in Fig. 4 (lower right panel) indicates that the peptides they represent are in some way related. They may result from a change in hydrophobicity (affecting the chromatography) and/or incomplete tryptic digestion possibly due to closely spaced cleavage sites. Furthermore, the presence of serine phosphorylation in this series of spots demonstrates that the peptide is serine- as well as tyrosine-phosphorylated. Given the low mobility of these peptides during electrophoresis, these peptides are likely to be large. To predict which tyrosines may be responsible for the novel EGF-induced tyrosine phosphorylation, we began by listing the theoretical peptides that
would result from trypsin proteolysis of STAT5b. Trypsin typically cleaves proteins C-terminal to lysine (K) and arginine (R) residues. We found that a total of greater than 80 potential peptides would result from trypsin digestion of STAT5b. Fig. 5 depicts the domain structure of STAT5b and the tryptic peptides that result from proteolysis of the SH2 domain through the C terminus of STAT5b. Tyr-699 is found here in a relatively short peptide (AVDGYVK). In addition, three tyrosines (Y725/740/743) are found in an unusually large peptide that is followed by two adjacent arginines (R). Furthermore, this peptide also contains the serine sites reported to be phosphorylated in response to prolactin (S715/730) (12). These characteristics (potential serine and tyrosine phosphorylation sites, large size, and potentially incomplete digestion pattern at the arginines) led us to investigate further this peptide as representing the series of five and three spots shown in Fig. 4. We predicted that one or more of the three tyrosines in this peptide would be responsible for the novel EGF-induced tyrosine phosphorylations.

Despite numerous attempts, we could not identify the phosphorylated amino acid(s) by Edman degradation because the peptides were of low abundance (probably because of low yield due to their relatively large size). Furthermore, unlike the single spot representing tyrosine 699, in this case the ^32P label was distributed among a number of spots. Thus, we decided to use site-directed mutagenesis, targeting the three tyrosines contained in the large peptide described in Fig. 5. As a first step toward determining whether any of these three tyrosines were novel phosphorylation sites, we reconstructed the STAT5b expression vector using a poly-histidine epitope tag (His) in place of the HA-epitope tag, which itself contains three tyrosines (YPYDVPDYA). As described under "Experimental Procedures," the His epitope tag also allowed purification using the Nickel-NTA magnetic agarose beads (Qiagen). As shown in Fig. 6, EGF induces the tyrosine phosphorylation of His-tagged Y699F STAT5b, ruling out the possibility that the phosphorylation detected previously was due to the three tyrosines in the HA-epitope. Also seen under these conditions is a constitutive level of tyrosine phosphorylation of Y699F STAT5b (Cont) that is not seen in the wild type control (His-STAT5b, Cont). A non-specific band in the anti-pTyr blot migrates just above STAT5b (open arrow) and is consistently seen (although at varying levels) in subsequent figures. As seen previously with the HA-tagged constructs, anti-STAT5b blotting demonstrates a shift in the wtSTAT5b protein upon activation by EGF, resulting in two additional STAT5b bands. However, a shift in the Y699F mutant is not as dramatic despite an EGF-induced tyrosine phosphorylation.

Using the His-tagged Y699F STAT5b, we mutated the three tyrosines, identified in Fig. 5 (725/740/743) to determine directly whether they were responsible for the novel EGF-induced phosphorylation. These constructs were transiently expressed in the 293 cells along with the EGFR. As described under "Experimental Procedures," nickel-NTA magnetic agarose beads (Qiagen) were used to isolate the His-tagged proteins. The filled arrow indicates the STAT5b band, and the open arrow indicates a nonspecific protein that binds to the nickel-agarose beads.

**FIG. 4.** Tryptic digests of wild type and Y699F STAT5b. GHR293 cells transiently expressing HA-tagged wtSTAT5b or Y699F mutant STAT5b were metabolically labeled with [32P]orthophosphate and treated with media (Cont), or 200 ng/ml GH or 100 ng/ml EGF. Anti-HA immunoprecipitates were analyzed by SDS-PAGE, and the band corresponding to STAT5b was excised from the blot and digested with trypsin. Tryptic peptides were then analyzed by two-dimensional chromatography/electrophoresis (as shown by arrows). The white circle represents Tyr-699 as determined by phosphoamino acid analysis and Edman degradation. In the lower right panel, the dark circle is phosphoserine and the brackets are as labeled and determined by phosphoamino acid analysis.

**FIG. 5.** Predicted tryptic peptides of STAT5b. Theoretical proteolysis of STAT5b by trypsin occurs after each lysine (K) and arginine (R). The peptides shown represent the region of STAT5b from the start of the SH2 domain through the C terminus. Tyrosines of interest are circled, and reported serine phosphorylation sites are underlined.

**FIG. 6.** His-tagged Y699F STAT5b is tyrosine-phosphorylated in response to EGF. The coding sequence for STAT5b was tagged with an epitope containing six histidines (His) and expressed in GHR293 cells along with the EGFR. As described under "Experimental Procedures," nickel-NTA magnetic agarose beads (Qiagen) were used to isolate the His-tagged proteins. The filled arrow indicates the STAT5b band, and the open arrow indicates a nonspecific protein that binds to the nickel-agarose beads.

**STAT5b Structure**
Tyrosine 725 or Tyr-740 can be phosphorylated in response to EGF and that mutation of one does not affect the total EGF-induced tyrosine phosphorylation of the Y699F mutant of STAT5b.

Because all the tyrosines mutated so far appear to play a role in the EGF-induced tyrosine phosphorylation of STAT5b, we mutated other tyrosines within the STAT5b sequence to determine the specificity of this effect. We chose to mutate tyrosine 405 (within the DNA-binding domain) and tyrosine 679 (not found in STAT5a and thus unique to STAT5b). Fig. 9 shows that neither of these mutations affects the ability of EGF to induce the tyrosine phosphorylation of the Y699F mutant of STAT5b (compare C to A). Again, mutation of tyrosines 725/740/743 in the context of Y699F completely abrogates EGF-induced tyrosine phosphorylation (Fig. 9E, top) and also unmasks the shift of the STAT5b band (bottom panel in Fig. 9E). We also mutated the only other tyrosine residue that is unique to STAT5b (Tyr-392) and found that this mutation did not affect EGF-induced tyrosine phosphorylation of the Y699F mutant of STAT5b (data not shown).

Finally, to determine the relative contribution of Tyr-699 versus the novel tyrosines (725/740/743) in the EGF-induced phosphorylation of STAT5b, we mutated Tyr-699 alone, the three tyrosines 725/740/743 together, or all four tyrosines (699/725/740/743). These mutations were compared with the wtSTAT5b and empty vector-expressing cells. As shown in Fig. 10B, EGF induces a major tyrosine phosphorylation of STAT5b, seen by anti-phosphotyrosine blotting, and a shift in the STAT5b band (bottom panel). As previously shown, when Tyr-699 is mutated there is an increase in the constitutive tyrosine phosphorylation of STAT5b as well as an EGF-induced tyrosine phosphorylation (Fig. 10C). Mutation of tyrosines 725/740/743 (Fig. 10D) does not result in any constitutive phosphorylation but clearly demonstrates an EGF-inducible tyrosine phosphorylation. Finally, the mutation of all four of these tyrosines (Fig. 10F) results in neither constitutive nor EGF-induced tyrosine phosphorylation above baseline levels (compare with Fig. 10A). The EGF-induced shift in the STAT5b band of the quadruple mutant, in the absence of any detectable tyrosine phosphorylation (lower panel of Fig. 10E), indicates that this shift may be due to serine phosphorylation or an unmasked conformational change.

To determine whether these novel sites of tyrosine phosphorylation are potentially present in physiologically relevant cell lines, we transiently transfected these constructs into the MDA-MB468 breast cancer cell line. As seen previously in the HEK293 model, a nonspecific band is seen in the anti-pTyr Western blot of nickel column-purified proteins (open arrow, Fig. 11). However, there is also a prominent EGF-induced tyrosine phosphorylation of both the Y699F and Y725F/Y740F/Y743F mutants of STAT5b, and lysates were isolated and analyzed as in Fig. 6. Tyr-699 is within the DNA-binding domain of STAT5b. Tyr-679 is one of two tyrosines unique to STAT5b (not in STAT5a). The filled arrow is STAT5b; the open arrow is a nonspecific band.
activation. We used mouse embryonic fibroblasts (MEFs) from STAT5a/b knockout mice to eliminate any background signal from endogenous STAT5 proteins. We investigated both DNA binding and transcriptional activation of the STAT5b tyrosine mutant constructs to gain insight into the role these tyrosines have in EGF-regulated gene transcription. Fig. 12A shows the results of a typical EMSA. When MEF cells are transiently transfected with STAT5b constructs, in addition to the EGFR, a shifted band results in lysates from both control and EGF-treated cells (lanes 1 and 2). This band (not seen in MEFs transfected with empty vector, data not shown) represents STAT5b. The presence of STAT5b in this band is confirmed by its supershift with anti-STAT5b antibody (lanes 3 and 4), indicating that these tyrosines are phosphorylated. Importantly, we do not see a band containing STAT5b, and the upper arrow indicates the band that is supershifted with anti-STAT5b antibody (lanes 12 and 14). To determine more directly whether the apparent increase in DNA binding of Y725F/Y740F/Y743F is relevant, we analyzed the ability of this mutant to regulate a STAT5 reporter construct. Using the MEF cells that do not express STAT5 proteins, there is no transcriptional activation induced in the cells that have been transfected with vector alone (not shown). However, transfection of wtSTAT5b into these cells results in an increase in the baseline level of luciferase activity and an EGF-induced luciferase activity of approximately 3-fold. In contrast, the Y699F mutant of STAT5b shows no response over vector alone, and is almost 100-fold less than that seen with wtSTAT5b, thus supporting the results from EMSA demonstrating that Tyr-699 is required not only for DNA binding but, as expected, for the EGF-induced transcriptional activation of STAT5b. In contrast, we found that the Y725F/Y740F/Y743F mutant caused a dramatic increase in the basal transcriptional activity of the STAT5b reporter (comparable to that seen with EGF-induced wtSTAT5). We have seen this dramatic increase in basal activity in response to transfection of Y725F/Y740F/Y743F not only in the MEF cells, but also in a COS cell transfection system (data not shown). EGF treatment of cells expressing the Y725F/Y740F/Y743F mutant of STAT5b results in a modest increase in transcriptional activity. Although not shown, but as expected, mutation of all four tyrosines (Y699F/Y725F/Y740F/Y743F) results in transcriptional activity, which is even lower than that seen with vector alone.

**DISCUSSION**

The role of the STAT proteins in signaling pathways activated not only by cytokine receptors but also by growth factor receptors and other membrane receptors has become under increasing study over the past few years (27). It is becoming ever more apparent that STATs play an important role not only in their originally characterized hematopoietic cell model but also in more widespread model of proliferation, apoptosis, transformation, and tumorigenesis (28, 29). In the studies presented here, we have demonstrated that, unlike cytokine (GH) activation of STAT5b, EGF-induced activation of STAT5b requires the overexpression of its receptor. This observation is important for two reasons: 1) This requirement for EGFR overexpression is not necessary for the EGF-induced activation of other pathways, such as Shc; and (2) many cancer cells, including aggressive breast cancer, overexpress the EGFR. Our analysis...
ysis of two breast cancer cell lines, one that overexpresses the EGF (MDA-MB468) and one that does not (MCF-7), demonstrates that STAT5b is activated by EGF in MDA-MB468 cells but not MCF-7. In other studies, we have analyzed the mechanisms involved in the pathway of EGF-dependent activation of STAT5b in EGF-overexpressing breast cancer cells. However, here we have characterized in detail the EGF-induced activation of STAT5b as a means toward potentially developing therapeutic interventions for cancer.

Our characterization of STAT5b activation in response to EGF has uncovered three previously unidentified tyrosine phosphorylation sites on STAT5b. Although our studies here have focused on the EGF-induced phosphorylation of these sites, our tryptic peptide maps (Fig. 4) as well as luciferase assays (not shown), suggest that these sites, although regulated differently, may also play a role in the GH activation of STAT5b. Therefore, our results may have application with respect to cytokine as well as growth factor activation of STAT5b. Furthermore, this mechanism of tyrosine phosphorylation may also apply to the EGF-induced activation of STAT5a, because the tyrosine phosphorylation of STAT5a (in addition to tyrosine 694) has been reported in EGF-overexpressing cells (30); although the sites were not identified.

Tryptic peptide mapping and site-directed mutagenesis allowed us to identify tyrosines 725, 740, and 743 in the C-terminal transactivation domain of STAT5b as EGF-induced phosphorylation sites. Our characterization of EGF-induced tyrosine phosphorylation, DNA binding, and transcriptional activation of a STAT5b allowed us to come to the following conclusions: 1) EGF can induce the phosphorylation of tyrosines 699, 725, 740, and 743 either together or individually; 2) mutation of Tyr-699 allows a constitutive tyrosine phosphorylation of the STAT5b protein but no detectable DNA binding or transcriptional activation; 3) stimulation by EGF leads to a further increase in the tyrosine phosphorylation of this Y699F mutant but no increase in transcriptional activity; 4) mutation of tyrosines 725/740/743 results in an apparent increase in DNA binding and a dramatic increase in the basal transcriptional activation (equal to that of EGF-induced wtSTAT5b); and 5) EGF stimulation of the Y725F/Y740F/Y743F mutant results in an increase in tyrosine phosphorylation (presumably at Tyr-699) and a modest increase in transcriptional activation (see Fig. 12B). Taken together, these data led us to propose a model in which Tyr-699 is absolutely required for transcriptional activation of STAT5b in response to EGF, whereas tyrosines 725/740/743 are in some way involved in the inhibition of basal transcription. The mechanism by which this inhibition occurs may involve binding of an inhibitory factor, or an effect (conformational or otherwise) on the function of the C-terminal transactivation domain of STAT5b. In support of this model, we find that the Y699F mutant of STAT5b has a very low transcriptional activity (almost 100-fold below baseline) supporting the fact that the intact tyrosines (725/740/743) are capable of inhibiting transcription even in this context. Therefore, there seems to be an intrinsic interplay between transcriptional activation of STAT5b through Tyr-699 and inhibition through tyrosines 725/740/743. Investigation of these mechanisms in growth factor as well as cytokine activation of STAT5b will be the topic of future investigation. Insight will be gained by initially identifying the kinase responsible for activating these novel tyrosine phosphorylations. Previous work by our group, as well as others, suggests that there are at least three obvious candidates, JAK1, the EGFR, and c-Src. Studies have shown that the JAK tyrosine kinases, although activated, are not required for STAT activation in response to growth factor stimulation but that the tyrosine kinase activity of the EGFR receptor itself is required (4, 5, 31). More recent studies have indicated that the c-Src tyrosine kinase is also involved in EGF activation of STAT5a and STAT5b (30, 32). Our work in model systems in which the c-Src kinase is overexpressed indicates that this kinase plays a role in EGF-induced tyrosine phosphorylation and transcriptional activation of STAT5b.

Furthermore, our tryptic mapping as well as anti-STAT5b blotting also indicate that EGF-induced serine phosphorylation plays a role in EGF-induced activation of STAT5b. Tryptic peptide mapping demonstrates a constitutive serine phosphorylation site in both wild type and the Y699F mutant of STAT5b (Fig. 4), as well as an EGF-induced serine phosphorylation seen in the series of related phosphorylated peptides. Also, in anti-STAT5b blotting of the Y699FY725FY740FY743F mutant, when there is no apparent tyrosine phosphorylation, we detect a dramatic shift in the STAT5b band with EGF treatment, as compared with the untreated lanes (Figs. 9 and 10). In other model systems, Waxman et al. (33) have identified that this shift is due to serine phosphorylation. These results together suggest that the EGF-induced serine phosphorylation is not dependent on EGF-induced tyrosine phosphorylation and that the shift due to this serine phosphorylation is unmasked when all four tyrosines in this same region of STAT5b are mutated and not phosphorylated. The role of serine phosphorylation in the transcriptional activation function of STAT5b and, perhaps, even the important sites of serine phosphorylation have yet to be characterized (34).

In conclusion, we have found that activation of the STAT5b transcription factor in response to EGF requires EGF receptor overexpression. Because EGF overexpression is known to occur in a number of cancers, including breast, ovary, and colon cancer (35), the mechanism of STAT5b tyrosine phosphorylation and transcriptional activation will provide important insight into the potential role of STAT5b in the process of tumorigenesis. There is increasing evidence that constitutively active STAT proteins, particularly STAT3 and STAT5b, contribute to oncogenesis (28). Thus, understanding the mechanism of this constitutive activation and the potential role of tyrosines 725/740/743 will have important implications for future therapeutic intervention.

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