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ERBB4/HER4 Potentiates STAT5A Transcriptional Activity by Regulating Novel STAT5A Serine Phosphorylation Events*[S]

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The epidermal growth factor receptor family member ERBB4 is required for mammary gland development and lactation. ERBB4 activities in the breast are mediated through the signal transducer and activator of transcription (STAT) family member STAT5A, and ERBB4 directly activates STAT5A, in part, through phosphorylation of STAT5A at the regulatory Tyr-694. Here we show that STAT5A regulation by ERBB4 is also mediated through STAT5A serine phosphorylation. Using a reverse-phase high performance liquid chromatography tandem mass spectrometry analysis of proteolytically digested STAT5A coexpressed with ERBB4, we identified STAT5A serine phosphorylations at the previously described Ser-779 and at the novel Ser-127/Ser-128. Immunohistochemistry of wild-type and *ERBB4*-null mammary glands at late pregnancy showed that ERBB4 expression was required for STAT5A phosphorylation at Ser-779. Independent serine-to-alanine residue substitutions in full-length STAT5A revealed that although STAT5A Ser-779 phosphorylation was dispensable for phosphorylation of STAT5A at Tyr-694 and subsequent DNA binding, Ser-779 was required to stabilize an interaction with ERBB4 and mediate ERBB4-induced STAT5A stimulation of gene expression. STAT5A Ser-127/Ser-128, on the other hand, was required for ERBB4-induced phosphorylation of Tyr-694, whereas Ser-779 and as yet unidentified tyrosine residues were phosphorylated in the absence of Ser-127/Ser-128. In addition, STAT5A S127A/S128A remained associated with ERBB4 but failed to bind DNA or activate transcription in response to ERBB4 coexpression. Our studies demonstrate that phosphorylation of STAT5A at Ser-127/Ser-128 and Ser-779 are obligatory events regulating ERBB4-mediated activation of STAT5A.

STAT5A, initially identified as a mammary differentiation factor, is a member of the signal transducer and activator of transcription (STAT)[1] family of transcription factors. STAT5A transcriptional activity is regulated through signals initiated at the cell surface by both cytokine and growth factor receptors, leading to the phosphorylation, nuclear translocation, and binding to γ-interferon activation sites in the promoters of target genes. In the mammary gland, STAT5A regulates multiple pregnancy-induced developmental events including specification of ductal epithelium to a secretory phenotype, epithelial terminal differentiation, and activation of milk gene expression during lactation (1–5).

STAT5A activity is regulated by phosphorylation events at specific residues through the actions of receptor tyrosine kinases, cytokine receptors, and non-receptor tyrosine kinases (6, 7). Phosphorylation of the regulatory Tyr-694 is indispensable for STAT5A transcriptional activity and has been used as a biochemical indicator of STAT5A transcriptional activation (8). In addition, constitutive serine phosphorylation of STAT5A at positions 725 and 779 has recently been reported, and convincing evidence indicates that phosphorylation of STAT5A at Ser-779 occurs within the mouse mammary gland during multiple developmental stages, including lactation (9, 10). Although a conserved residue substitution at STAT5A Ser-725 failed to impact prolactin (Prl)-induced β-casein promoter activity (9, 10), in one study a similar residue substitution at STAT5A Ser-779 suppressed Prl-induced STAT5A transcriptional activity (10). The lack of a dramatic influence of STAT5A serine phosphorylation on Prl-induced STAT5A transactivation raises the possibility that STAT5A serine phosphorylation modulates the activity of an alternative mammary gland signaling network.

The ERBB/epidermal growth factor receptor family of receptor tyrosine kinases play critical roles in normal development (11) and neoplastic transformation of the breast (12). Substantial *in vivo* evidence indicates that ERBB4, the final member of this receptor family to be discovered, is the essential mediator of STAT5A activation during epithelial differentiation at late pregnancy and lactation at parturition (4, 5, 13). Indeed, STAT5A phosphorylation at the regulatory Tyr-694 was abolished and the expression of STAT5A regulated milk genes was dramatically impaired in mice harboring mammary gland-specific deletions of both *ERBB4* alleles (4, 5). In tissue culture

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[S] The on-line version of this article (available at www.jbc.org) contains Supplementary Fig. S1, featuring a mass spectrometry analysis of STAT5A phosphopeptides.

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[1] The abbreviations used are: STAT, signal transducer and activator of transcription; MS/MS, tandem mass spectrometry; Prl, prolactin; PrlR, prolactin receptor; STAT5AS127/128A, STAT5A serines 127/128 mutated to alanine.

models, we have shown that ERBB4 physically interacts with and induces Tyr-694 phosphorylation of STAT5A in a STAT5A Src homology 2 domain-dependent manner (13). Furthermore, ligand-activated and proteolytically processed ERBB4 regulates STAT5A stimulation of the β-casein promoter by functioning as a STAT5A nuclear chaperone (14, 15). Accordingly, we have demonstrated by chromatin immunoprecipitation assay that ERBB4 binds with STAT5A at the endogenous β-casein promoter in the T47D breast cancer cell line (15). In addition to the unique function of ERBB4 as a STAT5A nuclear chaperone, ERBB4 induces phosphorylation of novel STAT5A tyrosine residues in addition to Tyr-694 (13). The critical role of ERBB4 as a unique regulator of STAT5A in breast cancer cell lines and the mouse mammary gland raises the possibility that ERBB4 is an important functional regulator of STAT5A serine phosphorylation. Indeed, phosphorylation of STAT5A at Ser-779 peaks at late pregnancy and early parturition (9, 10), coincident with the essential contribution of ERBB4 to STAT5A activation (4, 5).

In this study, we investigated the role of STAT5A serine phosphorylation on ERBB4-induced STAT5A stimulation of gene expression. Phosphopeptide mapping of STAT5A revealed that ectopic ERBB4 expression is associated with STAT5A phosphorylation at Ser-127/Ser-128, as well as at Ser-779. To determine the functional significance of these phosphorylation events, we incorporated independent base substitutions within STAT5A at Ser-127/Ser-128 and Ser-779 and characterized the influence of these mutations on ERBB4 regulation of STAT5A biological activity. In contrast to previous descriptions where STAT5A serine phosphorylation failed to influence Prl-regulated STAT5A signaling, serine phosphorylation of STAT5A had a significant impact on ERBB4 regulation of STAT5A activity. Moreover, our experiments identified distinct biological functions for STAT5A phosphorylation at Ser-127/Ser-128 and Ser-779 and underscore the role of ERBB4 as a regulator of unique STAT5A signaling mechanisms.

EXPERIMENTAL PROCEDURES

ERBB4 cDNA—The human ERBB4 cDNA used in these experiments has been sequenced in its entirety (16) and represents the JM-a isoform (17). This isoform retains both tumor necrosis factor α-converting enzyme (TACE) and γ-secretase recognition sequences and is therefore an ERBB4 isoform that undergoes complete proteolytic processing at the cell surface following ligand stimulation.

Tissue Culture and Transfections—HEK 293 cells were obtained from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 2 mM L-glutamine. The dramatic growth-inhibitory effect of ectopic ERBB4 expression (18) precluded analysis of ERBB4 and STAT5A in many common mammalian cell lines. We therefore performed all functional assays in the MCF-7 human breast cancer cell line MCF-7B, which was modified to stably overexpress BCL-2 (19) and is resistant to ERBB4-induced cell killing.[2] MCF-7B cells were cultured in minimum essential medium containing 10% fetal bovine serum, 1 mM sodium pyruvate, and 2 mM L-glutamine.

Transfections were performed on 2×10^5 cells in 35-mm tissue culture dishes with 1 μg of each transfected DNA by using FuGENE 6 transfection reagent (Roche Applied Science) as described by the manufacturer. In each experiment, transfected cells were harvested at 48 h post-transfection.

Plasmid Constructs—With the exception of mouse STAT5A harboring a residue substitution of S779A (9), the human ERBB4 and mouse STAT5A expression plasmids have been described elsewhere (13). The plasmid pEF-STAT5AS127/128A harboring alanine residue substitutions at serines 127 and 128 was generated in an NruI/BamHI fragment of pEF-STAT5A by PCR-mediated, site-directed mutagenesis using the upstream primer 5′-GCTGCAGAAGAAGG-3′ (nucleotides 241–254; GenBank™ accession number BC008998), the downstream primer 5′-GGGCAAACTGAGCT-3′ (nucleotides 602–589), and the mutation incorporating oligonucleotide primer pairs 5′-AATTGCGCAGCCCC-TGCTGGTGTCCTGGTT-3′ (nucleotides 413–442) and 5′-AGCAGG-GGCTGCGCAATTGTTGGCTTCGCG-3′ (nucleotides 430–401). The STAT5AS127/128A mutagenic primers were designed to generate a unique FspI site. The mutations were verified by sequencing.

Phosphopeptide Mapping of STAT5A—HEK 293 cells were cotransfected with mouse STAT5A and human ERBB4 expression vectors. At 48 h post-transfection, STAT5A was immunoprecipitated from cell lysates and separated by PAGE as described elsewhere (13). The resolving gel was stained with Gel Code Blue (Pierce), and the 95-kDa STAT5A band was excised and stored at −70 °C until use. The STAT5A sequence was evaluated using an in-house program, Enzyme Optimizer, for a dual enzyme strategy that would optimize for coverage of phosphorylated residues. The program considers factors that influence recovery and detection of a predicted peptide rather than simple protein coverage. The gel slices were subjected to in-gel tryptic and chymotryptic digestions after reduction and carboxyamidomethylation. The resultant digests were pooled just prior to liquid chromatography tandem mass spectrometry (MS/MS) injection. Phosphorylated peptide sequences were determined using a 75-μm reverse phase microcolumn (New Objective, Woburn MA) terminating in a custom nanoelectrospray source directly coupled to an LCQ DECA XP Plus quadrupole ion trap mass spectrometer (Thermo Electron, San Jose CA). Flow was nominally 200 nl/min. The ion trap repetitively surveyed the range *m/z* 395–1600, executing data-dependent MS/MS for peptide sequence information on the four most abundant ions in each survey scan. MS/MS spectra were acquired with a relative collision energy of 30% and an isolation width of 2.5 Da, and recurring ions were dynamically excluded. After data base correlation with the algorithm SEQUEST (32), phosphorylated peptides were confirmed by manual, *de novo* interpretation of the MS/MS spectra using the FuzzyIons program (20). When needed, a second targeted ion MS/MS experiment was conducted to increase detection sensitivity and spectrum quality. In this run, the predicted precursor mass-to-charge ratio (*m/z*) of the phosphopeptide was subjected to MS/MS for the entire chromatographic run, capturing the peptide of interest at the moment of its chromatographic elution.

STAT5A Phosphoserine779 Immunohistochemistry—Generation of paraffin-embedded mouse mammary glands at 18 days post-coitus from normal control mice (*ErbB4*$^{+/+}$*WAP-Cre*) and mice with conditional deletions of both *ERBB4* alleles (*ErbB4*$^{Flox/Flox}$*WAP-Cre*) has been described elsewhere (4). Immunohistochemical detection of the STAT5A nuclear antigen phosphorylated at Ser-779 was performed exactly as described previously (13) with the following modifications. STAT5A Ser(P)-779-specific antiserum (9) was diluted 1:100 and incubated on the tissue sections in the presence of 1 μM unphosphorylated peptide (9); the biotinylated goat anti-rabbit (Vector Labs) secondary antibody was diluted to 15 μg/ml. The diaminobenzidine tetrahydrochloride (DAB) substrate was prepared fresh before use (4). Sections were lightly counterstained in hematoxylin (Polysciences) according to the manufacturer's instructions, dehydrated in ethanol, cleared in xylene, and coverslipped with Permount (Fisher).

Western Blot Analysis—Immunoprecipitations from total cell lysates were separated on a 7.5% polyacrylamide gel, transferred to Hybond ECL (Amersham Biosciences) membrane, and analyzed by Western blot as described previously (13). Primary antibodies were anti-ERBB4 (Santa Cruz Biotechnology), anti-STAT5A (Santa Cruz Biotechnology), anti-P-Stat5 (Santa Cruz Biotechnology), and anti-phosphotyrosine (Santa Cruz Biotechnology). The STAT5A Ser(P)-779-specific antiserum was diluted 1:5000 and incubated in the presence of 2 μg/ml unphosphorylated peptide (9).

Luciferase Reporter Assay—Luciferase assays were performed exactly as described elsewhere (15). Each sample was performed in duplicate, and the entire experiment was performed at least three times. Statistically significant differences between data sets were determined using the paired Student's *t* test.

Electrophoretic Mobility Shift Assay—NIH3T3 cells were transfected with ERBB4, STAT5A, STAT5AS127/128A, or STAT5AS779A expression plasmids, and whole cell extracts were prepared at 48 h post-transfection as described elsewhere (9). STAT5A DNA binding activity was assayed by incubating 20 μg of whole cell lysate with gel shift binding buffer (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 2% glycerol, and 250 μg/ml poly(dI-dC) (Promega) for 10 min prior to the addition of a ^{32}P-5′-end-labeled oligonucleotide probe corresponding to the STAT5A binding sequence derived from the bovine β-casein promoter (5′-AGATTTCTAGGAAT-TCAATCC-3′) and further incubation at room temperature for 30 min.

[2] W. Long, G. A. Vidal, A. Naresh, W. C. Wimley, L. Marrero, C. I. Sartor, S. Tovey, T. G. Cooke, J. M. S. Bartlett, and F. E. Jones, unpublished observations.

TABLE I
STAT5A serine phosphorylation sites

Peptide	Position	Serine phosphorylation	
		Serine position	Phosphoserine detected/ peptides analyzed
EANNCSSPAGVLVDAMSQK	122–140	127/128	9/9
MDQAPSPVVCPQPHY	720–734	725	0/1[a]
RPMDSLDARLSPPAGLF	769–785	779	3/3

[a] Additional targeted analysis of peptides containing Ser-725 failed to detect phosphorylation of this site.

Lysates were run on a native 5% polyacrylamide gel for 1 h, dried, and exposed to x-ray film.

RESULTS

STAT5A Ectopically Coexpressed with ERBB4 Is Phosphorylated at Serines 127/128 and 779—When ectopically coexpressed with ERBB4, STAT5A is phosphorylated at the regulatory Tyr-694 (13, 21) in addition to as yet unidentified tyrosine residues (13). Recent evidence demonstrating that STAT5A is constitutively phosphorylated at serine residues raises the possibility that coexpressed ERBB4 and STAT5A may result in serine phosphorylation of STAT5A. We therefore performed a phosphoserine peptide analysis of STAT5A coexpressed with ERBB4 by microcapillary reverse-phase high performance liquid chromatography nanoelectospray MS/MS. This analysis revealed two unambiguous STAT5A serine phosphorylation events at Ser-127/Ser-128 and Ser-779 (Table I, Supplemental Fig. S1 (available in the on-line version of this article), and Fig. 2*A*). Interestingly, activation of STAT5A through Prl or growth hormone failed to result in phosphorylation of STAT5A at Ser-127/Ser-128 (9, 10, 22), suggesting that this phosphorylation event is unique to ERBB4 activation of STAT5A. Similar to our previous results (9), constitutive phosphorylation of STAT5A at Ser-725 (23) was not detected in our analysis of STAT5A phosphoserine residues. In summary, our data suggest that STAT5A regulation by ERBB4 may be partially mediated by a pathway involving serine kinases in addition to regulation by tyrosine phosphorylation.

ERBB4 Is Required for Phosphorylation of STAT5A at Ser-779 in Mammary Glands of Pregnant Mice—As an initial analysis to determine the functional significance of STAT5A serine phosphorylation during ERBB4/STAT5A signaling, we examined *ERBB4*-deficient mammary glands for expression of STAT5A phosphoserine 779. In previous reports, we and others have demonstrated that deletion of both *ERBB4* alleles in the mouse mammary gland results in the loss of STAT5A phosphorylation at Tyr-694 and lactational failure (4, 5). Peak phosphorylation of STAT5A at Ser-779 in the mouse mammary gland at late pregnancy (9) correlates with STAT5A Tyr-694 phosphorylation and the essential contribution of ERBB4 to breast function (4, 5). We used immunohistochemical analysis of ERBB4 deficient mammary glands at 18 days post-coitus to determine whether ERBB4 expression was required for STAT5A Ser-779 phosphorylation *in vivo*. High levels of STAT5A Ser-779 phosphorylation could be detected within the nuclei of mammary epithelium from wild-type mice (*ErbB4*$^{+/+}$*WAP-Cre*) (Fig. 1*A*, *arrowheads*). Despite normal levels of STAT5A protein expression (4), we failed to detect STAT5A Ser-779 phosphorylation in ERBB4-deficient mammary glands (*ErbB4*$^{Flox/Flox}$*WAP-Cre*) (Fig. 1*B*), thereby demonstrating that ERBB4 expression is necessary for STAT5A Ser-779 phosphorylation *in vivo*.

STAT5A Serine Phosphorylation Is Necessary for ERBB4-Induced STAT5A Transcriptional Activity—Most recently, we have demonstrated that the ectopic coexpression of STAT5A with ERBB4 results in transcriptional activation of a reporter gene fused to a β-casein promoter harboring STAT5A DNA binding sites (15). Here, by ectopically coexpressing ERBB4 with STAT5A harboring independent serine-to-alanine substitutions at Ser-127/Ser-128 and Ser-779 (STAT5AS127/128A and STAT5AS779A, respectively), we determined the contribution of STAT5A serine phosphorylation to ERBB4 regulation of STAT5A transactivation in a β-casein promoter luciferase reporter assay. In concordance with our previous report (15), independent expression of STAT5A or ERBB4 failed to stimulate β-casein promoter activity above vector control levels (Fig. 2*C*). Luciferase reporter activity was increased ~8-fold, however, when STAT5A was coexpressed with ERBB4 (Fig. 2*C*). Despite equivalent levels of STAT5A and ERBB4 expression (Fig. 2*B*), β-casein promoter activity was abolished when ERBB4 was coexpressed with STAT5AS127/128A or STAT5AS779A (Fig. 2*C*). Independent mutation of STAT5A Ser-127 or Ser-128 resulted in a STAT5A protein transcriptionally stimulated by ERBB4 (data not shown), suggesting that phosphorylation of one serine residue compensates for the loss of phosphorylation of the other site. Taken together, our results indicate that phosphorylation of STAT5A serine residues Ser-127/Ser-128 and Ser-779 is essential for ERBB4-induced STAT5A stimulation of the β-casein promoter.

STAT5AS127/128A and STAT5AS779A Interact with ERBB4, however Ser-127/Ser-128 Is Not Phosphorylated at the Regulatory Tyr-694—We have shown previously that ERBB4 and STAT5A physically interact, leading to phosphorylation of STAT5A at Tyr-694 and activation of the β-casein promoter

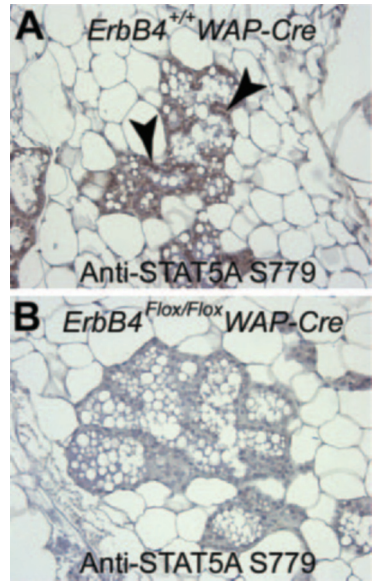

FIG. 1. **Phosphorylation of STAT5A at Ser-779 in mammary epithelium requires ERBB4 expression.** Wild-type (*ErbB4*$^{+/+}$*WAP-Cre*) (*A*) and *ERBB4*-null (ErbB4$^{Flox/Flox}$*WAP-Cre*) (*B*) mammary glands isolated from mice at 18 days pregnancy were fixed in 4% paraformaldehyde and embedded in paraffin, and 6-μm sections were stained by immunohistochemistry using an antibody directed against STAT5A phosphorylated at Ser-779. *Arrowheads* in *panel A* indicate nuclear accumulation of STAT5A phosphorylated at Ser-779 within the mammary secretory epithelium.

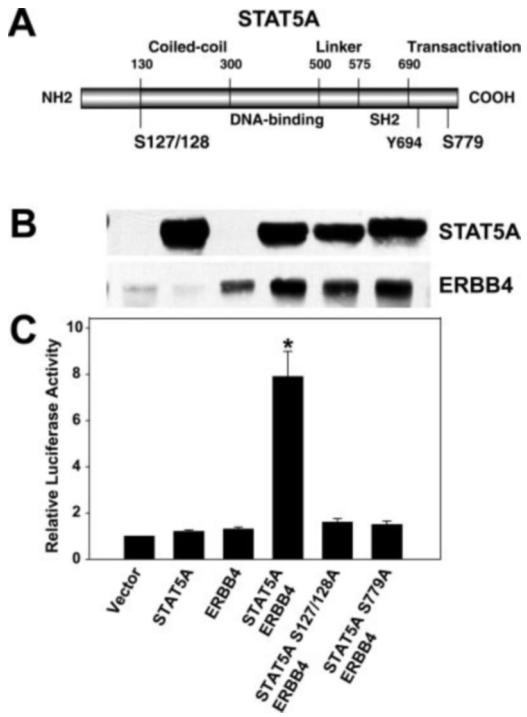

FIG. 2. **ERBB4-induced STAT5A transcriptional activity requires STAT5A phosphorylation at Ser-127/Ser-128 (*S127/128*) and Ser-779 (*S779*).** *A*, schematic of mouse STAT5A polypeptide indicating coiled-coil, DNA-binding, linker, and transactivation domains. The STAT5A Ser-127/Ser-128 and Ser-779 residues are located at the amino terminus/coiled-coil junction and transactivation domain, respectively. *Y694*, Tyr-694; *SH2*, Src homology 2. *B*, Western blot analysis of ERBB4 and STAT5A immunoprecipitations from MCF-7B cell lysates prepared from transfections with the indicated cDNAs. *C*, MCF-7B cells were co-transfected with the bovine β-casein promoter fused to luciferase and plasmids expressing the indicated cDNAs. Cell lysates were prepared at 48 h post-transfection, and luciferase activity was determined using standard methods. Results are reported as the fold increase in luciferase activity relative to β-casein promoter fused to luciferase and co-transfected with empty vector controls (mean ± S.E. of at least three experiments). *Asterisks* indicate ERBB4/STAT5A stimulation of the β-casein promoter significantly greater than each of the other treatments as determined by paired Student's *t* test ($p < 0.001$).

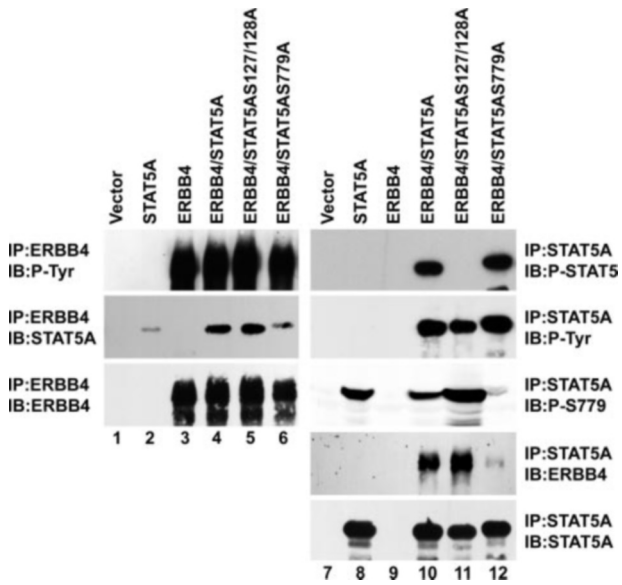

FIG. 3. **ERBB4-induced STAT5A Tyr-694 phosphorylation requires Ser-127/Ser-128, whereas STAT5A Ser-779 phosphorylation stabilizes STAT5A interaction with ERBB4.** HEK 293 cells were transfected with the indicated expression plasmids, and cell lysates were prepared at 48 h post-transfection. ERBB4 (*left sections*) or STAT5A (*right sections*) was immunoprecipitated (*IP*) from 1 mg of total lysate and analyzed by Western blot for ERBB4 (*IB:ERBB4*), STAT5A (IB:STAT5A), tyrosine phosphorylation (*IB:P-Tyr*), STAT5A phosphorylated at Tyr-694 (*IB:P-STAT5A*), and STAT5A phosphorylated at Ser-779 (*IB:P-Ser-779*).

(13, 15). To determine whether STAT5A phosphorylation at Ser-127/Ser-128 or Ser-779 has an effect on the interaction between STAT5A and ERBB4, we coexpressed ERBB4 with STAT5AS127/128A or STAT5AS779A in HEK 293 cells and performed a coimmunoprecipitation Western blot analysis. STAT5A and STAT5AS127/128A were coimmunoprecipitated with ERBB4 using ERBB4-specific (Fig. 3, *lanes 4* and 5) or STAT5A-specific (Fig. 3, *lanes 10* and 11) antibodies. Despite forming a stable interaction with ERBB4, STAT5AS127/128A was not phosphorylated at the regulatory Tyr-694 (Fig. 3, *lane 11*, *section* marked *IP:STAT5A IB:P-STAT5*), providing an explanation for the lack of STAT5AS127/128A transcriptional activity. Interestingly, STAT5AS127/128A retained ERBB4-regulated phosphorylation at the ERBB4-specific novel phosphotyrosine residue(s) (13) (Fig. 3, *lane 11*, *section* marked *IP:STAT5A IB:P-Tyr*) and Ser-779 (Fig. 3, *lane 11*, *section* marked *IP:STAT5A IB:P-S779*). In contrast, stable interaction of STAT5AS779A, with coexpressed ERBB4, was significantly impaired (Fig. 3, *lanes 6* and *12*). Phosphorylation of STAT5AS779A at Tyr-694 (Fig. 3, *lane 12*, *section* marked *IP:STAT5A IB:P-STAT5*) indicates an interaction with ERBB4; however, phosphorylation of STAT5A at Ser-779 appears to be required to stabilize the ERBB4/STAT5A complex. In summary, our findings suggest that phosphorylation of STAT5A at Ser-127/Ser-128 is necessary for subsequent ERBB4-mediated activation of STAT5A by Tyr-694 phosphorylation, whereas phosphorylation of STAT5A at S779A is required to stabilize an ERBB4/STAT5A complex.

STAT5A Ser-127/Ser-128, but Not Ser-779, Is Required for ERBB4-Induced STAT5A DNA Binding Activity—We have shown that ERBB4-induced STAT5A transcriptional activity is impaired by mutation of Ser-127/Ser-128 or Ser-779. We next determined if phosphorylation of these serine residues regulates ERBB4-induced STAT5A DNA binding. An electrophoretic mobility shift assay was performed utilizing a ^{32}P-labeled STAT5A DNA binding sequence derived from the bovine β-casein promoter and whole cell extracts prepared from NIH3T3 cells with transient ectopic expression of STAT5A, STAT5AS127/128A, or STAT5AS779A with or without ERBB4 coexpression. An electrophoretic mobility shift assay revealed that ERBB4 induces STAT5A DNA binding activity (Fig. 4, *lane 6*). A slight reduction in DNA binding was observed when ERBB4 was coexpressed with STAT5AS779A (Fig. 4, *lane 8*). In contrast, STAT5AS127/128A lacked ERBB4-induced DNA binding activity (Fig. 4, *lane 7*). This experiment was performed two times with similar results. Collectively, these results indicate that phosphorylation of STAT5A at Ser-127/Ser-128 is required for STAT5A DNA binding activity, whereas phosphorylation of STAT5A at Ser-779 is dispensable.

DISCUSSION

In this article we have identified novel STAT5A serine phosphorylation events essential for ERBB4-induced STAT5A transactivation. Based upon the experimental results presented here, we propose a model describing the influence of STAT5A serine phosphorylation on the regulation of STAT5A transactivation by ERBB4 (Fig. 5). Our results demonstrate, for the first time, the contribution of STAT5A phosphorylation at serines 127/128. This phosphorylation event is required for ERBB4-stimulated phosphorylation of STAT5A at the regulatory Tyr-694 and for the subsequent DNA binding of STAT5A (Fig. 5). Interestingly, STAT5A harboring a residue substitu-

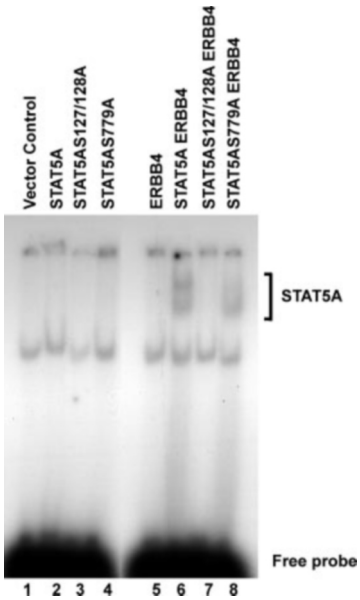

FIG. 4. **STAT5A Ser-127/Ser-128 phosphorylation is required for ERBB4-induced STAT5A DNA-binding.** NIH3T3 cells were transfected with the indicated expression plasmids, whole cell lysates were prepared at 48 h post-transfection, and 20 μg of lysate was incubated with a radiolabeled oligonucleotide corresponding to the STAT5A DNA-binding site of the bovine β-casein promoter. STAT5A DNA-binding activity was determined by an electrophoretic mobility shift assay.

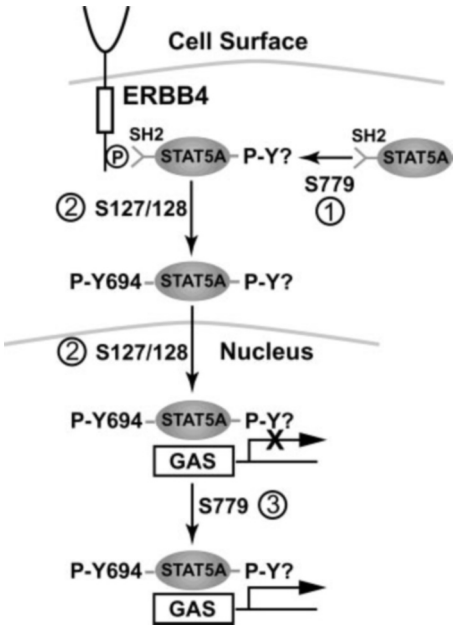

FIG. 5. **A model for the role of STAT5A serine phosphorylation during ERBB4-induced STAT5A activation.** *Step 1*, coexpression of ERBB4 and STAT5A results in a stable interaction between ERBB4 and STAT5A (13, 15) mediated by the STAT5A Src homology 2 domain (*SH2*) and stabilized by phosphorylation (*P*) of STAT5A at Ser-779 (S779). *Step 2*, although STAT5A lacking Ser-127/Ser-128 (*S127/128*) retains phosphorylation of Ser-779 and ERBB4-specific unidentified tyrosine residues (*P-Y?*), phosphorylation of STAT5A at Ser-127/Ser-128 is required for Tyr-694 phosphorylation (*P-Y694*) and subsequent DNA-binding of STAT5A. *Step 3*, DNA-bound STAT5A requires phosphorylation of Ser-779 for ERBB4-induced STAT5A stimulation of gene expression. *GAS*, γ interferon-activating site.

tion at Ser-127/Ser-128 still interacts with ERBB4 and retains phosphorylation at Ser-779 as well as uncharacterized, ERBB4-regulated (13, 24), tyrosine residues. We further show that although STAT5A is phosphorylated at Tyr-694 and binds DNA in the absence of Ser-779, phosphorylation of Ser-779 is required to stabilize an ERBB4/STAT5A complex and for ERBB4-induced STAT5A stimulation of the β-casein promoter (Fig. 5). Collectively, our results demonstrate that STAT5A serine phosphorylation events are critical regulators of the ERBB4/STAT5A signaling pathway.

The contribution of STAT5A serine phosphorylation to ERBB4/STAT5A signaling underscores the significant functional and mechanistic differences between ERBB4 and prolactin receptor (PrlR) regulation of STAT5A. In the developing breast, both ERBB4 and PrlR play essential roles as upstream mediators of STAT5A activation. PrlR appears to regulate STAT5A activation during early pregnancy, when this signaling pathway is required for cell specification during the ductal to secretory epithelial transition (2). ERBB4, on the other hand, regulates STAT5A activity at late pregnancy, contributing to epithelial differentiation and the initiation of lactation (4, 5). Despite STAT5A phosphorylation at multiple serine residues, including Ser-779, in cells treated with Prl, serine phosphorylation is dispensable for Prl-induced STAT5A stimulation of the β-casein promoter (9, 10). In contrast, STAT5A Ser-779 phosphorylation in the breast at late pregnancy requires ERBB4 expression, and this phosphorylation event is essential for ERBB4-induced STAT5A transcriptional activation. In addition, phosphorylation of STAT5A at Ser-127/Ser-128, unique to the ERBB4/STAT5A signaling axis, is essential for ERBB4-induced STAT5A stimulation of the β-casein promoter. Currently, the developmental function of STAT5A serine phosphorylation remains unresolved; however, one intriguing possibility is that STAT5A serine phosphorylation at late pregnancy mediates the mechanistic switch from PrlR to ERBB4 regulation of STAT5A activity. In addition, STAT5A serine phosphorylation may alter target gene selection and activation, further underscoring differences between ERBB4 and PrlR signaling.

At this point the kinase(s) regulating STAT5A serine phosphorylation in response to ERBB4 remains to be determined. One likely candidate is the serine/threonine kinase p21-activated kinase 1, which is regulated by members of the epidermal growth factor receptor family through the ERBB4 ligand heregulin (25). Significantly, transgenic expression of a dominant negative form of p21-activated kinase 1 in the developing mammary gland results in lobuloalveolar and lactational defects (26) coincident with identical phenotypes observed in *ERBB4*-null mammary glands (4, 5). Moreover, both ERBB4 and p21-activated kinase 1 are required for late pregnancy phosphorylation of STAT5A at Ser-779, and this phosphorylation event is required for maximal stimulation of the β-casein promoter induced by ERBB4 or p21-activated kinase 1 (26). Collectively, these results support a developmentally important signaling pathway involving coupled ERBB4 and Pak1 regulation of STAT5A in the mammary gland at late pregnancy.

Phosphorylation of STAT5A at Tyr-694 and subsequent DNA binding was thought to be sufficient to stimulate gene expression; however, our observations suggest that serine phosphorylation plays an important regulatory role. For example, the STAT5AS779A mutant was phosphorylated at Tyr-694 and bound DNA in response to ERBB4 coexpression, but this mutant failed to activate gene expression. We also show that STAT5A phosphorylation at Tyr-694 and transactivation activity requires Ser-127/Ser-128. STAT5AS779A retains phosphorylation at Tyr-694, implying that STAT5AS779A is also phosphorylated at Ser-127/Ser-128, excluding impaired Ser-127/Ser-128 phosphorylation as an explanation for the lack of STAT5AS779A transactivation activity. Thus, it seems likely

that STAT5A Ser-779 phosphorylation is required to recruit transcription coactivators critical for ERBB4-induced STAT5A stimulation of gene expression. For example, ERBB4 itself harbors transactivation activity (15, 27) and binds with STAT5A at the endogenous β-casein promoter (15). Therefore, the lack of potent STAT5AS779A transactivation activity may be a result of the suppressed interaction with ERBB4 observed in our experiments. We are currently investigating the contribution of STAT5A serine phosphorylation to the recruitment of ERBB4 and other transcriptional coactivators at STAT5A target promoters.

In summary, our findings provide the first description of STAT5A serine phosphorylation events critical for STAT5A function. Furthermore, we have identified a novel STAT5A serine phosphorylation event at Ser-127/Ser-128 critical for ERBB4-induced STAT5A phosphorylation at the regulatory Tyr-694 and for STAT5A-stimulated gene expression. In addition, the previously described STAT5A phosphorylation at Ser-779, which had minimal impact on PrlR regulation of STAT5A, is regulated by ERBB4 expression in mammary glands at late pregnancy and is essential for ERBB4 regulation of STAT5A transactivation activity. Thus, the ERBB4/STAT5A signaling axis appears to be mechanistically similar to STAT1, STAT3, and STAT4, which are functionally regulated by serine phosphorylation events (28–31).

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REFERENCES

1. Liu, X., Robinson, G. W., Wagner, K.-U., Garrett, L., Wynshaw-Boris, A., and Hennighausen, L. (1997) *Genes Dev.* **11,** 179–186
2. Miyoshi, K., Shillingford, J. M., Smith, G. H., Grimm, S. L., Wagner, K. U., Oka, T., Rosen, J. M., Robinson, G. W., and Hennighausen, L. (2001) *J. Cell Biol.* **155,** 531–542
3. Teglund, S., McKay, C., Schuetz, E., vanDeursen, J. M., Stravopodis, D., Wang, D., Brown, M., Bodner, S., Grosveld, G., and Ihle, J. N. (1998) *Cell* **93,** 841–850
4. Long, W., Wagner, K.-U., Lloyd, K. C. K., Binart, N., Shillingford, J. M., Hennighausen, L., and Jones, F. E. (2003) *Development* **130,** 5257–5268
5. Tidcombe, H., Jackson-Fisher, A., Mathers, K., Stern, D. F., Gassmann, M., and Golding, J. P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8281–8286
6. Coffer, P. J., Koenderman, L., and de Groot, R. P. (2000) *Oncogene* **19,** 2511–2522
7. Coffer, P. J., and Kruijer, W. (1995) *Biochem. Biophys. Res. Commun.* **210,** 74–81
8. Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) *EMBO J.* **13,** 4361–4369
9. Beuvink, I., Hess, D., Flotow, H., Hofsteenge, J., Groner, B., and Hynes, N. E. (2000) *J. Biol. Chem.* **275,** 10247–10255
10. Yamashita, H., Nevalainen, M. T., Xu, J., LeBaron, M. J., Wagner, K. U., Erwin, R. A., Harmon, J. M., Hennighausen, L., Kirken, R. A., and Rui, H. (2001) *Mol. Cell. Endocrinol.* **183,** 151–163
11. Stern, D. F. (2003) *Exp. Cell Res.* **284,** 89–98
12. Yarden, Y., and Sliwkowski, M. X. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 127–137
13. Jones, F. E., Welte, T., Fu, X.-Y., and Stern, D. F. (1999) *J. Cell Biol.* **147,** 77–87
14. Vidal, G. A., Naresh, A., Marrero, L., and Jones, F. E. (2005) *J. Biol. Chem.* **280,** 19777–19783
15. Williams, C. C., Allison, J. G., Vidal, G. A., Burow, M. E., Beckman, B. S., Marrero, L., and Jones, F. E. (2004) *J. Cell Biol.* **167,** 469–478
16. Plowman, G. D., Culouscou, J.-M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1746–1750
17. Elenius, K., Corfas, G., Paul, S., Choi, C. J., Rio, C., Plowman, G. D., and Klagsbrun, M. (1997) *J. Biol. Chem.* **272,** 26761–26768
18. Sartor, C. I., Zhou, H., Kozlowska, E., Guttridge, K., Kawata, E., Caskey, L., Harrelson, J., Hynes, N., Ethier, S., Calvo, B., and Earp, H. S., III (2001) *Mol. Cell. Biol.* **21,** 4265–4275
19. Burow, M. E., Weldon, C. B., Tang, Y., McLachlan, J. A., and Beckman, B. S. (2001) *J. Steroid Biochem. Mol. Biol.* **78,** 409–418
20. Chittum, H. S., Lane, W. S., Carlson, B. A., Roller, P. P., Lung, F. D., Lee, B. J., and Hatfield, D. L. (1998) *Biochemistry* **37,** 10866–10870
21. Olayioye, M. A., Beuvink, I., Horsch, K., Daly, J. M., and Hynes, N. E. (1999) *J. Biol. Chem.* **274,** 17209–17218
22. Park, S. H., Yamashita, H., Rui, H., and Waxman, D. J. (2001) *Mol. Endocrinol.* **15,** 2157–2171
23. Yamashita, H., Xu, J., Erwin, R. A., Farrar, W. L., Kirken, R. A., and Rui, H. (1998) *J. Biol. Chem.* **273,** 30218–30224
24. Jones, F. E., Golding, J. P., and Gassmann, M. (2003) *Cell Cycle* **2,** 555–559
25. Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J., and Kumar, R. (1998) *J. Biol. Chem.* **273,** 28238–28246
26. Wang, R. A., Vadlamudi, R. K., Bagheri-Yarmand, R., Beuvink, I., Hynes, N. E., and Kumar, R. (2003) *J. Cell Biol.* **161,** 583–592
27. Ni, C.-Y., Murphy, M. P., Golde, T. E., and Carpenter, G. (2001) *Science* **294,** 2179–2181
28. Eilers, A., Georgellis, D., Klose, B., Schindler, C., Ziemiecki, A., Harpur, A. G., Wilks, A. F., and Decker, T. (1995) *Mol. Cell. Biol.* **15,** 3579–3586
29. Kovarik, P., Stoiber, D., Novy, M., and Decker, T. (1998) *EMBO J.* **17,** 3660–3668
30. Ng, J., and Cantrell, D. (1997) *J. Biol. Chem.* **272,** 24542–24549
31. Visconti, R., Gadina, M., Chiariello, M., Chen, E. H., Stancato, L. F., Gutkind, J. S., and O'Shea, J. J. (2000) *Blood* **96,** 1844–1852
32. Eng, J., McCormack, A. L., and Yates, J. R., III (1994) *J. Am. Soc. Mass Spectrom.* **5,** 976–989