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Role of serine phosphorylation of Stat5a in prolactin-stimulated β-casein gene expression

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Abstract

Milk production remains suppressed in mammals during late pregnancy despite high levels of lactogenic polypeptide hormones. At parturition, associated with a precipitous fall in circulating progesterone, rising glucocorticoid levels synergize with prolactin to initiate copious milk production. This synergy is mediated at least in part through the coordinated activation of glucocorticoid receptors and transcription factor Stat5, particularly Stat5a. Here we show that two proline-juxtaposed serine residues within the transactivation domain of Stat5a are phosphorylated in the mammary gland during late gestation and lactation, and that these phosphorylation sites inhibit the transcriptional activity of Stat5a in the absence of glucocorticoid receptor costimulation. Specifically, transfection assays revealed that phosphorylation of residues S725 and S779 of Stat5a cooperatively suppressed prolactin-stimulated transcription from the β-casein promoter in both COS-7 kidney and MCF-7 mammary cells. This suppression was associated with shortened duration and reduced amplitude of nuclear DNA binding activity of wild type Stat5a relative to that of the serine phosphorylation-defective Stat5 mutant. However, costimulation of glucocorticoid receptors completely reversed the suppressive effect of Stat5a serine phosphorylation on β-casein gene transcription. We propose that serine phosphorylation within the transactivation domain may limit the activity of Stat5a in the absence of proper coactivation by glucocorticoid receptors. Published by Elsevier Science Ireland Ltd.

Keywords: Prolactin; Stat5 transcriptional regulation; Glucocorticoid receptors; Mouse mammary gland; β-casein promoter

1. Introduction

Transcription factors of the Stat family are central mediators of cytokine and hormone regulated cell growth and differentiation. Of the seven mammalian Stat genes, Stat1 and Stat2 are particularly important for antiproliferative and antiviral effects of interferons, whereas Stat3 is an oncogene that also regulates expression of acute phase response genes (Bromberg et al., 1999; Ihle, 1996). The highly homologous Stat5a and Stat5b proteins critically mediate antiapoptotic effects of cytokines in cells of hematopoietic origin, and are essential for normal hematopoiesis and development of the immune system (Mui et al., 1996; Iwatsuki et al., 1997; Rui et al., 1998; Moriggl et al., 1999; Socolovsky et al., 1999). Despite the approx. 95% amino acid homology between Stat5a and Stat5b and their redundant roles in regulating blood and immune cell function, the two transcription factors also appear to have evolved functional differences (Grimley et al., 1999). For instance, Stat5b is selectively important for growth hormone signaling (Udy et al., 1997), whereas Stat5a is

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particularly critical for prolactin (PRL)-induced mammary gland differentiation and milk protein gene expression (Liu et al., 1997).

The activity of Stat factors is rigorously controlled by tyrosine kinases. Phosphorylation of a positionally conserved tyrosine residue is required for dimerization, nuclear translocation, and subsequent binding of Stat5 to specific gene promoter elements (Darnell, 1997). In addition, serine kinases may also regulate the activity of Stat proteins. Specifically, phosphorylation of Stat1 and Stat3 at serine residue S727 within their transactivation domain is needed for maximal interferon-induced transcriptional activation (Zhang et al., 1995; Wen et al., 1995; Ng and Cantrell, 1997; Horvath and Darnell, 1996; Bromberg et al., 1996). Whereas serine phosphorylation of Stat1 and Stat3 was not important for DNA binding (Wen and Darnell, 1997), further work showed that phosphorylation of S727 mediated interaction between Stat1 and the nuclear minichromosome maintenance (MCM) protein-5 (Zhang et al., 1998), as well as with other nuclear coregulatory proteins (Korzus et al., 1998). Thus far, biological effects of serine phosphorylation of Stat5 have not been identified (Yamashita et al., 1998; Beuvink et al., 2000).

We recently demonstrated that a proline-directed serine kinase phosphorylated Stat5a and Stat5b on a shared Pro–Ser–Pro-motif within their transactivation domains, corresponding to serine residues S725 and S730 of mouse Stat5a and Stat5b, respectively (Yamashita et al., 1998). Phoshophoamino acid analysis suggested the existence of a second, major serine phosphorylation site unique to Stat5a (Yamashita et al., 1998), and work by Pircher and colleagues had provided indirect evidence of phosphorylation of a second proline-juxtaposed serine residue (S779) in mouse Stat5a (Pircher et al., 1999). More direct proof of phosphorylation of residue S779 was recently provided by Beuvink et al. (2000) using mass spectrometry. While that report showed that serine phosphorylation negatively affected DNA binding of Stat5a, no effect on transcriptional activity of Stat5a was detected. We now present novel evidence that both serine residues S725 and S779 are phosphorylated in mouse mammary gland during late gestation and lactation, and cooperatively suppress PRL-induced transcription from the β-casein promoter in the absence of glucocorticoid receptor coactivation. On the other hand, costimulation of glucocorticoid receptors, which are essential Stat5 coactivators needed for timely onset of milk production after parturition (Stocklin et al., 1996), could override the suppressive effect of Stat5a serine phosphorylation. Our new observations suggest that the modulatory effect of serine phosphorylation on Stat5a function is coactivator-dependent, and provide the first direct evidence for regulation of the biological activity of Stat5a by serine phosphorylation.

2. Materials and methods

2.1. Plasmids and mutants

Expression plasmid p3PRLR was constructed as described (Yamashita et al., 1998) and contains a 2.7-kb human PRL receptor cDNA (kindly provided by Paul A. Kelly, Institut National de la Santé et de la Recherche Médicale, Paris, France). Expression vectors for mouse Stat5a and Stat5b (pXM-Stat5a/b; kindly provided by Xiuwen Liu and Lothar Hennighausen, National Institutes of Health, Bethesda, MD), and generation of derivative mutants Stat5a-S725A and Stat5b-S730A also have been described previously (Yamashita et al., 1998). Two additional serine to alanine mutants Stat5a-S779A and Stat5a-S725A/S779A were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with oligonucleotide primers designed to alter serine residues to alanines (TCC to GCC). Before use, the DNA sequence of each mutant was verified. The genomic (-344 to -1) β-casein gene promoter linked to the luciferase reporter gene (pZZ1; kindly provided by Bernd Groner, Institute for Experimental Cancer Research, Freiburg, Germany), plasmid pCH110 containing the β-galactosidase gene under control of the simian virus 40 promoter, and human glucocorticoid receptor expression vector pRShGRα have been described previously (Stocklin et al., 1996; Liu et al., 1995; Hollenberg et al., 1985).

2.2. Cell Culture and Transfections

COS-7 cells (ATCC) were grown in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum, 2 mM L-glutamine and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), at 37 °C with 5% CO_2. MCF-7 cells were grown in RPMI-1640 medium containing 10% fetal calf serum, 2 mM L-glutamine and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), at 37 °C with 5% CO_2. For signal transduction studies, subconfluent COS-7 cells in 100 mm dishes were transfected with 2 μg of the PRL receptor construct (p3PRLR) and 2 μg of plasmid pXM-Stat5a, pXM-Stat5b, or corresponding mutants. For luciferase and β-galactosidase assays, FuGENE6 transfection reagent (Boeringer Mannheim Cat. No. 1814443) was used for transfection of both COS-7 cells and MCF-7 cells in six-well plates. Three microliters of FuGENE6 reagent, 0.25 μg of one of the plasmids pXM-Stat5a, pXM-Stat5b, or mutants of pXM-Stat5a or pXM-Stat5b, 0.25 μg of p3PRLR, 0.5 μg of pZZ1 and 0.1 μg of pCH110 encoding the β-galactosidase gene and/or 0.1 μg of the human glucocorticoid receptor expression vector pRShGRci were used.

2.3. [^{32}P]-*ortho*-phosphate labeling and phosphoamino acid analysis

COS-7 cells were transfected with plasmids encoding human PRL receptor and individual Stat5a mutants as indicated. After 24 h of culture in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), cells were starved for 12 h in serum-free DMEM. Cells were then washed twice with phosphate-free DMEM (Gibco, Cat. No. 11971-025), and incubated in the same medium supplemented with [^{32}P]-*ortho*-phosphate (0.75 mCi/ml; NEN) at 37 °C for 2 h. Cells were stimulated with or without 10 nM human PRL for 30 min, and were then analyzed and immunoprecipitated as described below. Proteins were eluted from protein A-Sepharose beads, separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE; 7.5% polyacrylamide), and transferred to PVDF membranes. Labeled proteins were visualized by autoradiography and analyzed by phosphoamino acid analysis using the Hunter Thin Layer Electrophoresis System (HLTE-7000) as described by Boyle et al. (1991). Briefly, samples were separated in first dimension buffer (7.8% acetic acid and 2.5% formic acid), applied to TLC plates (100 μM thick cellulose; Merck) and electrophoresed for 1 h at 1100 V. The second dimension was separated in 5% acetic acid and 0.5% pyridine at 1300 V for 25–45 min. Migration of the phosphorylated amino acid standards were detected by ninhydrin staining. Phosphoamino acid content was visualized by exposing the plates to X-ray film at −80 °C with intensifying screens.

2.4. Generation of site-specific antiStat5-phosphoserine antibodies

The phosphopeptide SRL[pS]PPAGL corresponding to amino acid residues 777–785 of human Stat5a was synthesized, conjugated to keyhole limpet hemocyanin and used as immunogen in rabbits (Genosys Inc., Woodlands, TX). Generation of a second antibody specific for phospho-Ser 725/730 of Stat5a/b has been described previously (Yamashita et al., 1998). For immunoblotting, blots were incubated for 16 h with either antiserum at 1:5000 dilution.

2.5. Solubilization of proteins, immunoprecipitation and immunoblotting

Cells were starved in serum-free medium for 16 h, then treated with or without 10 nM human PRL (NIDDK-hPRL-SIAFP-B2, AFP-2969A; a gift from A. F. Parlow at the National Pituitary Hormone Program), harvested and solubilized in lysis buffer containing 10 mM Tris–HCI, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium *ortho*-vanadate, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/ml aprotinin, 1 μg/ml pepstatin A and 2 μg/ml leupeptin as described (Kirken et al., 1997a). For immunoprecipitation from clarified cell lysates, polyclonal rabbit antisera (2 μl/ml) specific to peptides corresponding to the unique COOH-termini of Stat5a or Stat5b were used (Kirken et al., 1997b), and captured after incubation with protein A-Sepharose beads (Amersham Pharmacia). Immunoblotting was performed as described previously (Kirken et al., 1997a) using polyvinylidene difluoride (PVDF) membranes (Millipore), and antiphospho-Stat5a/b (Y694/Y699) mAb (UBI, Cat. No. 05-495; 0.5 μg/ml), site-specific anti-Stat5-phosphoserine antibodies (pS725/S730; 1:5000 dilution; and pS779; 1:5000 dilution) or polyclonal rabbit antisera of Stat5a and Stat5b (1:3000 dilution) as primary antibodies and horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG as secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham, Cat. No. RPN2106). Densitometric normalization and comparison of Stat5a phosphorylation in mammary glands from virgin and lactating mice from three independent experiments was performed using an Eagle Eye system (Stratagene, CA). The paired *t*-test was used for statistical analysis.

2.6. Collection and processing of mouse mammary glands

Female mice (C57/B16) were obtained from the animal facility at the National Cancer Institute (Frederick, MD). The fourth pair of mammary glands were collected from mature virgin mice and from mice at different stages of gestation (days 8, 14, and 18), lactation (days 5 and 10) and involution (6 h, 16 h, 24 h, and 3 days) and stored at −80 °C until processing. For each time point mammary glands from two mice were pooled (five for the virgin mice) and homogenized with an Ultraturrax homogenizer (Janke & Kunkel GmBH, Staufen, Germany) in lysis buffer (described above) at a ratio of 5 ml/g of tissue. Tissue homogenates were rotated end-over-end for 60 min at 4 °C, and insoluble material was pelleted at 12,000 × *g* for 30 min at 4 °C. The protein concentration of clarified tissue lysates was determined by simplified Bradford method (BioRad Laboratories, Hercules, CA). A total of 3.5 mg of protein was used for immunoprecipitation with either polyclonal rabbit antisera against Stat5a (2 μl) or Stat5b (2 μl) for 3 h at 4 °C. Antibody capture with protein A-Sepharose beads, SDS–PAGE and immunoblotting was performed as described for cell lysates above.

2.7. Luciferase and β-galactosidase assays

One day after transfection, cells were starved in serum-free medium and stimulated with or without 10 nM human PRL in the presence or absence of 100 nM dexamethasone 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 $MgSO_4$, 4 mM EGTA, 1 mM DTT) 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 360 μl of assay buffer containing 25 mM glycylglycine (pH 7.8), 15 mM potassium phosphate (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 2 mM ATP and 1 mM DTT. Luciferase activity of each sample was determined by measuring luminescence after injection of 200 μl of 1 mM luciferine as described (Yamashita et al., 1998). Three or four independent experiments with triplicate measurements were carried out as indicated in figure legends. Differences between treatments were compared by one-way analysis of variance followed by Scheffe's multiple range test.

2.8. Electrophoretic mobility shift assay (EMSA)

Cells were starved in serum-free medium for 16 h, then treated with or without 10 nM human PRL as indicated, pelleted by centrifugation and immediately solubilized in EMSA lysis buffer and analyzed as described earlier (Kirken et al., 1997a). [^{32}P]-labeled oligonucleotide probe corresponding to the PRL response element (5′agatttctaggaattcaaatc 3′) of the rat β-casein gene was used. Polyacrylamide gels (4%) containing 5% glycerol and 0.25 × TBE were prerun in 0.25 × TBE buffer at 4–10 °C for 1.5 h at 300 V. After loading of samples, the gels were run at room temperature for approx. 3 h at 250 V. Gels were dried by heating under vacuum and exposed to X-ray film.

2.9. DNA sequencing of cattle Stat5a

To verify the absence of a second serine phosphorylation site in cattle Stat5a, genomic DNA was purified from bovine muscle (Giants Supermarket, Bethesda, MD) and amplified by PCR for 25 cycles using the following primers: 5′:CGA CCA GGA TGG AGA ATT C 3′ (sense) and 5′: C ATG TGT ACA TGG GCT GCC 3′ (antisense). The product was applied to a 1% agarose gel, visualized by UV light, excised and recovered by MERmaid kit (BIO 101, Vista, CA). The recovered DNA was sequenced by primer extension with dye-labeled nucleotides with both sense and antisense primers and analyzed by ABI prism sequencer. The sequence was submitted to GenBank (Accession No. AF250911).

3. Results

3.1. Evaluation of extent of phosphorylation of serine residue S779 of Stat5a by mutagenesis and phosphoamino acid analyses

To quantitate the extent of phosphorylation of proline-juxtaposed serine residue S779 of Stat5a, we first tested the effect of converting residue S779 to alanine on overall Stat5a serine phosphorylation in a cellular reconstitution assay. COS-7 cells were transiently transfected with expression plasmids encoding PRL receptor and wild-type (WT) Stat5a, mutant Stat5a-S779A, or mutant Stat5a-S725A/S779A. Cells were metabolically labeled with [^{32}P]*ortho*-phosphate for 2 h and incubated with or without 10 nM PRL for 30 min. Individual Stat5a proteins were immunoprecipitated from cell lysates, separated by SDS–PAGE, and overall phosphate incorporation was compared by autoradiography (Fig. 1A, upper panel). Mutation of residue S779 to alanine led to a significant reduction of basal and PRL-stimulated phosphate incorporation, which was further reduced in the double mutant Stat5a-S725A/S779A (Fig. 1A, upper panel). Bands corresponding to full length, 94 kDa Stat5a isoforms were then excised and subjected to phosphoamino acid analysis (Fig. 1A, lower panel). The reduction in phosphate incorporation into mutant Stat5a-S779A was selective for serine (Fig. 1A, lower panel), providing strong evidence that S779 constituted a second serine phosphoacceptor site of Stat5a in addition to the previously established residue S725 (Yamashita et al., 1998). Combined mutation of S725 and S779 further reduced but did not completely eliminate phosphoserine from mutant Stat5a-S725A/S779A, suggesting either the existence of a single additional phosphorylation site within the molecule or minor phosphorylation of several bystander serine residues.

3.2. Analysis of tyrosine and serine phosphorylation of wild-type and Stat5 mutants by immunoblotting

To more directly determine whether residue S779 of Stat5a was a major phosphorylation site, we generated phosphospecific antibodies by immunizing rabbits with a phosphopeptide corresponding to this unique motif of Stat5a. We first examined the kinetics of phosphorylation of Y694, S725 and S779 of Stat5a in response to PRL treatment in COS-7 cells. Cells transfected with expression plasmids encoding PRL receptor and Stat5a-WT were serum-deprived for 16 h before exposure to PRL for various times up to 16 h. Immunoprecipitated Stat5 proteins were separated on SDS–PAGE and immunoblotted with appropriate phosphospecific antibody preparations. Unlike tyrosine residue Y694 of Stat5a, which was inducibly and transiently phosphory-

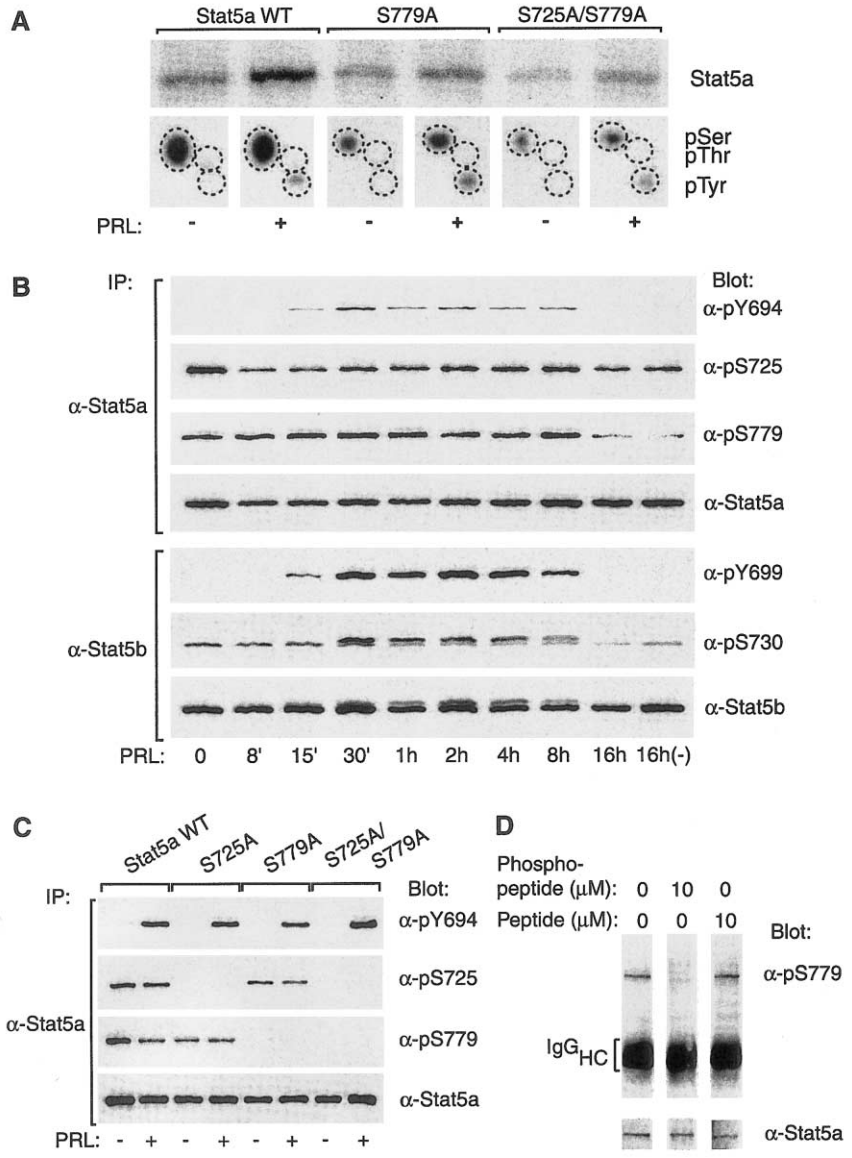

Fig. 1. Phosphorylation of Stat5a and serine-to-alanine mutants in COS-7 cells as determined by phosphoamino acid analysis and immunoblotting with phosphospecific antibodies. (A) Phosphoamino acid analysis of wild-type (WT) and serine-to-alanine mutants of Stat5a. Autoradiographies of immunoprecipitated wild-type and mutant forms of Stat5a from [^{32}P]*ortho*phosphate labeled COS-7 cells incubated with (+) or without (−) 10 nM PRL for 30 min at 37 °C are shown in the upper panel. The lower panel shows phosphoamino acid analysis of wild-type and mutant forms of Stat5a with (+) or without (−) PRL stimulation. Radioactive bands corresponding to Stat5a were excised and subjected to acid hydrolysis and thin layer electrophoresis, and phosphate incorporated into amino acids was visualized by autoradiography. Representative data from three independent experiments are shown. Migrational positions of phosphoserine (pSer), phosphothreonine (pThr), or phosphotyrosine (pTyr) are indicated. (B) Kinetic analysis of tyrosine and serine phosphorylation of Stat5a and Stat5b by immunoblotting. COS-7 cells transfected with the PRL receptor and wild-type (WT) Stat5a (panels 1 to 4 from top) or Stat5b (panels 5 to 7 from top) were starved in serum-free DMEM for 16 h, then incubated with medium without (−) or with (+) 10 nM PRL at 37 °C for up to 16 h as indicated. Lysates were immunoprecipitated (IP) with anti (α)-Stat5a or α-Stat5b antibodies. Parallel samples were blotted for either phosphotyrosine (α-pY694/pY699), site-specific phosphoserines (α-pS725/pS730 and α-pS779), or Stat5 levels (α-Stat5a or α-Stat5b). (C) Phosphotyrosine and phosphoserine content of Stat5a wild-type (WT) and serine-to-alanine mutants (Stat5a-S725A, S779A, and S725A/S779A) by immunoblotting. COS-7 cells transfected with PRL receptor and wild-type (WT) or mutant forms of Stat5a were starved in serum-free medium for 16 h, then exposed to medium without (−) or with (+) 10 nM PRL at 37 °C for 30 min. Lysates were immunoprecipitated (IP) with α-Stat5a antibodies. Parallel samples were blotted for either phosphotyrosine (α-pY694), site-specific phosphoserines (α-pS725 and α-pS779), or α-Stat5a. (D) Competition of immunogen for binding of anti-pS779 antiserum to phosphorylated Stat5a-WT. Stat5a-WT was immunoprecipitated from lysates of transfected COS-7 cells, and anti-pS779 antiserum (1:5000 dilution) was used for immunoblotting of three replicate samples in the absence or presence of either phosphopeptide immunogen (10 μM) or the unphosphorylated form of the same peptide (10 μM) as indicated (upper panel). Reblotting for Stat5 protein levels are shown in the lower panel.

lated in response to PRL (Fig. 1B, panel 1), both serine residues S725 and S779 were constitutively phosphorylated when Stat5a was expressed in COS-7 cells (Fig. 1B, panels 2 and 3). In parallel experiments, phosphorylation of Stat5b on Y699 showed PRL-inducible kinetics similar to that of Stat5a (Fig. 1B, panel 5). In contrast to Stat5a serine phosphorylation of S725 and S779, phosphorylation of S730 of Stat5b was only weakly constitutive but was further induced by PRL with kinetics parallel to tyrosine phosphorylation (Fig. 1B, panel 6). Levels of Stat5a and Stat5b remained constant over the observation period (Fig. 1B, panels 4 and 7, respectively).

3.3. Phosphospecific antibodies show that phosphorylation of residues S725 and S779 of Stat5a may occur independently, and is not directly tied to inducible tyrosine phosphorylation

Phosphoamino acid analysis had demonstrated reduced levels of serine phosphorylation in mutant Stat5a-S779A when expressed in COS-7 cells, and Stat5a-WT showed immunoreactivity to antibodies raised against a corresponding phosphopeptide. To specifically demonstrate phosphorylation of serine residue S779, and test the mutual interdependence of phosphorylation of S725 and S779, immunoblotting with phosphospecific Stat5a antibodies were used in combination with mutagenesis. PRL receptors and Stat5a-WT or either of mutants Stat5a-S725A, -S779A, or -S725A/S779A were expressed in COS-7 cells, and serum-deprived cells were treated with or without PRL for 30 min. Immunoblotting demonstrated that each mutant maintained normal capacity of PRL inducible phosphorylation of Y694 (Fig. 1C, upper panel). Furthermore, whereas mutant Stat5a-S779A retained normal levels of constitutive phosphorylation of residue S725, immunoreactivity was selectively lost in mutants Stat5a-S725A and Stat5a-S725A/S779A which both lack the phosphoacceptor hydroxyl group (Fig. 1C, second panel from top). Correspondingly, mutant S725A retained constitutive phosphorylation of S779, whereas immunoreactivity of the anti-phospho-S779 antibody was selectively absent from mutants Stat5a-S779A and Stat5a-S725A/S779A (Fig. 1C, third panel from top). Expression levels of individual mutants were comparable (Fig. 1C, bottom panel).

To further validate the specificity of the new anti-pS779-Stat5a antiserum, we tested the ability of the phosphopeptide immunogen and the corresponding unphosphorylated peptide to displace binding of the antiserum to phosphorylated Stat5a-WT by immunoblotting. Stat5a-WT was expressed in COS-7 cells, immunoprecipitated, and separated by SDS–PAGE. Three parallel samples were blotted with anti-pS779-Stat5a antiserum in the absence or presence of either phosphopeptide SRL[pS]PPAGL (10 μM) or unphosphorylated peptide SRLSPPAGL (10 μM) as indicated (Fig. 1D, upper panel). This analysis established that phosphorylated, but not unphosphorylated peptide, could displace binding of the antibody to phosphorylated Stat5a. Equal protein loading was verified by reblotting with anti-Stat5a antiserum (Fig. 1D, lower panel). These results supplemented the evidence of specific recognition by the antiserum of the phosphorylated residue S779 of Stat5a.

From the studies using phosphospecific antibodies presented above, we conclude that S779 is a second major proline-directed serine phosphorylation site within the transactivation domain of Stat5a in addition to S725. Furthermore, phosphorylation of residues S779 and S725 occurred independently, and was not directly affected by the tyrosine phosphorylation state of Stat5a.

3.4. Examination of serine phosphorylation of Stat5a in mammary gland during gestation

Due to the paucity of reported biological effects of Stat5 serine phosphorylation, we examined the pattern of Stat5 serine and tyrosine phosphorylation during mammary gland differentiation and lactation. Stat5a is particularly important for this physiological process as evidenced by gene targeting studies in mice (Liu et al., 1997). A previous report had documented that Stat5a was phosphorylated on S779 during gestation and lactation (Beuvink et al., 2000) but a systematic analysis of both proline-directed serine phosphorylation sites of Stat5a during the mammary gestation cycle has not been carried out. Initial examination of Stat5a immunoprecipitated from mammary glands of either virgin or lactating mice showed that Stat5a was markedly phosphorylated on both S725 and S779 in tissue from lactating but to a lesser extent in virgin animals (Fig. 2A). Mouse mammary gland tissue at various stages of gestation cycle was then collected and Stat5a serine phosphorylation status of residues S725 and S779 was determined. As shown in Fig. 2B, residues S725 and S779 were coordinately phosphorylated during gestation and remained serine phosphorylated during lactation. The observation that both serine residues of Stat5a were phosphorylated during gestation was consistent with a biological role of Stat5a serine phosphorylation during mammary gland differentiation and lactogenesis. In parallel, Stat5b was also serine phosphorylated on its solitary serine phosphorylation site S730 (Fig. 2B). Furthermore, serine phosphorylation of both Stat5 isoforms was rapidly turned off during mammary gland involution. The observed phosphorylation of residues S725 and S779 of Stat5a, the Stat5 isoform most critical for milk production, suggested its involvement as a physiologically relevant control mechanism for mammary gland differentiation.

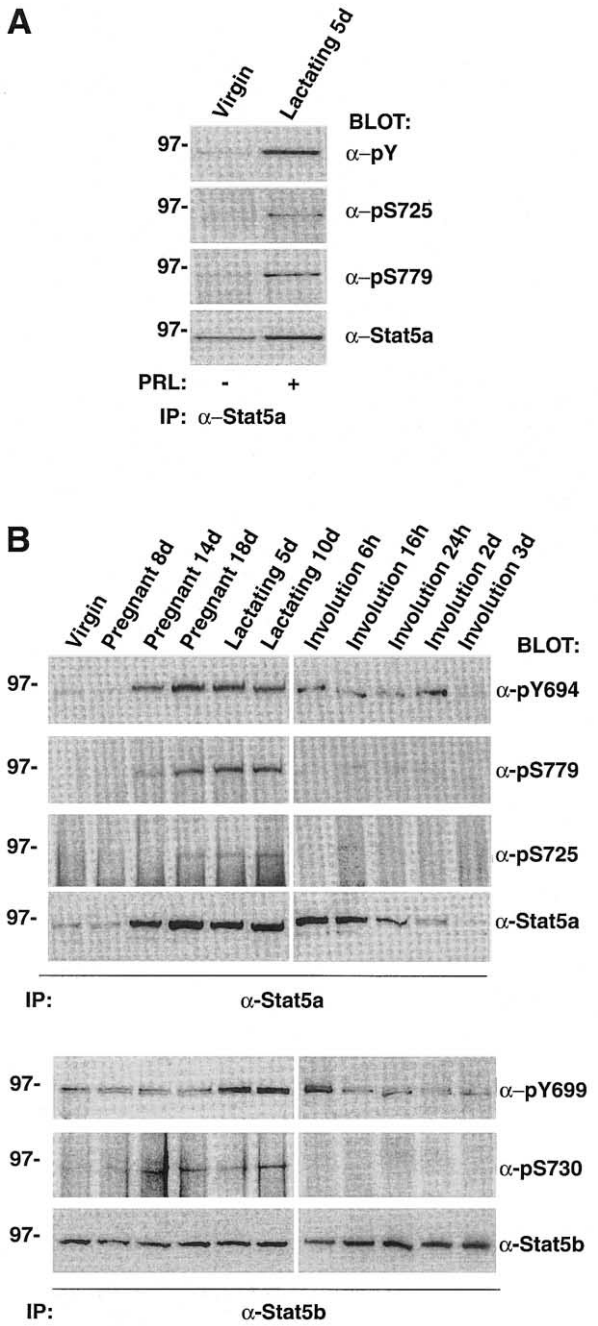

Fig. 2. Serine phosphorylation of Stat5a and Stat5b in mouse mammary gland during gestation cycle. (A) Initial analysis of phosphorylation of serine residues S725 and S779 of mouse Stat5a in mammary tissue from virgin and lactating mice. Tissues were harvested from virgin mice or mice at day 5 of lactation. Stat5a was immunoprecipitated from tissue homogenates that had been normalized with respect to total protein concentration. After separation by SDS–PAGE, phosphorylation of individual residues were detected in replicate samples by immunoblotting with phospho-Stat5 specific antibodies to pY694, pS725, or pS779. Levels of Stat5a were also detected by antibodies to Stat5a. (B) Temporal profile of serine phosphorylation of Stat5a and Stat5b in mouse mammary gland during the gestation cycle. Stat5a or Stat5b were immunoprecipitated from tissue homogenates of mouse mammary gland at various stages of the gestation cycle. Phosphorylation of Stat5 on tyrosine and serine residues levels was detected by phosphospecific antibodies.

In general, serine phosphorylation of Stat5a and Stat5b paralleled that of the conserved tyrosine residue over the pregnancy cycle. Whereas the increase in tyrosine and serine phosphorylation of Stat5b during pregnancy and lactation occurred without any increase in Stat5b protein levels, increased phosphorylation of Stat5a during pregnancy and lactation was accompanied by a marked increase in levels of Stat5a. To more directly assess the increase in Stat5a phosphorylation that was not due to increased levels of Stat5a protein, phosphoprotein immunoblots were analyzed by densitometry and normalized for Stat5 protein levels. Samples of mammary glands of virgin and lactating mice from three independent experiments were used. Overall, after normalization there was a twofold increase in phosphorylation of residues Y694, S725, and S779 of Stat5a in lactating mammary glands over that detected in virgin mammary glands. More specifically, mean normalized values $\pm$ SEM were 47 ± 14 and 98 ± 4 for pY694 in virgin and lactating mammary glands, respectively ($n = 3$, $P < 0.05$). The corresponding values in virgin and lactating mammary glands were 29 ± 5 and 69 ± 3 for pS725 ($n = 3$, $P < 0.05$), and 16 ± 4 and 39 ± 8 for pS779 ($n = 3$, $P < 0.05$). Therefore, normalization to Stat5a protein levels during pregnancy cycle suggested that Stat5a serine and tyrosine phosphorylation increased by approximately a factor of two from virgin to lactating state. Regardless of the absolute increase in Stat5 phosphorylation, the present study verified that both serine residues S725 and S779 of Stat5a are phosphorylated during pregnancy and lactation.

3.5. Phosphorylated serine residues S725 and S779 of Stat5a cooperate to negatively regulate PRL-induced transcription in the absence of glucocorticoid receptor coactivation

To analyze the effect of serine phosphorylation of Stat5a on transcriptional activity, we used a luciferase reporter gene under control of the Stat5a-responsive β-casein gene promoter (Yamashita et al., 1998). This reporter gene has been used extensively for studies of transcriptional activity of Stat5 (Stocklin et al., 1996). The plasmid encoding the β-casein reporter gene was transfected into COS-7 cells along with expression plasmids encoding the PRL receptor and wild-type Stat5a or mutant proteins. A constitutively expressed β-galactosidase gene was also included to compensate for differences in transfection efficiencies. Luciferase activity was measured in extracts of cells that had been incubated in the absence or presence of PRL for 16 h (Fig. 3A and B). Wild-type Stat5a mediated a highly consistent 35-fold induction of reporter gene expression in response to PRL stimulation. Mutant Stat5a-S779A mediated a response comparable to that of Stat5a-WT,

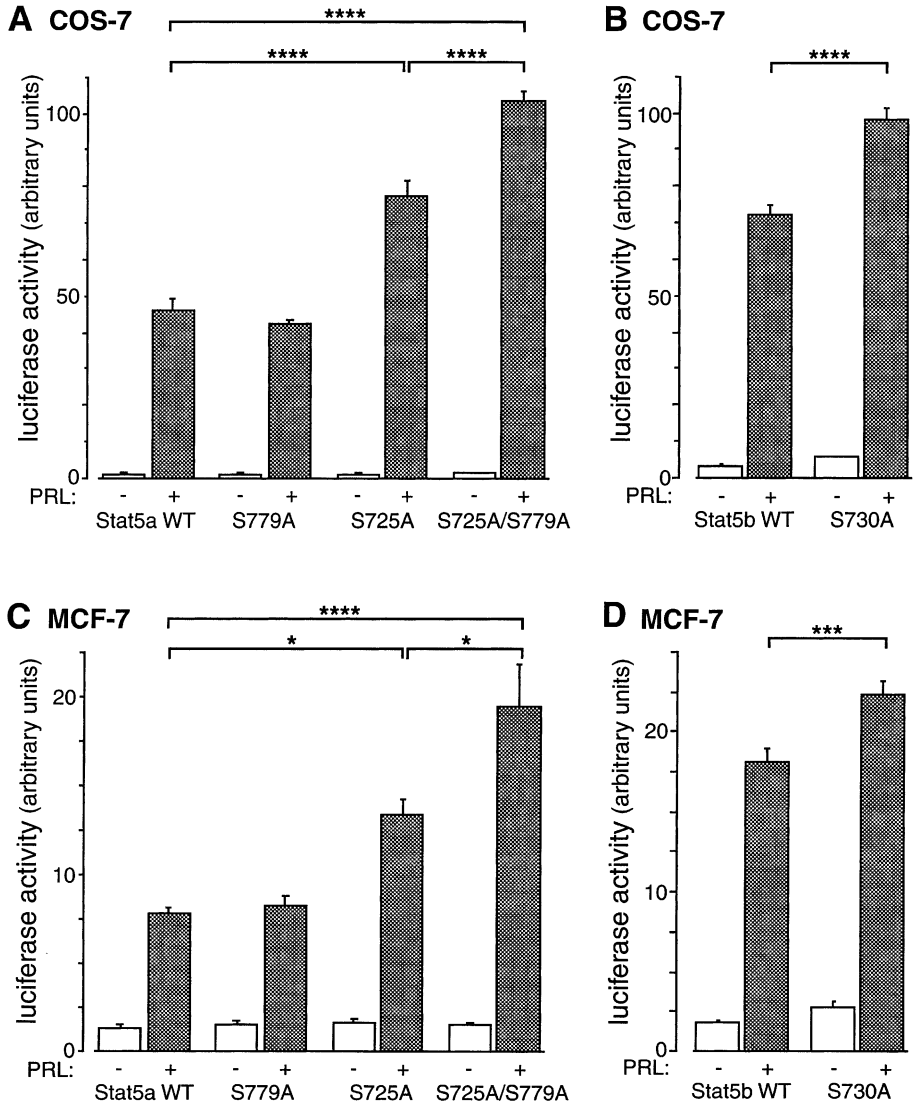

Fig. 3. PRL-inducible β-casein gene activation by wild-type (WT) and mutants of Stat5a or Stat5b in COS-7 cells (A, B) or in MCF-7 cells (C, D). Cells were transfected with a β-casein-luciferase reporter gene, the PRL receptor, WT or serine-to-alanine mutants of Stat5a and Stat5b, and a β-galactosidase gene under the control of the Simian virus 40 promoter. Cells were treated with (+) or without (−) 10 nM PRL for 16 h in serum-free DMEM. 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. Differences between treatments were compared by one-way analysis of variance followed by Scheffe's multiple range test (****$P < 0.0001$;***$P < 0.001$; **$P < 0.0l$; *$P < 0.05$).

whereas Stat5a-S725A demonstrated a moderate, 1.7-fold elevation of PRL-stimulated activity ($P < 0.0001$, Fig. 3A). This increased PRL-stimulated activity of Stat5a-S725A was further enhanced when both serine sites were converted to alanine, as demonstrated by a consistent, 2.2-fold increase in PRL-stimulated transcription mediated by mutant Stat5a-S725A/S779A ($P < 0.0001$, Fig. 3A). The elevated activity of the double mutant Stat5a-S725A/S779A above that of either mutant with a single serine substitution therefore suggested functional cooperativity between the two serine phosphorylation sites. Increased activity associated with Stat5a serine mutants was limited to PRL-induced transcription, while there was no consistent effect on basal transcription rate. The observed gain of function associated with combined mutation of two Stat5a serine phosphorylation sites indicated that serine phosphorylation negatively affected transcriptional activity of Stat5a. Stat5b, which lacks a second, proline-juxtaposed serine phosphorylation site, only moderately gained activity upon mutation of the phosphorylation site (25%; $P < 0.0001$; Fig. 3B), providing new evidence of functional and regulatory differences between Stat5a and Stat5b.

To test whether serine phosphorylation suppressed Stat5a-mediated β-casein gene transcription in cells

other than COS-7 cells, the more physiologically relevant MCF-7 human mammary epithelial cell line was selected. Initially we expected to take advantage of the endogenous PRL receptors expressed in MCF-7 cells (Biswas and Vonderhaar, 1987), but transfection of both PRL receptor and Stat5a plasmids was required in order to detect β-casein gene induction (data not shown). A transfection approach identical to that used in COS-7 cells was therefore taken, and Stat5a-WT was observed to mediate a sixfold PRL-induced stimulation of transcription (Fig. 3C). Importantly, when tested in MCF-7 cells, Stat5a serine mutants exhibited activation profiles similar to those observed in COS-7 cells. Specifically, mutant Stat5a-S779A showed wild-type activity, whereas the activity of Stat5a-S725A was increased by approx. 60% ($P < 0.01$). Also in MCF-7 cells this moderate augmentation was further enhanced by combined mutation of both serine phosphorylation sites, as evidenced by a 2.5-fold increase in PRL-stimulated transcriptional activity of mutant Stat5a-S725A/S779A ($P < 0.0001$; Fig. 3D). Thus, the two serine phoshorylation sites cooperated to negatively regulate transcriptional activity of Stat5a also in MCF-7 mammary cells. Extending the observations from COS-7 to a human mammary cell line supported the physiological relevance of a regulatory effect of Stat5a serine phosphorylation.

3.6. Analysis of DNA binding activities of Stat5a mutants

It was recently suggested that serine phosphorylation of Stat5a suppressed binding to the GAS element of the β-casein promoter, although no effect on the transcriptional activity of Stat5a was reported (Beuvink et al., 2000). Specifically, mutant Stat5a-S725A/S779A was shown to display elevated DNA binding activity (Beuvink et al., 2000). Furthermore, it had previously been established that a C-terminal portion of the transactivation domain of Stat5a contained a regulatory element that inhibited DNA binding (Moriggl et al., 1996). It was therefore of relevance to examine whether the negative effect of serine phosphorylaton on transcriptional activity of Stat5a-S725A/S779A could be explained in terms of a modulated strength of nuclear DNA binding activity. It was also of interest to determine to what extent DNA binding associated with complete removal of the transactivation domain of Stat5a could be attributed to loss of the two serine phosphorylation sites.

To specifically compare the strength of PRL-induced nuclear DNA-binding activity of wild-type Stat5a to that of mutants Stat5a-S725A, Stat5a-S779A, Stat5a-S725A/S779A, and the C-terminal truncation mutant Stat5a-Δ713, nuclear extracts from transfected cells were tested for binding to an oligonucleotide probe corresponding to the Stat5 response element of the β-casein gene promoter (Fig. 4). When reconstituted in PRL-responsive COS-7 cells, there was a consistent increase in the DNA-binding activities of serine-phosphorylation-defective Stat5a mutants in nuclear extracts of PRL-stimulated cells (Fig. 4). This conclusion was

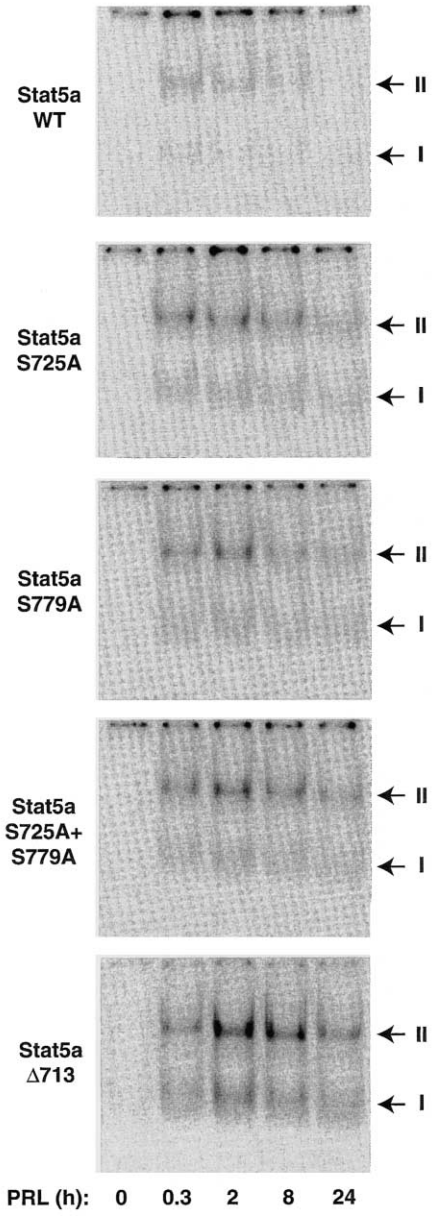

Fig. 4. PRL-inducible DNA binding activities of wild-type (WT) and mutants of Stat5a in COS-7 cells. COS-7 cells that had been transfected with the PRL receptor and Stat5a wild-type (WT) or serine-to-alanine mutants (Stat5a-S725A, S779A, or S725A/S779A), or a mutant Stat5aΔ713, which lacks the entire transactivation domain and hence both phosphoserine sites, were starved in serum-free DMEM for 16 h, then incubated with (+) or without (−) PRL (10 nM) from 0 to 24 h. Equal amounts of nuclear protein extracts were used for gel shift analysis with ^{32}P-labeled β-casein gene promoter probe. Roman numerals I and II indicate two previously characterized prolactin-inducible Stat5-DNA complexes (Yamashita et al., 1998; Kirken et al., 1997a,b) .

based on evaluation of both signal amplitude and duration in the presence of continuous PRL stimulation for up to 24 h (Fig. 4). Specifically, WT Stat5a showed little if any DNA binding after 24 h, while elevated DNA binding activity was especially pronounced in the doubly serine phosphorylation-defective mutant Stat5a-S725A/S779A. Based on these results and the observations by Beuvink et al. (2000) we suggest that the negative effect of serine phosphorylation of Stat5a is mediated by reduced DNA binding activity. Furthermore, loss of serine phosphorylation contributed to a large extent to the overall increased DNA binding activity associated with removal of the entire transactivation domain (Fig. 4, bottom panel; Moriggl et al., 1996).

3.7. Glucocorticoid receptor (GR) costimulation reversed the inhibitory effect of Stat5a serine phosphorylation

Whereas a negative effect of serine phosphorylation on Stat5a function may help prevent premature milk production by suppressing its transcriptional activity during late gestation, Stat5a continued to be serine phosphorylated during lactation when the key synergism between Stat5 and glucocorticoid receptors stimulates abundant milk production (Houdebine et al., 1985). The synergy between Stat5 and glucocorticoid receptors has been shown to be of direct importance for transcription of the β-casein gene (Moriggl et al., 1996). We therefore tested whether GR costimulation would overcome the suppressive effect of phosphorylation of Stat5a on serine residues.

Plasmids encoding GR and PRL receptor were cotransfected with plasmids encoding either Stat5a-WT or one of the three Stat5a serine mutants into COS-7 cells, which do not express functional levels of GR. PRL-induced β-casein reporter gene expression was compared for each form of Stat5a in the absence and presence of the GR agonist, dexamethasone (Dex). Activation of GR alone had little effect on β-casein gene expression, but coactivation of GR enhanced PRL-induced gene transcription from approx. 35-fold to approx. 170-fold (Fig. 5A). In the presence of GR without Dex, Stat5a-serine mutants responded functionally to PRL the same way as described above, showing a consistent cooperative suppression of activity in response to phosphorylation of the two serine residues (Fig. 5A). However, when GR was activated in parallel, serine phosphorylation status of Stat5a no longer suppressed PRL-induced transcription rate. Thus, costimulation of GR could override the negative effect of phosphorylation of Stat5a serine residues on PRL-induced β-casein gene transcription in COS-7 cells. Likewise, the inhibitory effect of serine phosphorylation of Stat5b was also masked by GR activation (Fig. 5B).

We then broadened the analysis of the interaction between GR-costimulation and Stat5a serine phosphorylation on PRL-induced β-casein gene transcription in MCF-7 mammary cells. MCF-7 cells express abundant levels of endogenous GR (Horwitz et al., 1978), and preparatory experiments established that transfection of GR into MCF-7 cells was not needed, because endogenous GR levels were sufficient to mediate marked Dex-dependent synergy with Stat5. In MCF-7 cells transfected with PRL receptors and Stat5a-WT, Dex-cotreatment elevated PRL-induced gene transcription from six-fold to 30-fold (Fig. 5C). Importantly, also in MCF-7 cells GR-coactivation masked the inhibitory effect of Stat5a serine phosphorylation, as evidenced by activation profiles of Stat5a mutants remarkably similar to those observed in reconstituted COS-7 cells (Fig. 5C). Finally, synergy between GR and Stat5b was also independent of Stat5b serine phosphorylation of S730 (Fig. 5D). Collectively, these experiments established that the inhibitory effect of Stat5a serine phosphorylation on transcription of the β-casein gene was reversed by GR costimulation in both COS-7 kidney cells and MCF-7 mammary cells.

4. Discussion

The present study demonstrated that two proline-directed serine phosphorylation sites within the transactivation domain of Stat5a interact to modulate Stat5a function. Specifically, the two phosphoserine sites cooperated to suppress PRL-induced transcription from a genomic β-casein gene promoter when tested in both COS-7 and MCF-7 cells. The data furthermore showed that Stat5a serine phosphorylation negatively affected amplitude and duration of nuclear DNA binding activity. The fact that glucocorticoid receptor costimulation could override the suppressive effect of Stat5a serine phosphorylation on transcription revealed a novel facet of the synergism between Stat5a and GR, and suggested that the regulatory role of Stat5a serine phosphorylation is coactivator-dependent. However, the mechanism by which GR overcomes this inhibitory effect remains to be determined. It is possible that GR, which physically associates with Stat5 as a coactivator (Stocklin et al., 1996; Moriggl et al., 1996), disrupts Stat5a interactions with other candidate molecular partners such as CrkL (Fish et al., 1999). Alternatively, association with GR may influence phosphoserine-dependent recruitment of nuclear Stat5 tyrosine phosphatases or Stat5 proteases. The close proximity of phosphoserine residues S725 of Stat5a and S730 of Stat5b to the recently identified cleavage site for a nuclear Stat5 serine protease (Lee et al., 1999) could indicate a regulatory role of serine phosphorylation in Stat5 proteolysis.

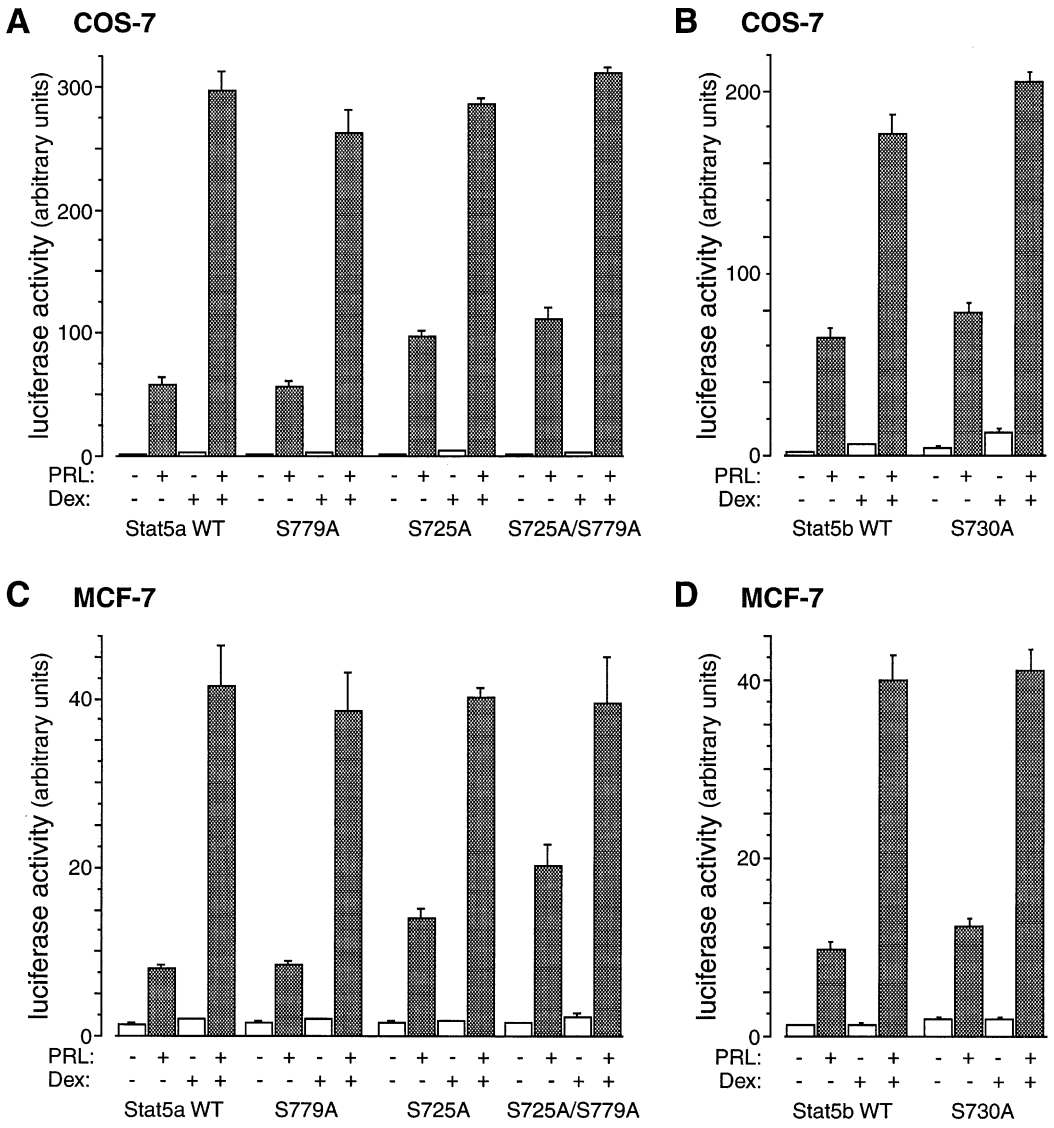

Fig. 5. Glucocorticoid receptor costimulation reversed the suppressive effect of Stat5a serine phosphorylation on β-casein reporter gene activation in COS-7 cells (A, B) and in MCF-7 cells (C, D). COS-7 cells or MCF-7 cells were transfected with a β-casein-luciferase reporter gene, PRL receptor, the glucocorticoid receptor, WT or mutants of Stat5a or Stat5b, and a β-galactosidase gene under the control of the Simian virus 40 promoter. Cells were treated with (+) or without (−) PRL (10 nM) in the presence (+) or absence (−) of dexamethasone (Dex; 100 nM) for 16 h in serum-free DMEM. 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 three independent experiments are presented, and S.E. values are indicated by bars.

The present work is focused on the specific context of Stat5a regulation of the β-casein promoter, and it will be important to extend the analysis to other Stat5 responsive promoters relevant to mammary physiology in order to establish more firmly a general role of serine phosphorylation of Stat5a in mammary gland differentiation and lactogenesis. However, based on our data we propose as a working model that serine phosphorylation may serve to suppress maximal activation of Stat5a during late gestation, and that proper coactivation of GR will override this suppression and thus facilitate timely onset of milk synthesis after parturition. Indirect support for a suppressive effect of Stat5a serine phosphorylation on milk production comes from the observation that among mammals, bovine Stat5a is unique in that it lacks the second C-terminal serine phosphorylation site that corresponds to S779 of mouse Stat5a, and contains instead a proline residue (Table 1). As part of this work we have verified by independent sequencing of cattle Stat5a DNA that a point mutation has indeed resulted in a serine to proline mutation (LeBaron et al., 2000; GenBank accession No. AF250911), possibly due to selective breeding for higher milk yield. A third cattle Stat5a DNA sequence has since been submitted that further confirmed the loss of the second phosphorylation site (Seyfert et al., 2000;

Table 1
Loss of COOH-terminal proline-directed serine phoshorylation site in Stat5a of cattle

Mouse	765	ELLRRPMDSLDARL	SP	P.AGLFTSARSSLS	793
Rat	765	ELLRRPMDSLDPRL	SP	P.AGLFTSARSSLS	793
Pig	766	ELLRRPMDSLDSRL	SP	P.AGLFTSARGSLS	794
Human	766	ELLRRPMDSLDSRL	SP	P.AGLFTSARGSLS	794
Sheep	766	ELLRRPNGQSGP.L	SP	PPAGLFTPARGSLS	794
Cattle	766	ELLRRPMDSLEPSL	PP	P.TGLFTPGRGSLS	794

Serine residue corresponding to S779 of mouse Stat5a is conserved in Stat5a of rat, pig, human, sheep but not in cattle.

GenBank accession No. AJ237937). Future work is needed to specifically determine whether cattle Stat5a is transcriptionally more active than Stat5a from other species.

The molecular mechanisms involved in suppressing Stat5a transcriptional activity through phosphorylation of residues S725 and S779 may involve reduced stability of DNA binding. In the present work, mutation of Stat5a-S779 to alanine alone did not notably affect PRL-induced β-casein gene transcription, and dual mutation of both proline-directed phosphoserine sites was needed to detect a regulatory involvement in this experimental model. While our demonstration of a suppressive effect of Stat5a serine phosphorylation on DNA binding activity is consistent with recent data by Beuvink et al. (2000), that study did not detect a parallel suppression of transcriptional activity as reported here. However, this apparent discrepancy may be due to more optimized transfection conditions and more effective prolactin-induced β-casein gene induction by Stat5a-WT achieved in the present study, which was approx. 35-fold compared to two- to three-fold in the previous study. Furthermore, while we have limited this initial study of Stat5a serine phosphorylation to the β-casein promoter, a broader side-by-side analysis of the effects of Stat5a serine phosphorylation in various cell types, signaling systems and gene promoters is now warranted. The coactivator-dependent inhibitory role of Stat5a serine phosphorylation revealed by the present study certainly suggests the existence of further conditional effects.

Until now the lack of evidence for biological involvement of Stat5a serine phosphorylation has made it difficult to justify efforts to identify Stat5a serine kinase(s). Whether Stat5a is phosphorylated on S725 and S779 by the same proline-directed serine kinase, or whether the two sites are substrates for distinct serine kinases also needs to be determined. Our previous data indicated that residue S725 is not a direct substrate for ERK1/2, but possibly an indirect target (Yamashita et al., 1998). Another report presented evidence to suggest that S779 of mouse Stat5a is a substrate and/or docking site for ERK1/2 (Pircher et al., 1999), reporting that ERKs bind and phosphorylate a glutathione-Stat5a fusion protein in vitro but not the corresponding S779A mutant. Thus, it is possible that the two serine phosphorylation sites within the Stat5a transactivation domain are targets for separate proline-directed serine kinases. The availability of phosphospecific antibodies should facilitate the identification of physiological Stat5 serine kinases.

We have previously pointed out differences in phosphoserine regulation between Stat5a and Stat5b (Yamashita et al., 1998; Kirken et al., 1997b). The existence of a unique second serine phosphorylation site within the transactivation domain of Stat5a that is not present in Stat5b further underscores this differential regulation of Stat5a and Stat5b by serine kinase(s). It is of direct relevance that Kazansky and colleagues recently reported that nuclear accumulation of Stat5b but not Stat5a could be induced by Src tyrosine kinase activation in a prolactin-independent manner, a functional difference that was due to structural determinants within the C-terminus of the Stat5 proteins (Kazansky et al., 1999). The second, unique serine phosphorylation site and its flanking sequences within the transactivation domain of Stat5a may provide a clue to this functional difference between Stat5a and Stat5b.

Acknowledgements

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