Differential Control of the Phosphorylation State of Proline-juxtaposed Serine Residues Ser\textsuperscript{725} of Stat5a and Ser\textsuperscript{730} of Stat5b in Prolactin-sensitive Cells

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Transcription factors of the Stat family are controlled by protein kinases. Phosphorylation of a positionally conserved tyrosine residue is obligatory for Stat dimerization, nuclear translocation, and specific DNA binding. Studies of Stat1 and Stat3 have suggested that serine phosphorylation may also regulate function. We now identify serine residues located in a conserved PSP motif of Stat5a (Ser\textsuperscript{725}) and Stat5b (Ser\textsuperscript{730}) as major phosphorylation sites, using mutagenesis, phosphoaminoo acid analysis, and site-specific anti-Stat5-phosphoserine antibodies. Unexpectedly, phosphorylation control of this PSP motif differed between the highly homologous Stat5a and Stat5b proteins. Whereas Ser\textsuperscript{725} of Stat5a was constitutively phosphorylated both in COS-7 cells and Nb2 lymphocytes, phosphorylation of Ser\textsuperscript{730} of Stat5b was markedly stimulated by prolactin. The data also suggested the existence of a second major serine phosphorylation site in Stat5a. Interestingly, constitutive phosphorylation of the PSP motif was suppressed by PD98059 but not by staurosporine under conditions in which both agents inhibited mitogen-activated protein kinases. Furthermore, pretreatment of cells with staurosporine, PD98059, H7, or wortmannin did not prevent either Stat5a or Stat5b from becoming maximally serine-phosphorylated after prolactin exposure. We propose that two pathways regulate Stat5 serine phosphorylation, one that is prolactin-activated and PD98059-resistant and one that is constitutively active and PD98059-sensitive and preferentially targets Stat5a. Finally, phosphorylation of the PSP motif of Stat5a or Stat5b was not essential for DNA binding or transcriptional activation of a β-casein reporter gene in COS-7 cells, suggesting that serine kinase control of Stat5 activity differs from that of Stat1 and Stat3.

Although information on the involvement of Stat proteins in the etiology and progression of disease is still limited, fundamental knowledge of the function and regulation of Stat proteins is expected to have important clinical and pharmaceutical significance. There are currently seven known Stat genes (2). Among these, Stat1 and Stat2 are important for the antiproliferative and antiviral effects of interferons, whereas Stat3 regulates acute phase response genes (2). Furthermore, the highly homologous Stat5a and Stat5b proteins (3–6) are essential mediators of prolactin and growth hormone effects (7, 8) and have been implicated in cytokine control of apoptosis, growth, and differentiation (9–11). Of particular clinical relevance, constitutively activated Stat5 is associated with several hematopoietic malignancies (12, 13), and both Stat5a and Stat5b were selectively downregulated in T lymphocytes from patients with human immunodeficiency virus infection (14) and in T and B lymphocytes from immunocompromised tumor-bearing mice (15).

The activity of Stat factors is under strict regulation by protein tyrosine kinases. Phosphorylation of a positionally conserved tyrosine residue is obligatory for dimerization, nuclear translocation, and subsequent binding of Stats to specific promoter sequences (1). Recently, studies have suggested that serine kinases also regulate the activity of some Stat proteins. Specifically, serine residue 727 constitutes a major phosphorylation site in Stat1α and Stat3 and is important for interferon-induced nuclear translocation, DNA binding, and maximal transcriptional activation (16–18). Substitution of Ser\textsuperscript{727} of Stat1α with alanine abolished interferon-γ and induced an antiviral state and growth arrest of cells (19, 20).

The serine kinases responsible for Stat1 and Stat3 phosphorylation have not yet been identified. However, amino acid Ser\textsuperscript{727} is located within a consensus PXSP phosphoacceptor site for mitogen-activated protein kinase (MAPK)\textsuperscript{1} (21, 22), and experimental evidence supports a role of the MAPK p42ERK2 in Stat1α and Stat3 phosphorylation (17, 18, 23). On the other hand, MAPK-independent serine phosphorylation of Stat3 also has been reported (24, 25). Stat5a and Stat5b were recently shown to become phosphorylated on serine residues after activation by prolactin or interleukin 2 (26, 27), but the phosphoacceptor sites have not yet been identified. Stat5 transcription factors lack the conserved PMSP motif of Stat1 and Stat3. However, serine residues Ser\textsuperscript{725} of Stat5a and Ser\textsuperscript{730} of Stat5b

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PRL, prolactin; PAGE, polyacrylamide gel electrophoresis.

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are candidate phosphorylation sites because they are located within a positionally homologous PSP motif. In contrast to Stat1 and Stat3, Stat5α and Stat5β proteins do not appear to be substrates of extracellular signal-regulated kinases (ERKs) (27–29), raising the possibility that Stat5 proteins are regulated differently than other Stats.

To identify serine phosphorylation sites of Stat5α transcription factors, a series of serine mutants of Stat5α and Stat5β were analyzed using a COS-7 cell reconstitution system. COS-7 cells do not express detectable levels of prolactin receptors, Stat5a, or Stat5b and have been used for studies of prolactin receptor-mediated β-casein gene regulation by several laboratories (30–32). We now report that the proline-juxtaposed serine residue Ser725 of Stat5α and Ser730 of Stat5β are major phosphorylation sites, based on phosphoamino acid analysis and the generation of site-directed anti-Stat5- phosphoserine antibodies. Unexpectedly, phosphorylation control of this PSP motif differed between the highly homologous Stat5α and Stat5β proteins, and phosphoamino acid analysis suggested the existence of a second major serine phosphorylation site unique to Stat5α.

The data also indicated that two distinct pathways lead to serine phosphorylation of the PSP motif of Stat5β, one PD98059-resistant, prolactin-activated pathway, and one PD98059-sensitive, constitutive pathway. Because phosphorylation of the PSP motif of Stat5α or Stat5β was not essential for PD98059-sensitive, constitutive pathway. Because phosphorylation of the PSP motif of Stat5α or Stat5β was not essential for PD98059-sensitive, constitutive pathway.

**Materials and Methods**

**Plasmids and Mutants—**Expression vectors for mouse Stat5α (pXM-Stat5α) and mouse Stat5β (pXM-Stat5β) were kindly provided by Xiaowen Liu and Lothar Hennighausen (National Institutes of Health, Bethesda, MD) (31). The (-344 to –1) β-casein gene promoter linked to the luciferase reporter gene (pZ21, kindly provided by Bernd Groner, Institute for Experimental Cancer Research, Freiburg, Germany) and the plasmid pCH110 containing the β-galactosidase gene under the control of the simian virus 40 promoter have been described previously (30, 31). Plasmid pSPRLR containing a 2.7-kb human prolactin receptor cDNA kindly provided by Paul A. Kelly (Institut National de la Santé et de la Recherche Médicale, Paris, France) was constructed by cloning into the EcoRI site of pcDNA3 expression vector (Invitrogen). Mutants of Stat5α and Stat5β were prepared from double-stranded DNAs using the QuikChange site-directed mutagenesis kit (Stratagene) with oligonucleotide primers designed to alter serine residues to alanines or tyrosines to phenylalanines. The following mutants of mouse Stat5α were expressed in COS-7 cells: Ser721→Tyr (TAC to TAT) corresponding to amino acid residues 721–729 of human Stat5α or 726–734 of human Stat5β was synthesized, conjugated to keyhole limpet hemocyanin, and used as an immunogen in rabbits (Genosys Inc., Woodlands, TX). The antiserum was first precleared by passing over a resin of immobilized, unphosphorylated peptide, followed by affinity purification using immobilized, phosphorylated peptide. For immunoblotting, blots were incubated for 1 h with antibodies at a concentration corresponding to 1:3000 dilution of original serum.

**Generation of Site-specific Anti-Stat5-Phosphoserine Antibodies—**To generate peptide DQAP[pS]PAVC corresponding to amino acid residues 721–729 of human Stat5α or 726–734 of human Stat5β was synthesized, conjugated to keyhole limpet hemocyanin, and used as an immunogen in rabbits. Cells were preincubated at 37 °C for 30 min and then lysed and immunoprecipitated as described above. Proteins were eluted from protein-A-Sepharose beads, separated on SDS-PAGE (7.5% polyacrylamide), and transferred to polyvinylidene difluoride membranes. Labeled proteins were visualized by autoradiography and analyzed by phosphoamino acid analysis as described earlier (33). Bands corresponding to Stat5α or Stat5β proteins were excised and exposed to limited hydrolysis in 6 N HCl for 60 min. Samples were then dried, resuspended in water with phosphoamino acid standards, and spotted onto a thin layer cellulose acetate gel. One-dimensional thin layer electrophoresis was performed at 1500 V for 40 min in buffer containing pyridine-acetic acid:water at a 10:100:1890 ratio. Standards were visualized with ninhydrin, and samples were analyzed by autoradiography.

**Electrophoretic Mobility Shift Assay—**Cells were treated with or without 10 nM human prolactin as indicated, pelleted by centrifugation, and immediately solubilized in electrophoretic mobility shift assay lysis buffer and analyzed as described earlier (26). 32P-Labeled oligonucleotide corresponding to the prolactin response element (5′-attttgaattttgaatcataac-3′) of the rat β-casein gene was used. Polyacrylamide gels (4%) containing 5% glycerol and 0.25 × Tris borate/EDTA were prerun in 35 mM Tris/3 mM Na2EDTA/1 mM EDTA for 60 min. Samples were then run at 120 V for 1.5 hr at 300 V. After loading of samples, the gels were run at room temperature for ~3 h at 250 V. Gels were dried by heating under vacuum and exposed to x-ray film (X-Omat, Eastman Kodak Co.).

Luciferase and β-Galactosidase Assays—One day after transfection, COS-7 cells were stimulated with prolactin for 16 h and harvested. Cells were washed twice with PBS and lysed in Triton/glycylglycine lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 1 mM dithiothreitol) and centrifuged at 12,000 × g for 5 min at 4 °C. Supernatants were used for luciferase and β-galactosidase assays. For luciferase assays, 100 μl of cell lysate were mixed with 100 μl of assay buffer containing 25 mM glycylglycine, 7.8 mM MgSO4, 15 mM potassium phosphate, pH 7.8, 15 mM EGTA, 2 mM MgSO4, 2 mM ATP, and 1 mM dithiothreitol. Luciferase activity of each sample was determined by measuring luminescence after injection of 200 μl of 1 mM luciferine. Assays for β-galactosidase were carried out using Galacto-Light Plus (Tropix, Inc.). Two hundred microliters of reaction buffer were added to 20 μl of cell lysate and incubated at room temperature for...
whereas inducible tyrosine phosphorylation levels were therefore much less pronounced in Stat5a than in Stat5b.

The extent of inducible serine phosphorylation of Stat5a was selective for serine residues (Fig. 2A). The highly homologous DNA binding domains, Src homology domain 3 regions (SH3), and Src homology domain 2 regions (SH2) are indicated (96% amino acid identity). Nonhomologous regions of Stat5a and Stat5b are hatched. The COOH-terminal regions of Stat5a and Stat5b containing the SH2 docking site were inducible phosphorylated (Fig. 2B). The most conserved docking site for SH2 binding domains, Src homology domain 3 (SH3), and the Src homology domain 2 (SH2) are indicated (96% amino acid identity). Nonhomologous regions of Stat5a and Stat5b are hatched.

RESULTS

Targeting of Putative Phosphoacceptor Sites in Stat5a and Stat5b—Sequence alignments of various Stat proteins in the region corresponding to the phosphorylated serine Ser727 of Stat1 and Stat3 showed that whereas Stat1, Stat3, and Stat4 have the classical MAPK phosphorylation site PXSP (21, 22), Stat5a and Stat5b contain instead a conserved PSP motif (Fig. 1A). A previous alignment based solely on the sheep Stat5 sequence did not recognize this difference (17). Peptide phosphorylation studies have suggested that PSP motifs are less efficient phosphoacceptors for ERKs than PXSP motifs (21). It was therefore of particular relevance to determine whether Stat5 PSP motifs were phosphorylation sites. To specifically analyze the involvement of the PSP serine residues of Stat5a and Stat5b, mutants Stat5a-S725A and Stat5b-S730A were generated (Fig. 1B). In addition, mutants Stat5a-S710A and Stat5b-S715A were also made based on the conserved nature and proximity of these serine residues to the dimerization and trans-activation domains (Fig. 1B).

Effect of Alanine Substitution of Ser725 of Stat5a and Ser730 of Stat5b on Prolactin-inducible Stat5 Serine Phosphorylation as Judged by Phosphoamino Acid Analysis—COS-7 cells were transiently transfected with expression plasmids for the prolactin receptor and wild-type or mutant forms of Stat5a or Stat5b. The isolated Stat5a or Stat5b proteins were immunoprecipitated from corresponding cell lysates, separated by SDS-PAGE, and visualized by autoradiography (Fig. 2, A and B, lower panels). Wild-type Stat5a showed significantly higher levels of constitutive phosphorylation than wild-type Stat5b when expressed in COS-7 cells (Fig. 2, A and B, upper panels). This constitutive phosphorylation of Stat5a was selective for serine residues (Fig. 2A, lower panel). The extent of inducible serine phosphorylation was therefore much less pronounced in Stat5a than in Stat5b, whereas inducible tyrosine phosphorylation levels were comparable.

The effect of mutation of Ser730 on Stat5b serine phosphorylation was dramatic, because inducible phosphoserine levels were reduced almost to background levels in mutant Stat5b-S730A (Fig. 2B). This analysis provided the first evidence that Ser730 is the predominant serine phosphorylation site of Stat5b. Furthermore, substitution of S715 of Stat5b with alanine led to constitutively elevated phosphoserine levels of mutant Stat5b-S715A (Fig. 2B). However, this hyperphosphory-
lated state did not prevent mutant Stat5b-S715A from becoming inducibly tyrosine-phosphorylated in response to prolactin. To address whether Ser725 was the hyperphosphorylated residue in the Stat5b-S715A mutant, we converted Ser725 of this mutant to alanine and generated the double mutant Stat5b-S715A,S730A. This co-mutation completely abolished the constitutive phosphorylation and reduced inducible serine phosphorylation to the low levels observed in the Stat5b-S730A mutant (Fig. 2B). This result was consistent with Ser730 as the hyperphosphorylated site of Stat5b-S715A and supported the notion that Ser730 is a predominant serine phosphorylation site of Stat5b. Furthermore, although of unknown significance, stoichiometrically low levels of phosphothreonine were also induced in Stat5b but not in Stat5a.

The corresponding phosphoamino acid analysis of the Stat5a-S725A mutant showed high constitutive phosphoserine levels, which suggested the existence of an additional site distinct from Ser725 in Stat5a (Fig. 2A, lower panel). However, a significant and consistent reduction in phosphoserine content of Stat5a-S725A compared with wild-type Stat5a indicated that Ser725 of Stat5a was also a phosphorylation site. No changes in phosphorylation were observed in mutant Stat5a-S710A, in contrast to the increased levels of constitutive serine phosphorylation in the matching Stat5b-S715A mutant. Finally, no constitutive or inducible phosphotyrosine could be detected in mutants Stat5a-Y694F and Stat5b-Y699F (Fig. 2A and B), consistent with the key regulatory role of these positionally conserved tyrosine residues (30). On the other hand, both tyrosine mutants were constitutively phosphorylated on serine, showing that serine phosphorylation of both Stat5a and Stat5b can occur independently of tyrosine phosphorylation. Thus, we conclude from these phosphoamino acid analyses that Ser730 of Stat5b is a predominant and inducible phosphorylation site, and that the corresponding Ser725 of Stat5a was constitutively phosphorylated. Finally, phosphoamino acid analysis suggested that a second phosphoserine site existed in Stat5a.

**Analysis of Phosphotyrosine and Phosphoserine Content of Stat5 Mutants by Immunoblotting**—To directly establish whether the PSP motif is phosphorylated in both Stat5a and Stat5b, we generated site-specific anti-Stat5-phosphoserine antibodies using a phosphorylated peptide corresponding to this shared motif. Consistent with marked constitutive phosphophorylation of Ser725 of wild-type Stat5a, these antibodies showed strong reaction to wild-type Stat5a but not mutant Stat5a-S725A or the double mutant Stat5a-S710A,S725A (Fig. 3A, middle panel). Constitutive serine phosphorylation of Ser725 was also verified in mutants Stat5a-S710A and Stat5a-Y694F.

Examination of Stat5b mutants with the same antiphosphoserine antibodies gave a similar pattern, except that basal phosphorylation of Ser730 was much lower and was inducible by prolactin, consistent with phosphoamino acid data. No immunoreaction toward proteins carrying the Stat5a-S725A or Stat5b-S730A mutations was observed, attesting to the specificity of the anti-Stat5-phosphoserine antibodies. Parallel examination of tyrosine phosphorylation states of Stat5 proteins in prolactin-treated COS-7 cells by anti-phosphotyrosine immunoblotting also corroborated the phosphoamino acid analyses and showed that Stat5 wild-type proteins and the various Ser-to-Ala mutants were tyrosine-phosphorylated equally well in response to prolactin stimulation (Fig. 3, A and B, upper panels). Similarly, immunoblotting confirmed that mutants Stat5a-Y694F and Stat5b-Y699F did not become tyrosine-phosphorylated on additional sites. Immunoblotting also verified that each of the mutants was expressed at comparable levels in COS-7 cells. We conclude from these experiments that the PSP motif is a major serine phosphorylation site in both Stat5a and Stat5b.

**Analysis of DNA Binding Activities of Stat5 Mutants**—The ability of wild-type proteins and mutants Stat5a-S725A and Stat5b-S730A to bind to an oligonucleotide probe corresponding to the prolactin response element of the β-casein gene promoter was evaluated (Fig. 4, A and B). Using prolactin-responsive COS-7 cells, mutants Stat5a-S725A and Stat5b-S730A were found to be fully capable of forming DNA complexes with a 32P-labeled β-casein promoter probe and were at least as efficient as wild-type proteins over the duration of the 20-h test period (Fig. 4, A and B). Similarly, mutants Stat5a-S710A and Stat5b-S715A were also fully capable of binding DNA, whereas no induction of DNA binding was observed with the tyrosine mutants Stat5a-Y694F and Stat5b-Y699F (data not shown), consistent with the critical role of these tyrosine residues for Stat5 activation (30). We therefore concluded that the serine phosphorylation state of the PSP motif is not essential for Stat5 DNA binding.

**Assessment of Transactivation Potential of Mutants Stat5a-S725A and Stat5b-S730A**—Expression plasmids encoding the prolactin receptor, wild-type or mutant Stat5 proteins and a β-casein gene promoter-luciferase reporter gene were cotransfected into COS-7 cells. A constitutively expressed β-galactosidase gene was also included to compensate for differences in transfection efficiencies. Luciferase activities were measured in extracts of cells incubated in the absence or presence of prolactin for 16 h (Fig. 5, A and B). The wild-type Stat5a and Stat5b proteins both mediated a highly consistent 2.5-fold induction of the reporter gene after prolactin stimulation, whereas mutants Stat5a-Y694F and Stat5b-Y699F were completely inactive as previously reported (30). Each of the three Stat5a serine mutants, S725A, S710A, and S710A,S725A, showed signals comparable to those of the wild-type Stat5a (Fig. 5A). Likewise, mutant Stat5b-S730A was also equally as efficient as wild-type Stat5b to induce luciferase activity (Fig.
both Stat5a and Stat5b (Fig. 6, C). Basal transcription levels were also not affected by mutation of the serine residue of the PSP motif of either Stat5a or Stat5b. In contrast, the inducible lucerase activities were reduced in prolactin-treated cells transfected with Stat5b-S715A or Stat5b-S715A,S730A. Indeed, neither Stat5b-S715A nor Stat5b-S715A,S730A mediated significant prolactin-induced lucerase activity. Further studies are needed to establish why mutant Stat5b-S715A exhibits reduced ability to activate transcription of the β-casein gene. More importantly, we conclude that phosphorylation of the conserved PSP motif of Stat5a and Stat5b is not critical for their inducible transactivation of a β-casein reporter gene in COS-7 cells. It remains possible that serine phosphorylation influences the transcriptional activity of Stat5 toward other responsive genes or in the presence of cofactors other than those available in COS-7 cells.

Effect of Protein Kinase Inhibitors on Constitutive and Prolactin-regulated Serine Phosphorylation of Stat5a and Stat5b in Nb2 Lymphocytes—To examine Stat5 serine phosphorylation in cells with endogenous prolactin receptors and to begin pharmacological characterization of the prolactin-activated Stat5 serine kinase, quiescent Nb2 lymphocytes were pre-treated with a series of kinase inhibitors for 30 min before they were incubated with or without prolactin (100 nM) for another 15 min. The inhibitors used included staurosporine (500 nM), PD98059 (100 μM), H7 (100 μM), and wortmannin (100 nM). Immunoblotting for phosphotyrosine and phosphoserine levels were done in parallel on immunoprecipitated Stat5a (Fig. 6A) and Stat5b (Fig. 6B) proteins. Control cells incubated without inhibitors revealed a similar distinction in serine phosphorylation between Stat5a and Stat5b in Nb2 cells as that observed in COS-7 cells, with the PSP motif constitutively serine-phosphorylated in Stat5a and highly inducible in Stat5b. Furthermore, whereas none of the inhibitors significantly antagonized PRL-induced tyrosine or serine phosphorylation of Stat5a or Stat5b in Nb2 cells, the MAPK/ERK kinase inhibitor PD98059 significantly reduced basal serine phosphorylation levels in both Stat5a and Stat5b (Fig. 6, A and B), and significantly inhibited PRL-induced activation of MAPK (Fig. 6C). H7 had a similar but less marked effect on basal Stat5 phosphoserine levels (Fig. 6, A and B). In contrast, staurosporine, which also markedly inhibited PRL-induced MAPK activation under these conditions (Fig. 6C), had little or no effect on constitutive Stat5a or Stat5b phosphoserine levels (Fig. 6, A and B). The fact that staurosporine pretreatment (500 nM) for 30 min did not block subsequent PRL-induced Stat5 tyrosine phosphorylation may suggest that maximally effective conditions have not been achieved for this combined inhibitor of tyrosine and serine kinases. However, we have observed that pretreatment of Nb2 cells for 30 min with staurosporine up to 2 μM consistently blocks PRL-induced MAPK activation and tyrosine phosphorylation of Stat1 and Stat3 but not Stat5 and Jak2.2 It is possible that longer preincubation times with staurosporine will block constitutive Stat5 serine phosphorylation. On the other hand, in light of the comparable sensitivity of MAPK to PD98059 and staurosporine under the conditions tested, the constitutively active Stat5 serine kinase appeared, at least in relative terms, more resistant to staurosporine than to PD98059. Additional

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Fig. 4. Prolactin-inducible DNA binding activities of wild-type (WT) and mutants of Stat5a (A) and Stat5b (B). COS-7 cells transfected with the prolactin receptor and Stat5a or Stat5b wild-type (WT) or serine to alanine mutants (Stat5a-S725A and Stat5b-S730A) were starved with serum-free Dulbecco’s modified Eagle’s medium for 16 h and incubated with (+) or without (−) PRL (10 nM) for 0–20 h, and cell extracts were prepared for gel shift analysis with 32P-labeled β-casein promoter probe. Markers indicate migrational positions of Stat5a or Stat5b.

Fig. 5. Prolactin-inducible β-casein gene activation by wildtype (WT) and mutants of Stat5a (A) and Stat5b (B). COS-7 cells were transfected with a β-casein-luciferase reporter gene, the prolactin receptor, mutants of Stat5a and Stat5b, and a β-galactosidase gene under the control of the simian virus 40 promoter. Cells were treated with (+) or without (−) PRL (10 nM) for 16 h. Luciferase and β-galactosidase activities in cell extracts were determined, and the ratios of the luciferase to β-galactosidase activities are shown. The mean values of four independent experiments are presented, and S.E. values are indicated by bars. Note interassay consistency; no normalization to controls was made. Differences between unstimulated and prolactin-stimulated levels were compared by one-way analysis of variance followed by Scheffe’s multiple range test (***, p < 0.001; **, p < 0.01; *, p < 0.05).
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The present study identified the conserved PSP motif of Stat5 transcription factors as a major phosphorylation site that can be differentially modulated between Stat5a and Stat5b. Whereas serine residue Ser272 of Stat5a was constitutively phosphorylated in both COS-7 cells and Nb2 lymphocytes and could not be further phosphorylated by prolactin treatment, the corresponding Ser272 residue of Stat5b was only weakly phosphorylated under basal conditions but was highly responsive to prolactin. Interestingly, constitutive serine phosphorylation of Stat5a Ser272 was suppressed when cells were pretreated with the MAPK/ERK kinase inhibitor PD98059. However, the drug did not inhibit prolactin-induced phosphorylation of the PSP motif of either Stat5a or Stat5b. Collectively, these observations suggested that two pathways regulate Stat5 serine phosphorylation, one sensitive to PD98059 and one that is insensitive and stimulated by prolactin. Furthermore, the prolactin-activated Stat5a serine kinase was also resistant to several other inhibitors, including staurosporine, H7, and wortmannin. It remains to be established whether the prolactin-activated pathway and the constitutive pathway regulate two distinct Stat5 serine kinases, or whether the two pathways converge on one Stat5 serine kinase that by itself is insensitive to PD98059, staurosporine, H7, and wortmannin.

Recent studies of Stat3 have also suggested two serine phosphorylation pathways, a growth factor-activated, MAPK-dependent pathway and an interleukin 6-activated, MAPK-independent pathway (24). There is currently no direct evidence suggesting that Stat5 proteins can be substrates for MAPK. In fact, several reports and the present study argue against a role for MAPK in prolactin and interleukin 2 stimulation of Stat5 transcription factors (27–29). Furthermore, because PD98059 and staurosporine both inhibited MAPK, but only PD98059 blocked constitutive Stat5a serine phosphorylation, we also propose that the constitutive Stat5a serine phosphorylation pathway does not require MAPK. Finally, previous in vitro peptide kinase assays have shown that the PSP sequence of Stat5a is a relatively poor substrate for MAPK when compared with the PSXP-sequence of Stat1 and Stat3 (21, 22). Thus, it appears likely that individual Stats are phosphorylated by different serine kinases with at least some degree of selectivity.

It is still controversial whether a serine phosphorylation site other than Ser272 exists in Stat3 (17, 24). Interestingly, in the present study phosphoamino acid analyses showed stoichiometrically significant levels of residual constitutive serine phosphorylation of Ser272, as well as mutated Tyr679 to phenylalanine (see Fig. 1B). Tyr679 is the only conserved tyrosine residue in Stat5b that is not present in Stat5a, and phosphorylation of a second tyrosine residue of Stat3, Tyr656, was recently demonstrated (35). However, each of these Stat5b mutants behaved normally with regard to inducible mobility retardation (data not shown). We therefore conclude that inducible phosphorylation of conserved serine residues Ser35, Ser45, Ser132, Ser427, and Ser748 and tyrosine residues Tyr679, which are unique to Stat5b and not present in Stat5a, does not contribute to the unique Stat5b gel mobility shift. Furthermore, because the phosphoamino acid analyses showed stoichiometrically significant residual levels of constitutive serine phosphorylation of the Stat5a-S725A mutant but not Stat5b-S730A, it is possible that a second constitutively phosphorylated serine residue in Stat5a may be responsible for this structural difference between Stat5a and Stat5b. Further work is needed to resolve this distinction between Stat5a and Stat5b mobility.

**DISCUSSION**

**Fig. 6. Effect of protein kinase inhibitors on prolactin-induced serine phosphorylation of Stat5a and Stat5b in Nb2 lymphocytes.** A and B, quiescent Nb2 cells were preincubated with dimethylsulfoxide (Ctrl, 500 µM) or wortmannin (100 µM) for 30 min before stimulation with (+) or without (−) prolactin for 15 min. Lysates were immunoprecipitated with anti-α-Stat5a (A) or α-Stat5b (B). Parallel samples were blotted for phosphoamino acid (ps-PY, upper panels), site-specific phosphoamino acid (ps-PS725 and ps-PS730, middle panels), α-Stat5a (A, lower panel), or α-Stat5b (B, lower panel). C, inhibition of prolactin-induced activation of MAPK by PD98059 and staurosporine. Quiescent Nb2 cells were preincubated with dimethylsulfoxide (Ctrl), PD98059 (100 µM), or staurosporine (500 µM) for 30 min before stimulation with (+) or without (−) prolactin for 15 min. Samples from whole cell lysates were separated by SDS-PAGE with blotted with antibodies to phospho- and total MAPK (upper panel). Parallel samples were blotted with anti-pan ERK antibodies (lower panel).

studies are needed to extend this initial pharmacological characterization of Stat5 serine kinase activities, and the anti-Stat5-phosphoserine antibodies will allow the development of specific in vitro enzyme assays. At present, we conclude that constitutive Stat5 serine phosphorylation is sensitive to PD98059, whereas the PRL-activated serine kinase is not sensitive to PD98059, staurosporine, H7, or wortmannin.

**Assessment of Phosphorylation Sites Contributing to the Unique Stat5b Gel Mobility Shift—As observed previously (26, 27) and seen in Fig. 3, A and B (lower panels), Stat5b but not Stat5a undergoes a pronounced mobility shift in SDS-PAGE after activation. Phosphorylation often causes gel mobility retardation of proteins in SDS-PAGE, and the mobility shift of Stat5b disappeared after in vitro phosphatase treatment of Stat5b (28, 34). The lack of an inducible shift of Stat5a could not simply be attributed to constitutive phosphorylation of Ser272, because the Stat5a-S725A mutant showed wild-type migration on SDS-PAGE (Fig. 3). On the other hand, mutation of Ser270 to alanine only led to a small, 15–20% reduction in the shift of mutants Stat5b-S730A and Stat5b-S715A, S730A (Fig. 3B; data not shown). In contrast, mutation of conserved tyrosine residues Tyr669 completely abolished the prolactin-induced mobility shift (Fig. 3B). To test the possibility that the unique mobility shift of Stat5b could be attributable to a combined phosphorylation of Tyr669 and a Stat5b-specific tyrosine or serine that required prephosphorylation of Tyr669, we individually substituted with alanines each of the five unique serine residues that are conserved across species in Stat5b but not present in Stat5a (Ser35, Ser45, Ser132, Ser427, and Ser748), as well as mutated Tyr679 to phenylalanine (see Fig. 1B). Tyr679 is the only conserved tyrosine residue in Stat5b that is not present in Stat5a, and phosphorylation of a second tyrosine residue of Stat3, Tyr656, was recently demonstrated (35). However, each of these Stat5b mutants behaved normally with regard to inducible mobility retardation (data not shown). We therefore conclude that inducible phosphorylation of conserved serine residues Ser35, Ser45, Ser132, Ser427, and Ser748 and tyrosine residues Tyr679, which are unique to Stat5b and not present in Stat5a, does not contribute to the unique Stat5b gel mobility shift. Furthermore, because the phosphoamino acid analyses showed stoichiometrically significant residual levels of constitutive serine phosphorylation of the Stat5a-S725A mutant but not Stat5b-S730A, it is possible that a second constitutively phosphorylated serine residue in Stat5a may be responsible for this structural difference between Stat5a and Stat5b. Further work is needed to resolve this distinction between Stat5a and Stat5b mobility.

**FIG. 6.**

**Effect of protein kinase inhibitors on prolactin-induced serine phosphorylation of Stat5a and Stat5b in Nb2 lymphocytes.** A and B, quiescent Nb2 cells were preincubated with dimethylsulfoxide (Ctrl, 500 µM), PD98059 (100 µM), or wortmannin (100 µM) for 30 min before stimulation with (+) or without (−) prolactin for 15 min. Lysates were immunoprecipitated with anti-α-Stat5a (A) or α-Stat5b (B). Parallel samples were blotted for phosphoamino acid (ps-PY, upper panels), site-specific phosphoamino acid (ps-PS725 and ps-PS730, middle panels), α-Stat5a (A, lower panel), or α-Stat5b (B, lower panel). C, inhibition of prolactin-induced activation of MAPK by PD98059 and staurosporine. Quiescent Nb2 cells were preincubated with dimethylsulfoxide (Ctrl), PD98059 (100 µM), or staurosporine (500 µM) for 30 min before stimulation with (+) or without (−) prolactin for 15 min. Samples from whole cell lysates were separated by SDS-PAGE with blotted with antibodies to phospho- and total MAPK (upper panel). Parallel samples were blotted with anti-pan ERK antibodies (lower panel).
phosphorylation of the Stat5a-S725A mutant but not of the corresponding Stat5b-S730A mutant. This suggested the existence of a second major serine phosphorylation site unique to Stat5a, although minor phosphorylation of several bystander serine residues has not been ruled out. Nonetheless, the observed differential phosphorylation of Stat5a and Stat5b supports the notion that these two homologous genes have evolved into structurally and functionally discernible proteins (26, 27). Stat5a and Stat5b differ the most in their COOH-terminal domains, which also contain the phosphorylated PSP motif (see Fig. 1B). Stat5a and Stat5b can undergo both homodimerization and heterodimerization and bind to similar DNA response elements (26). On the other hand, Stat5a and Stat5b may also form distinct complexes to DNA (26). Consistent with distinct, but overlapping functions of Stat5a and Stat5b, the phenotypes of mice deficient in either gene are relatively normal (7, 8). However, Stat5b is unable to compensate for Stat5a deficiency in the mammary gland, resulting in a loss of prolactin-induced milk production (7). Conversely, Stat5b null mice have specific growth hormone-signaling defects, resulting in stunted growth and liver dysfunction (8). The current work adds to a series of dissimilarities and suggests regulatory and structural differences between the highly homologous Stat5a and Stat5b proteins.

The present demonstration of regulated serine phosphorylation of Stat5 proteins extends documented serine phosphorylation to include four of the seven known Stat transcription factors. In addition, Stat4 is a candidate serine kinase target based on indirect evidence using serine kinase inhibitors (36). A general pattern of serine phosphorylation of Stat transcription factors is therefore emerging, although further work is needed to firmly establish the biological role of serine modulation. An initial report demonstrated a positive effect of Stat1 and Stat3 serine phosphorylation on transcriptional activation (17). Subsequent work has suggested a positive effect of serine phosphorylation on DNA binding (16), although this has been disputed (37). Finally, a recent paper suggested that serine phosphorylation negatively regulates tyrosine phosphorylation of Stat3 (24). The present data did not support a role for serine phosphorylation of the PSP motif in modulating DNA binding or transcriptional activity of Stat5, nor was there any negative effect of serine phosphorylation on Stat5 tyrosine phosphorylation. The current evidence for a second major serine phosphoacceptor site in Stat5a suggests that additional work is needed to understand the functional role of serine phosphorylation of Stat transcription factors. Motivated in part by recent studies, which have suggested a role of constitutively activated or serine phosphorylated Stat3 in the progression of several cancers, including chronic lymphocytic leukemias, breast tumors, and transformed fibroblasts (38, 39), we are currently examining the effect of serine phosphorylation on the transcriptional activities of Stat5a and Stat5b on additional Stat5-responsive genes as well as in cells other than COS-7.

In summary, we have demonstrated that serine residues Ser725 of Stat5a and Ser730 of Stat5b are major phosphorylation sites. Unexpectedly, phosphorylation control of this PSP motif differed between the highly homologous Stat5a and Stat5b proteins, and phosphoamino acid analysis suggested the existence of a second, major serine phosphorylation site unique to Stat5a. Although further studies are needed to establish the functional role of regulated Stat5 serine phosphorylation, the data suggested the existence of two pathways leading to serine phosphorylation of the PSP motif of Stat5, one PD98059-resistant, prolactin-activated pathway and one PD98059-sensitive, constitutive pathway. As more is learned about the involvement of Stat transcription factors in pathogenesis and progression of disease, the ability to pharmacologically manipulate individual Stat family members is expected to have important clinical consequences.