

Serine Phosphorylation of GH-Activated Signal Transducer and Activator of Transcription 5a (STAT5a) and STAT5b: Impact on STAT5 Transcriptional Activity

SOO-HEE PARK, HIROKO YAMASHITA, HALLGEIR RUI, AND DAVID J. WAXMAN

Division of Cell and Molecular Biology, Department of Biology, Boston University (S.-H.P., D.J.W.), Boston, Massachusetts 02215; and Department of Pathology, Uniformed Services University of the Health Sciences (H.Y., H.R.), Bethesda, Maryland 20814

Signal transducer and activator of transcription 5b (STAT5b), the major liver-expressed STAT5 form, is phosphorylated on both tyrosine and serine in GH-stimulated cells. Although tyrosine phosphorylation is known to be critical for the dimerization, nuclear translocation, and activation of STAT5b DNA-binding and transcriptional activities, the effect of STAT5b serine phosphorylation is uncertain. Presently, we identify Ser⁷³⁰ as the site of STAT5b serine phosphorylation in GH-stimulated liver cells. We additionally show that the serine kinase inhibitor H7 partially blocks the GH-stimulated formation of (Ser,Tyr)-diphosphorylated STAT5b without inhibiting STAT5b nuclear translocation. Evaluation of the functional consequences of STAT5b serine phosphorylation by mutational analysis revealed an approximately 50% decrease in GH-stimulated luciferase reporter gene activity regulated by an isolated STAT5-binding site when STAT5b Ser⁷³⁰ was mutated to alanine and under conditions where STAT5 DNA-binding activity was not diminished. No decrease in GH-stimulated

reporter activity was seen with the corresponding STAT5a-Ser⁷²⁵Ala mutant; however, a decrease in reporter activity occurred when the second established STAT5a serine phosphorylation site, serine 779, was additionally mutated to alanine. Unexpectedly, STAT5a-Ser^{725,779}Ala and STAT5b-Ser⁷³⁰Ala displayed approximately 2-fold higher GH- or PRL-stimulated transcriptional activity compared with wild-type STAT5b when assayed using an intact β -casein promoter-luciferase reporter. Finally, STAT5b-stimulated gene transcription was abolished in cells treated with H7, but in a manner unrelated to the inhibitory effects of H7 on STAT5b Ser⁷³⁰ phosphorylation. These findings suggest that the effects of STAT5b and STAT5a serine phosphorylation on STAT-stimulated gene transcription can be modulated by promoter context. Moreover, in the case of STAT5a, phosphorylation of serine 779, but not serine 725, may serve to regulate target gene transcriptional activity. (*Molecular Endocrinology* 15: 2157-2171, 2001)

SIGNAL TRANSDUCER AND activator of transcription (STAT) factors are signal transducers that mediate the effects of a broad range of hormones and cytokines on target gene expression (1, 2). STAT protein activation is catalyzed by a cell surface receptor-associated tyrosine kinase of the Janus kinase (JAK) family, which phosphorylates the STAT protein on a single C-terminal region tyrosine in response to hormone or cytokine stimulation. Tyrosine-phosphorylated STATs undergo rapid homo- or heterodimerization associated with STAT translocation to the

nucleus, where the dimeric, DNA-binding STAT activates target gene transcription. Dephosphorylation catalyzed by a phosphotyrosine-specific phosphatase (3) terminates STAT signaling and returns the inactivated STAT protein to the cytosol.

In addition to this JAK-catalyzed tyrosine phosphorylation reaction, STAT proteins may undergo serine phosphorylation in a manner that can be cell type and stimulus dependent (4-6). In the case of STAT1, -3, and -4, serine phosphorylation is directed at a conserved PMSP motif located about 20-30 amino acids C-terminal of the conserved tyrosine phosphorylation site. This phosphorylation can be stimulated by cytokine treatment and may be catalyzed by a downstream kinase, e.g. a cytokine- or growth factor-activated MAPK. Basal serine phosphorylation of STAT proteins has also been observed (for a review, see Ref. 7). Mutation of the conserved PMSP serine phosphorylation site (e.g. STAT1-Ser⁷²⁷, STAT3-Ser⁷²⁷, or STAT4-Ser⁷²¹) decreases cytokine-induced transcriptional activity, supporting the hypothesis that serine phosphorylation is required for maximal transcriptional ac-

Abbreviations: anti-pS730 antibody, Antibody specific for phosphoserine 730 of STAT5b and phosphoserine 725 of STAT5a; anti-pY699 antibody, antibody specific for tyrosine 699 or 694 of STAT5b and STAT5a, respectively; JAK, Janus kinase; PMSP motif, Pro-Met-Ser-Pro; PSP, Pro-Ser-Pro; STAT, signal transducer and activator of transcription; TST buffer, 10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM Na₂P₂O₇, 50 mM NaF, 1 mM Na₃VO₄, 1% Triton X-100, and 1 mM phenylmethylsulfonylfluoride.

Site-specific STAT5 mutants are designated using the single letter amino acid code; thus, STAT5b-S730A corresponds to STAT5b with Ser⁷³⁰ mutated to alanine.

tivity of these three STATs and may modulate cytokine responses (4, 8, 9), at least on some promoters (10). Serine phosphorylation can also negatively regulate cytokine-induced tyrosine phosphorylation in the case of STAT3 (11). STAT nuclear translocation and DNA-binding activity are generally not affected by STAT serine phosphorylation, suggesting that serine phosphorylation modulates STAT's intrinsic transcriptional potential, which is mediated by a C-terminal *trans*-activation domain just downstream of the serine phosphorylation site.

Serine phosphorylation of STAT5a and the closely related (>90% identical) STAT5b (12) has been observed in cells and tissues stimulated with STAT5-activating ligands such as GH (13–15), PRL (16, 17), and IL-2 (18). In PRL-stimulated cells, both STAT5 forms are phosphorylated on a conserved serine residue (STAT5a-Ser⁷²⁵ and STAT5b-Ser⁷³⁰) located within a PSP sequence, which corresponds in location to the PSMP serine phosphorylation sequence of STAT1, -3, and -4 (19). STAT5a is additionally phosphorylated at a second site, recently identified as serine 779 (20, 21). STAT5 serine phosphorylation may in part be mediated by the MAPK cascade, as suggested by binding interactions between STAT5a and the MAPKs ERK1 and ERK2 (21) and by the inhibition of constitutive, but not PRL-inducible, STAT5a serine 725 phosphorylation in Nb2 lymphocytes by the MAPK pathway inhibitor PD98059 (19). Functional studies of the effects of serine phosphorylation on STAT5's transcriptional activity have not provided a consistent picture. In the case of IL-2-activated STAT5 (STAT5a and/or STAT5b), cytokine-stimulated reporter gene activity is abolished in cells treated with the serine kinase inhibitor H7, which blocks STAT5 serine phosphorylation (18). Similarly, GH-activated STAT5a activity is blocked by inhibition of MAPK activity (22), which may play a role in STAT5a serine phosphorylation (21). In contrast, no difference in PRL-stimulated STAT5 reporter gene activity was seen when cells were transfected with serine to alanine mutant forms of STAT5b (Ser⁷³⁰ to Ala) or STAT5a (Ser⁷²⁵ mutated to Ala, Ser⁷⁷⁹ to Ala, or the Ser^{725,779}Ala double mutant) compared with the corresponding wild-type STAT5 forms (19, 20). Delayed tyrosine dephosphorylation was, however, reported for STAT5a-S725A in cells stimulated with PRL (20). These findings raise the possibility that the consequences of STAT5 serine phosphorylation may vary with the activating hormone or cytokine, or perhaps with the target gene used to evaluate the impact of STAT5 serine phosphorylation. These and related issues are investigated in the present study, where we evaluate the functional consequences of STAT5 serine phosphorylation in GH-stimulated cells using site-specific mutants of STAT5a and STAT5b. Our findings reveal that GH induces the same pattern of STAT5 serine phosphorylation as that previously reported for PRL. Moreover, we report that serine phosphorylation can modulate the transcriptional

activity of both STAT5a and STAT5b in a promoter-dependent manner.

RESULTS

Serine Phosphorylation of STAT5b in GH-Stimulated Cells

STAT5a and STAT5b carry out distinct functions in endocrine target tissues. STAT5a is the principal mediator of mammopoietic and lactogenic signaling stimulated by PRL, whereas STAT5b is an important determinant of sexual dimorphic liver gene expression induced by GH (23). To ascertain whether these distinctive biological roles of STAT5a and STAT5b, might in part reflect their differential serine phosphorylation in response to GH and PRL, we first investigated whether GH induces phosphorylation of STAT5b on the same serine residue (Ser⁷³⁰) as that reported previously for PRL (19).

Initial experiments were carried out using COS-1 cells transfected with GH receptor and either STAT5b or the site-specific mutant STAT5b-S730A and then stimulated with GH. Western blotting with anti-STAT5b antibody revealed multiple protein bands, which were previously identified as differentially phosphorylated forms of STAT5b (Fig. 1A). STAT5b band 0 (Fig. 1A) migrates as a doublet of bands, neither of which appears to be phosphorylated, as shown previously by phosphatase treatment experiments, whereas STAT5b band 1a corresponds to serine-phosphorylated STAT5b (14). This latter conclusion is supported by the absence of STAT5b band 1a in unstimulated cells transfected with STAT5b-S730A (Fig. 1A, lane 3 vs. lane 1). STAT5b band 2, previously identified as STAT5b phosphorylated on both tyrosine and serine, is the major GH-induced phosphorylated form of wild-type STAT5b (lane 2). In contrast, STAT5b-S730A was converted to a doublet of proteins after GH treatment (Fig. 1A, lane 4 vs. lane 3). Both bands of the doublet were phosphorylated on tyrosine, as shown by immunoprecipitation with anti-STAT5b antibody followed by antiphosphotyrosine 4G10 Western blotting (data not shown; also see below). The lower band corresponds in mobility to STAT5b phosphorylated on tyrosine alone, *i.e.* STAT5b band 1, whereas the upper band of the doublet remains unidentified (band X). The STAT5b doublet dominates in STAT5b-S730A-transfected cells treated with GH and is not further converted to the doubly phosphorylated STAT5b band 2, presumably because of the block in the secondary serine phosphorylation on residue 730 (see below). The precise relationship between STAT5b-S730A bands 1 and band X is uncertain. The two proteins may correspond to the tyrosine-phosphorylated counterparts of the two marginally resolved STAT5b protein forms seen in unstimulated cells (both designated band 0; Fig. 1A, lanes 1 and 3).

Western blotting using phospho-STAT5-specific antibodies (anti-pS730-STAT5b and anti-pY699-STAT5b) further supported these STAT5 band identifications (Fig. 1A, lanes 5–9). Thus, STAT5b was basally phosphorylated on Ser⁷³⁰ (band 1a; Fig. 1, *middle panel*, lane 6), and GH stimulated the formation of STAT5b phosphorylated on both Ser⁷³⁰ and Tyr⁶⁹⁹ (band 2; Fig. 1, lane 7). STAT5b Ser⁷³⁰ phosphorylation was blocked in cells transfected with STAT5b-S730A (Fig. 1, lanes 8 and 9, *middle panel*), whereas GH stimulated Tyr⁶⁹⁹ phosphorylation of STAT5b-S730A to form a band that migrated just below band 2 (band 1/band X; Fig. 1, *upper panel*, lane 9). [Of note, STAT5b bands 0, 1 and X are poorly resolved in the *right panel* of Fig. 1A (*c.f.* lanes 1 and 4).]

We next investigated whether GH induces phosphorylation of STAT5b on Ser⁷³⁰, *e.g.* via a GH-stimulated serine kinase, and whether phospho-Ser⁷³⁰-STAT5b serves as a substrate for the GH-stimulated tyrosine phosphorylation reaction. These studies were carried out using the GH-responsive liver cell line CWSV-1, where all the components required for GH-induced STAT5b tyrosine and serine phosphorylation are expressed endogenously (14, 24). CWSV-1 cells were stimulated with GH for varying periods of time, and cell extracts were prepared and analyzed by immunoprecipitation with anti-STAT5b antibody, followed by sequential probing with the antibodies shown in Fig. 1B. As reported previously (14), STAT5b is basally phosphorylated on serine in CWSV-1 cells (*c.f.* presence of STAT5b band 1a in unstimulated cells; Fig. 1B, lane 1, *lower panel*). At least a portion of this phosphorylation is on Ser⁷³⁰, as indicated by the reactivity of band 1a with phospho-Ser⁷³⁰-specific anti-STAT5 antibody (anti-pS730; *upper panel*). Moreover, GH-induced tyrosine phosphorylation of Ser⁷³⁰-phosphorylated STAT5b was readily detectable, as revealed by the appearance of the pS730-reactive STAT5b, band 2 (lane 2). The GH-stimulated increase in total pS730-STAT5b normalized to total STAT5b immunoreactivity (lane 2 vs. lane 1, *lower portion* of Fig. 1B) suggests that STAT5b Ser⁷³⁰ can be phosphorylated by a GH-stimulated serine kinase in addition to the basal Ser⁷³⁰ kinase activity. Both the basal and the GH-stimulated STAT5b Ser⁷³⁰ kinase activity are partially inhibited by the serine kinase inhibitor H7 (Fig. 1B, lanes 7 and 8 vs. lanes 1 and 2). This conclusion is supported by densitometric analysis of pS730-STAT5b immunoreactivity normalized to total STAT5b protein (*lower portion* of Fig. 1B). Moreover, the specific phospho-Ser⁷³⁰ content of STAT5b is seen to decline back to the basal level from its peak 30 min after GH stimulation.

We cannot determine from the above data whether the diphosphorylated STAT5b (band 2) is preferentially formed by GH-stimulated tyrosine phosphorylation of preexisting phospho-Ser⁷³⁰-STAT5b (band 1a) or by tyrosine phosphorylation of STAT5b band 0, followed by a secondary, GH-stimulated Ser⁷³⁰ phosphorylation of a STAT5b band 1 intermediate. Support for the latter possibility is provided by Western blot analysis of GH-stimulated CWSV-1 extracts using antibody specific for

STAT5b phosphotyrosine 699. Figure 1C shows that GH stimulates the transient formation of pY699-STAT5b at 5 min, followed by conversion to band 2 in an apparent serine phosphorylation reaction (lanes 3 and 4 vs. lane 2). In cells treated with H7, this secondary conversion to STAT5b band 2 is partially blocked. This finding supports the partial inhibition of GH-stimulated S730 phosphorylation shown in Fig. 1B (note the persistence in Fig. 1C of the pY699-STAT5b-immunoreactive doublet, even at 40–80 min; lanes 11–13 vs. single band at 20 min in lane 15 in the absence of H7). Densitometric analysis of the phospho-Tyr⁶⁹⁹ signals normalized to total STAT5b protein verified that H7 treatment slows down the decay in STAT5b signaling, as shown previously (24).

Transcriptional Activity of Site-Specific STAT5 Serine Mutants

To ascertain the functional significance of GH-stimulated STAT5 serine phosphorylation, serine to alanine mutations were introduced at the conserved PSP serine phosphorylation site of both STAT5 forms (STAT5a Ser⁷²⁵ and STAT5b Ser⁷³⁰). The effects of these site-specific mutations on GH-stimulated, STAT5-dependent gene transcription were evaluated in transfection experiments using a luciferase reporter gene driven by four copies of a STAT5-binding site derived from the promoter of the rat *ntcp* gene (25). These studies were carried out in the liver cell line HepG2, which has low endogenous GH receptor and STAT5, but serves as a useful model for STAT reporter gene studies and for expression of GH-regulated liver promoters (25, 26). Wild-type STAT5a and wild-type STAT5b *trans*-activated *ntcp* promoter activity in these cells by 32- to 40-fold after GH stimulation (Fig. 2A). STAT5a-Y694F and STAT5b-Y699F, which are mutated at the established STAT5 tyrosine phosphorylation site, were inactive, consistent with the absolute requirement of STAT5 tyrosine phosphorylation for activation of gene transcription. By contrast, mutation of the PSP serine phosphorylation site had a more subtle effect on STAT5-dependent transcriptional activity. No significant change in *ntcp* promoter activity was seen with STAT5a-S725A compared with wild-type STAT5a, whereas a substantial (~50%) reduction in activity was seen with the corresponding STAT5b-S730A mutant (Fig. 2A). This effect was observed in each of four independent HepG2 transfection experiments and was confirmed in a second cell model, transfected COS-1 cells (Table 1).

EMSA analysis verified that GH activated the DNA-binding activity of both STAT5b and STAT5b-S730A, as determined using a β -casein promoter STAT5-binding site probe. Similarly, GH activated STAT5a and STAT5a-S725A DNA-binding activity (Fig. 2B). By contrast, mutation of the STAT5 tyrosine phosphorylation site abolished STAT5 DNA-binding activity, as anticipated (Fig. 2B, lanes 4 and 10). We conclude that phosphorylation of STAT5b at Ser⁷³⁰ is not required for DNA-binding activity, but is required to achieve full

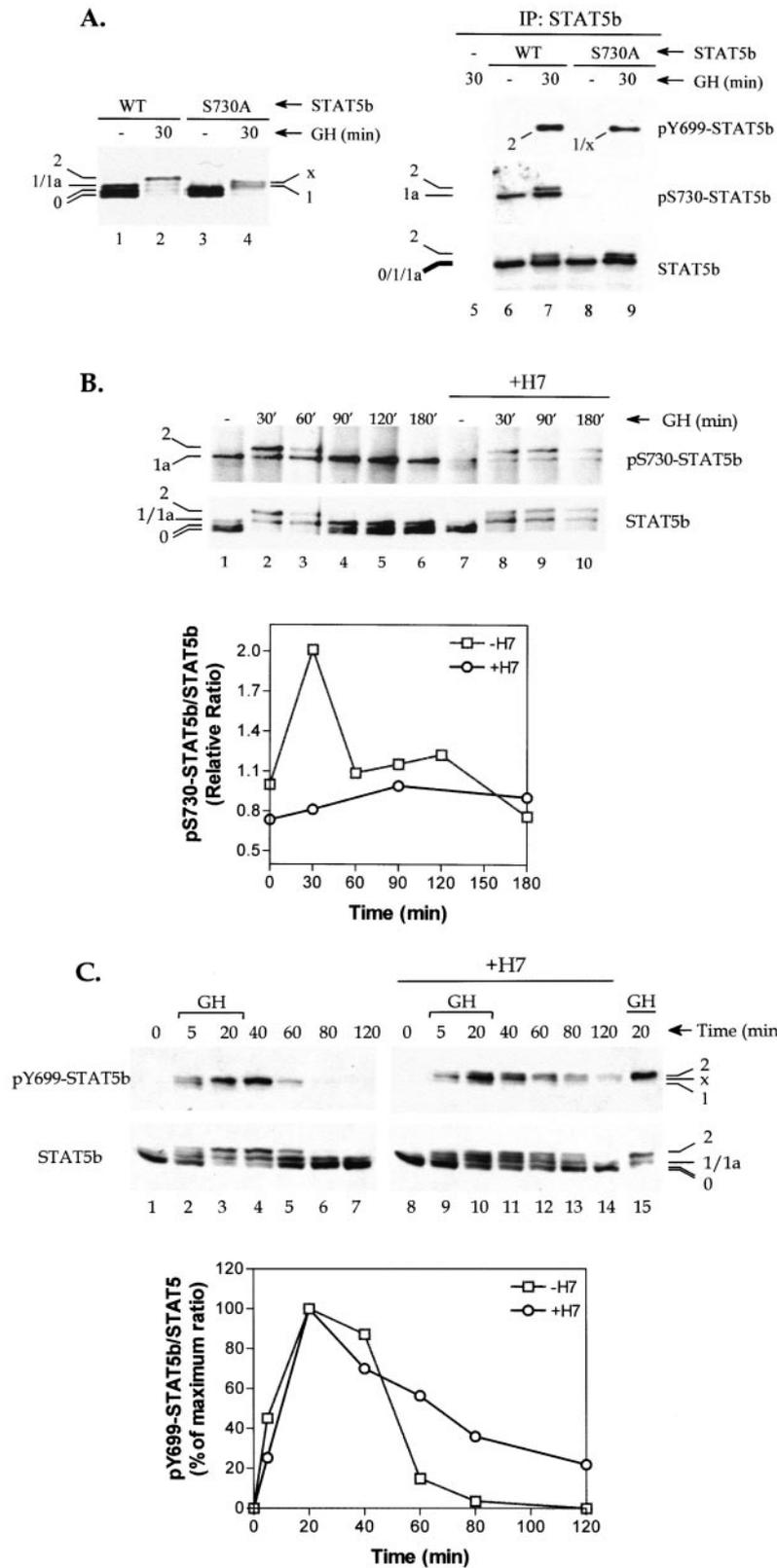


Fig. 1. GH-Stimulated Phosphorylation of STAT5b on Ser⁷³⁰ and Tyr⁶⁹⁹
 A, COS-1 cells were transiently transfected with wild-type (wt) STAT5b or STAT5b-S730A as indicated. Thirty-six hours later, cells were either treated with 200 ng/ml GH for 30 min or were untreated. Total cell extracts were analyzed on Western blots probed directly with anti-STAT5b antibody (lanes 1–4) or analyzed by immunoprecipitation with anti-STAT5b antibody followed

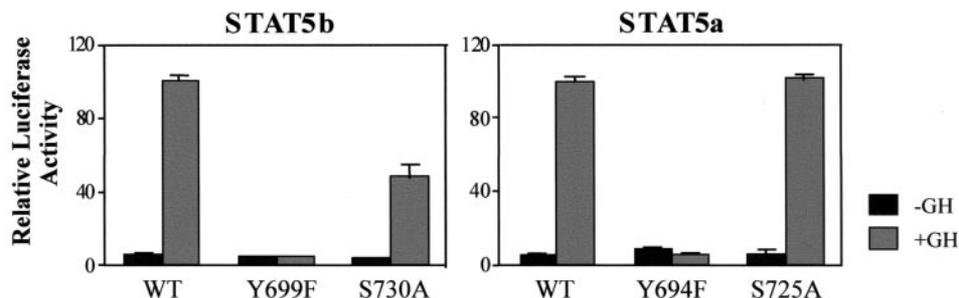
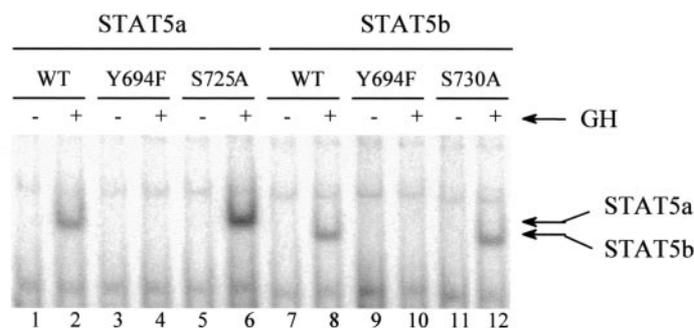
A: 4xNTCP-Luc**B: EMSA**

Fig. 2. Requirement of Ser⁷³⁰ Phosphorylation of STAT5b for Maximal Transcriptional Activation of *ntcp* Reporter Gene in HepG2 Cells

A, Expression plasmids (200 ng) encoding STAT5a, STAT5b, or the indicated Tyr to Phe or Ser to Ala STAT5 mutants were transfected into HepG2 cells together with the firefly luciferase reporter *4xNTCP-Luc* (200 ng). *pRL-tk-Luc* plasmid (*Renilla* luciferase) was used as an internal standard for transfection efficiency. Twenty-four hours later, the cells were either treated with GH for 18–20 h or were untreated. Total cell extracts were prepared, and relative luciferase activity was assayed as described in *Materials and Methods*. Data shown are the mean \pm SD ($n = 3$), with wild-type STAT5 expression plasmid activity set at 100. B, EMSA analysis showing gel-shift complex formed by STAT5a or STAT5b or the indicated STAT5 mutants using a β -casein probe. Samples were stimulated with GH overnight, followed by the addition of a fresh aliquot of GH 30 min before harvesting the cells for EMSA analysis. Samples were normalized for STAT5 protein content (determined by Western blotting), with the exception of the sample in lane 6, which had a high STAT5a protein content. STAT5b EMSA complexes migrate faster than STAT5a EMSA complexes, as shown previously (41).

by sequential Western blotting using anti-pS730-STAT5b and anti-pY699-STAT5b antibody (lanes 5–9). Band 0 represents nonphosphorylated STAT5b, bands 1 and 1a represent monophosphorylated forms of STAT5b (tyrosine and serine phosphorylated, respectively), and band 2 represents STAT5b phosphorylated on both tyrosine and serine. Band X was shown to be a tyrosine-phosphorylated form of STAT5b; its serine phosphorylation status could not be determined. STAT5b (and STAT5a) were not detected in untransfected COS-1 cells (lane 5). STAT5b bands 0, 1, and X were poorly resolved in the gel shown in lanes 5–9. Protein recovery was low in the samples shown in lanes 2 and 4. B, CWSV-1 cells were treated with 200 μ M H7 beginning 1 h before GH treatment. Cells were then stimulated with GH in the continued presence of H7 for the times indicated (lanes 7–10). Control CWSV-1 cells were treated with GH in the absence of H7 (lanes 1–6). Total cell extracts were analyzed by immunoprecipitation with anti-STAT5b antibody, followed by sequential Western blotting with anti-pS730 STAT5 and anti-STAT5b antibodies. C, Control CWSV-1 cells (lanes 1–7 and 15), or cells pretreated with H7 for 1 h (lanes 8–14) were stimulated with GH for 20 min. Incubation in the absence of GH (but in the continued presence of H7) was continued up to 120 min. Shown is a Western blot of cell extracts, probed sequentially with anti-pY699-STAT5b and anti-STAT5b antibodies. In view of the low STAT5b protein recovery in several of the samples in B and C, the specific pSer⁷³⁰ content and the specific pTyr⁶⁹⁹ content of STAT5b (pS730-STAT5b or pY699-STAT5b normalized to total STAT5b protein) were determined by densitometric quantitation using ImageQuant software. These normalized data are graphed as a function of time of GH stimulation at the *bottom* of B and C, respectively. The basal pSer⁷³⁰ level in the absence of GH was set at 1.0 in B, and the maximal pY699-STAT5b signals at 20 min were set at 1.0 in C.

Table 1. Effect of STAT5 Ser to Ala Mutations on 4x-*ntcp*-Luciferase Reporter Gene Activity

	Fold Activation (n = 3–4; mean ± sd)	
	HepG2 cells	COS-1 cells
A. STAT5b		
WT	40 ± 7	11 ± 3
S730A	20 ± 6 ^a	6 ± 3 ^a
B. STAT5a		
WT	32 ± 12	38 ± 7
S725A	26 ± 9	29 ± 9
S779A	21 ± 4	27 ± 3
S(725,779)A	15 ± 4 ^b	12 ± 2 ^a

HepG2 and COS-1 cells were transfected with GH receptor and STAT5a, STAT5b, or the indicated STAT5 mutants. GH-stimulated *ntcp* reporter gene activity was assayed as described in *Materials and Methods*. Data shown are fold activation values (+GH/–GH) calculated from three or four independent sets of transfection experiments.

^a Significant differences from wild-type, $p < 0.05$.

^b Decrease in fold activation did not reach statistical significance ($p \leq 0.2$) compared with wild-type.

activation of *ntcp* promoter activity, as demonstrated in both HepG2 and COS-1 cells. In the case of STAT5a, phosphorylation of the corresponding Ser⁷²⁵ is not required for maximal transcriptional activity.

Role of STAT5a Ser⁷⁷⁹ Phosphorylation

In addition to Ser⁷²⁵ phosphorylation, STAT5a can be constitutively phosphorylated at a second site, identified as Ser⁷⁷⁹ (20, 21). To investigate the significance of Ser⁷⁷⁹ phosphorylation, we prepared and then assayed the transcriptional activity of STAT5a expression plasmids containing a serine to alanine mutation at position 779 alone, or serine to alanine mutations at positions 725 and 779 in combination. Figure 3A shows that GH-stimulated *ntcp* promoter activity was significantly lower in the case of the double serine mutant STAT5a construct in both HepG2 and COS-1 cells. Moreover, the fold stimulation of promoter activity was substantially decreased (32- to 38-fold for wild-type STAT5a vs. 12- to 15-fold for STAT5a-S725,779A; Table 1), despite the expression of the STAT5a mutant protein at a level at least as great as wild-type STAT5a (Fig. 3B, lanes 7–10 vs. lanes 3 and 4). A less dramatic decrease in transcriptional activity was seen in the case of STAT5a-S779A (Table 1), indicating that phosphorylation of STAT5a on Ser⁷⁷⁹ in the context of phosphorylation on Ser⁷²⁵ is required for full STAT5a transcriptional activity. Phospho-STAT5a analysis confirmed that mutation of the STAT5a serine phosphorylation sites did not alter tyrosine phosphorylation of STAT5a at Tyr⁶⁹⁹ (Fig. 3B, middle panel, lanes 8, 10, and 12 vs. lane 4). Moreover, mutation of Ser⁷⁷⁹ did not decrease phosphorylation of Ser⁷²⁵ (Fig. 3B, upper panel, lanes 9 and 10 vs. lanes 3 and 4).

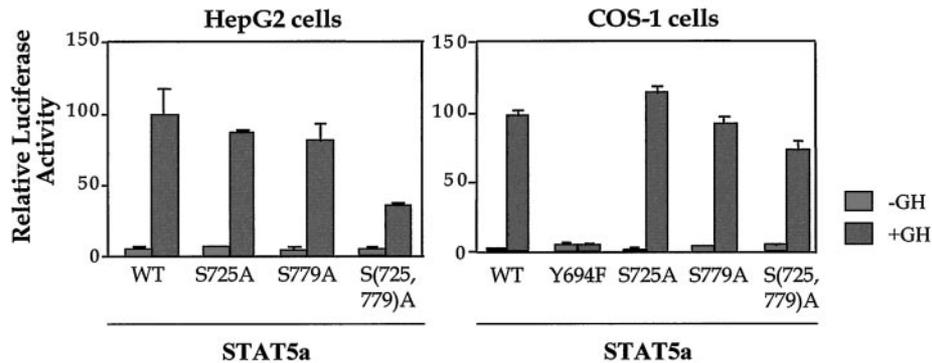
STAT5 serine phosphorylation can be stimulated by PRL and by a variety of other activators of cytokine receptor signaling pathways. To determine whether the consequences of STAT5 serine phosphorylation differ between receptors, we examined the effects of the STAT5 serine mutations on *ntcp* promoter activity in cells cotransfected with PRL receptor and treated with PRL. Figure 4A (left panel) shows that PRL activation of STAT5a or STAT5b led to a substantial increase in *ntcp* reporter activity. This gene activation was reduced in cells transfected with the serine-mutated STAT5 forms, STAT5a-S725,779A and STAT5b-S730A, just as it was in GH-stimulated cells.

Impact of STAT5 Serine Mutations on β -Casein Promoter Activity

We next investigated whether target gene promoter context may influence the effect of the STAT5 serine mutations on the STAT's transcriptional activity. For these experiments we used the reporter construct pZZ1 (27), which is comprised of 344 nucleotides of the rat β -casein proximal promoter linked to a luciferase reporter gene. This promoter contains a STAT5-binding site with the same core sequence (TTC-TTG-GAA) as the *ntcp* promoter STAT site used in all of the experiments presented above, but is flanked by a non-consensus STAT5 site; combined, the pZZ1 STAT sequence constitutes a strong tetrameric STAT5-binding site (28, 29). Figure 4A (right panel) shows that β -casein promoter activity is strongly activated in COS-1 cells transfected with PRL receptor together with either STAT5a or STAT5b and stimulated overnight with PRL. However, in sharp contrast to the reduced transcriptional activity obtained in parallel experiments with the *ntcp* promoter-based luciferase reporter (left panel), β -casein promoter transcriptional activity was increased to an approximately 2-fold higher level in cells transfected with either STAT5a-S725,779A or STAT5b-S730A compared with the corresponding wild-type STAT5 proteins. A similar stimulatory effect of the STAT5 serine mutations on β -casein promoter activity was observed in GH receptor-transfected COS-1 cells stimulated with GH (data not shown; also see below).

The differential effect of STAT5 serine mutation on *ntcp* vs. β -casein promoter activity shown in Fig. 4A suggests that the effects of a mutational block in STAT5 serine phosphorylation may be influenced by promoter context. To further investigate this finding, we examined the effects of the STAT5b S730A mutation on reporter gene activity in cells stimulated with GH for times ranging from 3–22 h. As shown in Fig. 4B, mutation of Ser⁷³⁰ to alanine led to a decrease in GH-stimulated *ntcp* reporter activity assayed at each time point. By contrast, this same mutation increased β -casein promoter activity when assayed 22 h after GH addition, as seen in Fig. 4A in the case of PRL stimulation, but had no significant impact when the cells were assayed 3, 5, or 8 h after GH addition (Fig.

A: 4xNTCP-Luc



B: Phospho-STAT5a

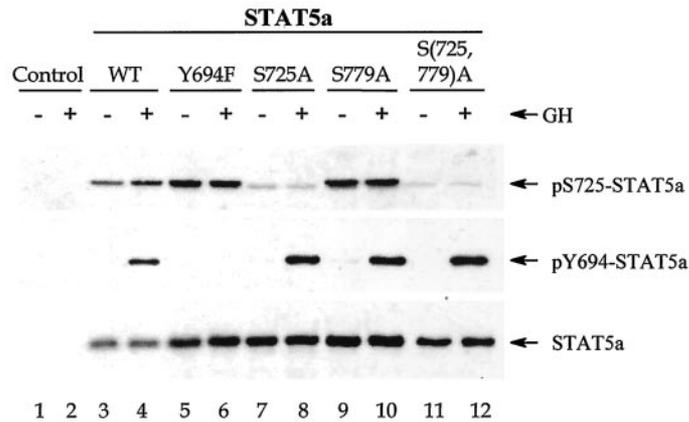


Fig. 3. Requirement of STAT5a Serine Residues 725 and 779 for Full Activation of *ntcp* Promoter Activity in COS-1 and HepG2 Cells

A, Relative luciferase activity was determined in cells transfected with the indicated STAT5a expression plasmids as described in Fig. 2. B, Shown is a STAT5a Western blot of COS-1 cell extracts transfected as described in A and treated with GH, as indicated. Cell extracts were immunoprecipitated with anti-STAT5a antibody followed by sequential Western blotting with anti-pS725-STAT5a, anti-pY694-STAT5a, and anti-STAT5a antibody. STAT5a was not detected in untransfected COS-1 cells (lanes 1 and 2). Anti-pS725-STAT5a cross-reacted with a minor band migrating just below pS725-STAT5a (c.f. upper blot of B, lanes 7, 8, 11, and 12).

4C). Analysis of extracts prepared from GH-stimulated cells revealed a higher level of Tyr⁶⁹⁹ phosphorylation in the STAT5b-S730A-transfected cells (Fig. 5A, upper panel, lanes 8–14 vs. lanes 1–7). Correspondingly, a higher DNA-binding activity was obtained at all time points examined (Fig. 5B). This increased activity of STAT5b-S730A largely reflects a higher level of STAT5b-S730A protein expression (Fig. 5A, lower panel, lanes 8–12 vs. lanes 1–5). Consequently, total cellular STAT5 DNA-binding activity is higher in the case of the STAT5b mutant (Fig. 5B), and as a result there is a differential in the DNA-binding activity found in STAT5b-transfected cells vs. STAT5b-S730A-transfected cells that is maximal 22 h after GH stimulation (Fig. 5C). This finding provides an explanation for the

increased *β-casein* promoter activity of STAT5b-S730A at this time point (c.f. Fig. 4C).

Inhibitory Effect of H7 on STAT5b Activity

The serine kinase inhibitor H7 has previously been used to support the proposed importance of STAT serine phosphorylation for STAT transcriptional activity (8, 18). We therefore investigated whether the reporter-dependent changes in STAT5b activity seen with STAT5b-S730A could also be observed in cells treated with H7, which partially inhibits this serine phosphorylation reaction (c.f. Fig. 1). Table 2 shows, however, that H7 treatment of COS-1 cells transfected with wild-type STAT5b leads to a complete inhibition

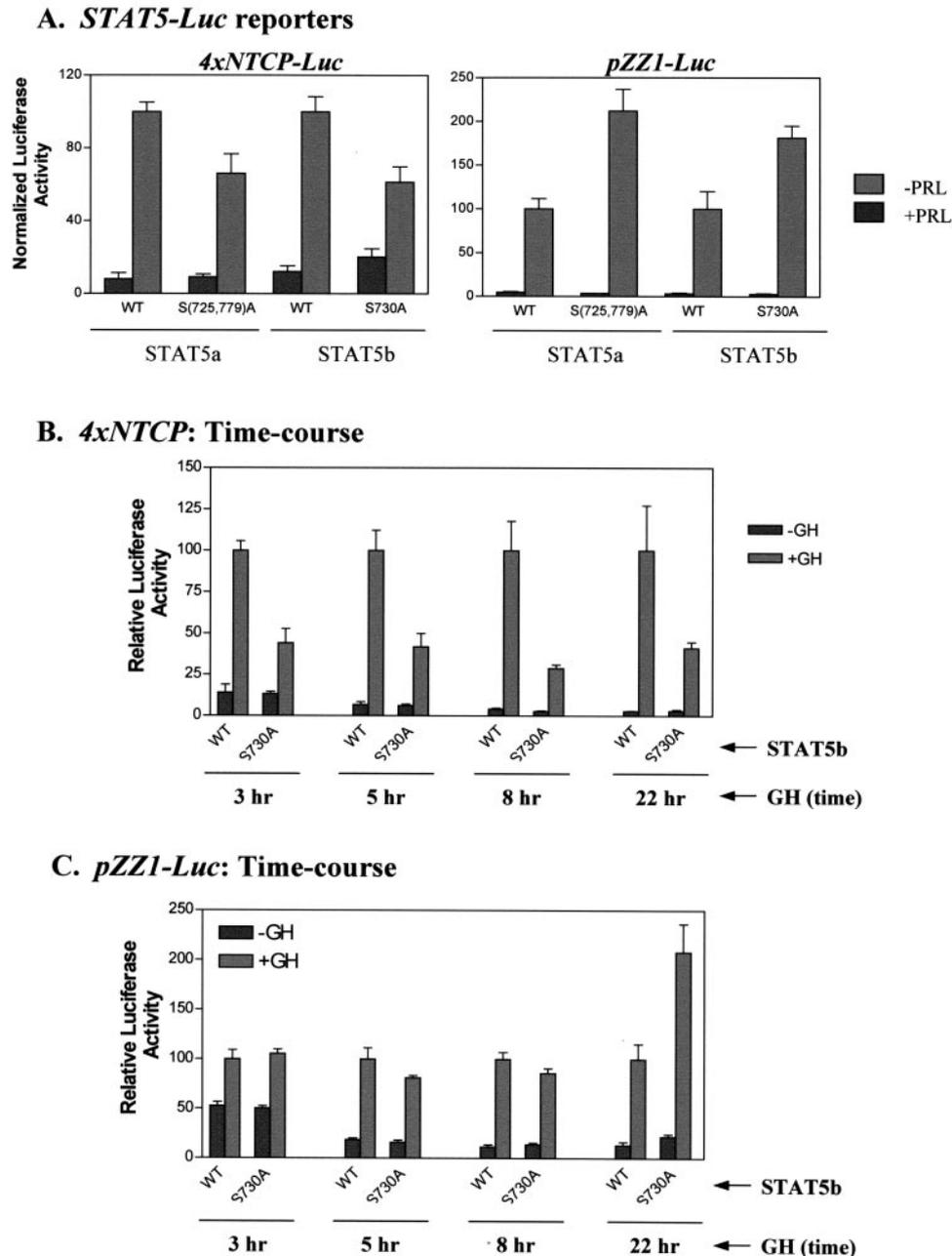
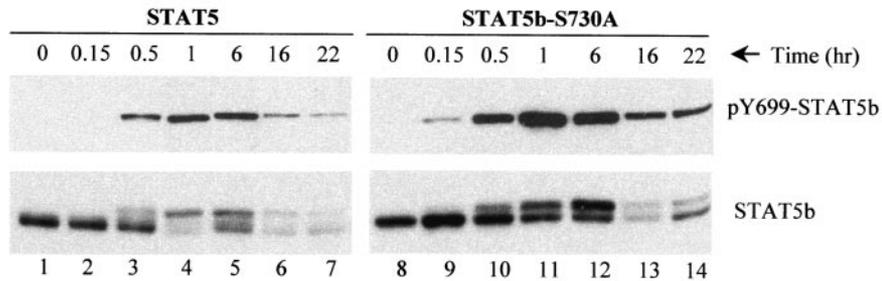


Fig. 4. Influence of STAT5 Serine Mutations on PRL- or GH-Stimulated STAT5 Activity Assayed with β -casein and *ntcp* Reporters
 STAT5 reporter plasmid, *4xNTCP-Luc* or *pZZ1-Luc*, was cotransfected in COS-1 cells with wild-type STAT5a or STAT5b or the indicated Ser to Ala mutated STAT5 expression plasmid. Cells were transfected with PRL receptor for PRL-activated reporter activity (A) or with GH receptor for GH-activated reporter activity (B) and the internal control plasmid pRL-tk-Luc. Twenty-four hours after transfection, cells were stimulated with 10 nM PRL for 18–20 h (A). For the time-course study (B and C), the cells were stimulated with GH for times ranging from 3–22 h. For all three panels, relative luciferase activities were then determined as described in Fig. 2. Values shown are the mean \pm SD ($n = 3$).

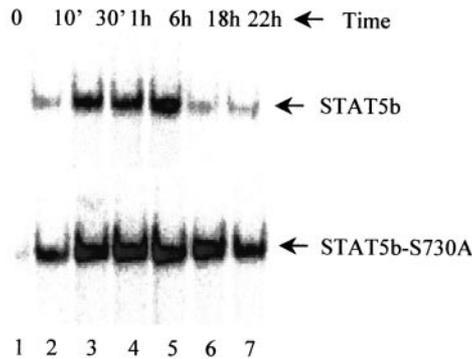
of GH-stimulated, STAT5b-dependent transcription of the *ntcp*-luciferase reporter gene, in contrast to the 50% activity decrease seen with the STAT5b-S730A mutant. Moreover, H7 treatment led to a similar inhibition of the transcriptional activity of STAT5b-S730A, demonstrating that the inhibitory action of H7 is unrelated to its effects on STAT5b serine phosphorylation.

Complete inhibition of STAT5b and STAT5b-S730A-stimulated transcription was also observed using the pZZ1 reporter (Table 2). Finally, a similar inhibitory effect of H7 on STAT5b-S730A transcriptional activity was obtained in cells stimulated with GH for a shorter time period (3 h; *c.f.* 6 h GH stimulation in Fig. 5A; data not shown).

A. WB analysis



B. EMSA analysis using β -casein probe



C. STAT5-DNA Complex

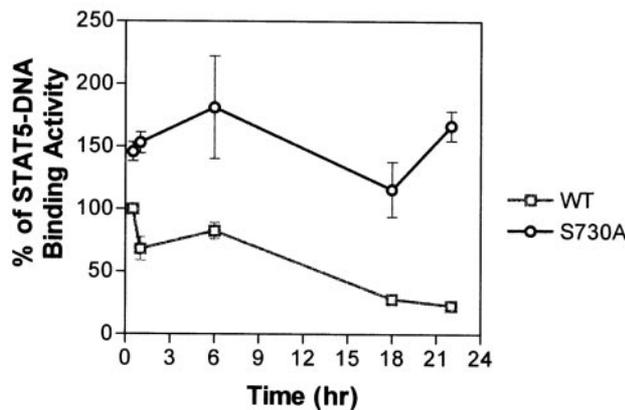


Fig. 5. Mutation of STAT5b Ser⁷³⁰ Results in Higher Cellular STAT5b Protein Levels and DNA-Binding Activity
 COS-1 cells were transiently transfected with plasmids encoding GH receptor and either wild-type or S730A-mutated STAT5b. Thirty hours later the cells were treated with GH as indicated. Cell extracts were prepared and analyzed on Western blots probed with antibody to pY699-STAT5b and STAT5b protein (A) or on EMSA gels (β -casein probe; B). C, PhosphorImager quantitation of EMSA band intensities, with the data normalized to the EMSA signal observed 30 min after GH stimulation. Graphed are the mean \pm SD for data points from 30 min to 22 h of GH stimulation based on two independent experiments, one of which is shown in B. The 30-min data point for wild-type STAT5b is set at 100.

Mechanistic studies revealed that H7 did not block STAT5b tyrosine phosphorylation (Fig. 1C) or DNA-binding activity (data not shown). Moreover, H7 treatment did not impair nuclear translocation of STAT5b

protein in its tyrosine-phosphorylated form, as revealed by immunofluorescence microscopy using anti-STAT5b and anti-pY699-STAT5b antibodies (Fig. 6). We conclude that H7 inhibits STAT5b transcriptional

Table 2. Serine kinase inhibitor H7 fully inhibits STAT5 reporter activity

STAT5b	Relative Luciferase Activity							
	4xNTCP-Luc				pZZ1-Luc			
	-H7		+H7		-H7		+H7	
	-GH	+GH	-GH	+GH	-GH	+GH	-GH	+GH
WT	7 ± 1	100 ± 3	8 ± 2	7 ± 1	33 ± 9	100 ± 3	34 ± 5	38 ± 5
S730A	4 ± 1	60 ± 4	4 ± 1	4 ± 1	26 ± 7	67 ± 5	24 ± 1	25 ± 4

HepG2 cells were transfected with GH receptor and a STAT5 reporter (either 4xNTCP-Luc or pZZ1-Luc, as indicated) together with either wild-type STAT5b or STAT5b-S730A for 24 h. The cells were then pretreated with H7 for 1 h and stimulated with GH in the continued presence of H7, as indicated, for an additional 6 h. Relative luciferase reporter activity was then determined (mean ± SD; n = 3). GH-stimulated activity in the absence of GH was set at 100. The reduced transcriptional activity of STAT5b-S730A with pZZ1-Luc seen at 6 h in the absence of H7 in this experiment (relative activity, 67% of wild-type STAT5b) is consistent with the 5-h data point shown in Fig. 4C.

activity at a stage that is downstream of the STAT5b nuclear translocation and DNA-binding steps.

DISCUSSION

STAT5a can be constitutively phosphorylated on serine at two sites, Ser⁷²⁵ and Ser⁷⁷⁹, whereas STAT5b, which lacks the COOH-terminal peptide sequence corresponding to Ser⁷⁷⁹, can be phosphorylated at a single serine, Ser⁷³⁰, in a manner that is inducible by either PRL (19) or GH (this report). Whereas mutation of STAT5a Ser⁷²⁵ had no significant effect on STAT5 transcriptional activity, mutation of the corresponding STAT5b Ser⁷³⁰ is presently shown to modulate STAT5b's transcriptional activity. This modulation is manifest as a decrease in transcription driven by a promoter sequence containing four copies of a STAT5-binding site derived from the *ntcp* gene, but leads to a time-dependent increase in transcription from a proximal promoter fragment of the *β-casein* gene. This latter increase is associated with a higher cellular level of STAT5 DNA-binding activity in the case of the Ser⁷³⁰-mutated STAT5b. These findings indicate that the functional consequences of STAT5b serine phosphorylation can vary from one promoter to the next, suggesting that STAT5b serine phosphorylation may serve as a mechanism to differentially modulate the expression of STAT5 target genes. This promoter-dependent effect of STAT5b Ser⁷³⁰ phosphorylation appears to reflect a change in STAT5b's intrinsic transcriptional activity, insofar as the decrease in *ntcp*-luciferase reporter activity observed with Ser⁷³⁰-mutated STAT5b occurred in cells where there was an increase in STAT5 DNA-binding activity. Conceivably, serine phosphorylation may modulate interactions between STAT5b and other transcription factors bound to the same promoter, or perhaps may influence the recruitment of STAT-interacting coactivator and corepressor proteins (30, 31), which is likely to occur in a promoter-dependent manner. Indeed, interferon- γ -activated STAT1 interacts

with the nuclear factor minichromosome maintenance-5 in a phospho-Ser⁷²⁷-dependent manner (9). Finally, changes in the *trans*-activation potential of STAT5 can also be achieved by mutations elsewhere in the C-terminal region (e.g. increased activity of STAT5a-T757V) (32).

The increased DNA-binding activity of STAT5b-S730A compared with wild-type STAT5b appears to be due at least in part to increased expression and/or stability of the Ser⁷³⁰-mutated STAT5 protein. This raises the possibility that phosphorylation of Ser⁷³⁰ in the wild-type protein may enhance the turnover of STAT5b. Although the apparent increase in signaling of STAT5b-S730A at longer times of GH stimulation could additionally involve a decrease in the rate of STAT5b tyrosine dephosphorylation, as was suggested earlier for STAT5a mutated at serine 725 and/or 779 (20), this has not been established. Independent of the mechanism underlying this effect, the increased cellular DNA-binding activity of STAT5b-S730A compared with wild-type STAT5b appears to account for the enhanced *β-casein* reporter activity seen at longer times after GH stimulation. Nevertheless, *ntcp* reporter activity was decreased under these same conditions, highlighting the intrinsic differences in the effects of Ser⁷³⁰ mutation on transcription of the two STAT5-responsive reporter genes, discussed above.

Of the two STAT5a serine phosphorylation sites, residues 725 and 779, Ser⁷⁷⁹ appears to be more important for maximal *ntcp* promoter activity. This is supported by the decrease in *ntcp* reporter activity in cells transfected with STAT5a-S779A, but not in cells transfected with STAT5a-S725A. This decrease cannot be explained by an effect of the Ser⁷⁷⁹ mutation on the phosphorylation of STAT5a on Tyr⁶⁹⁴ or Ser⁷²⁵ (Fig. 3B). Both STAT5a serine residues are likely to be important, however, as suggested by the more substantial decrease in *ntcp* reporter activity displayed by the double mutant, STAT5a-S725,779A. Similarly, mutation of Ser⁷⁷⁹, alone or in combination with Ser⁷²⁵, led to an increase in *β-casein* promoter activity, a

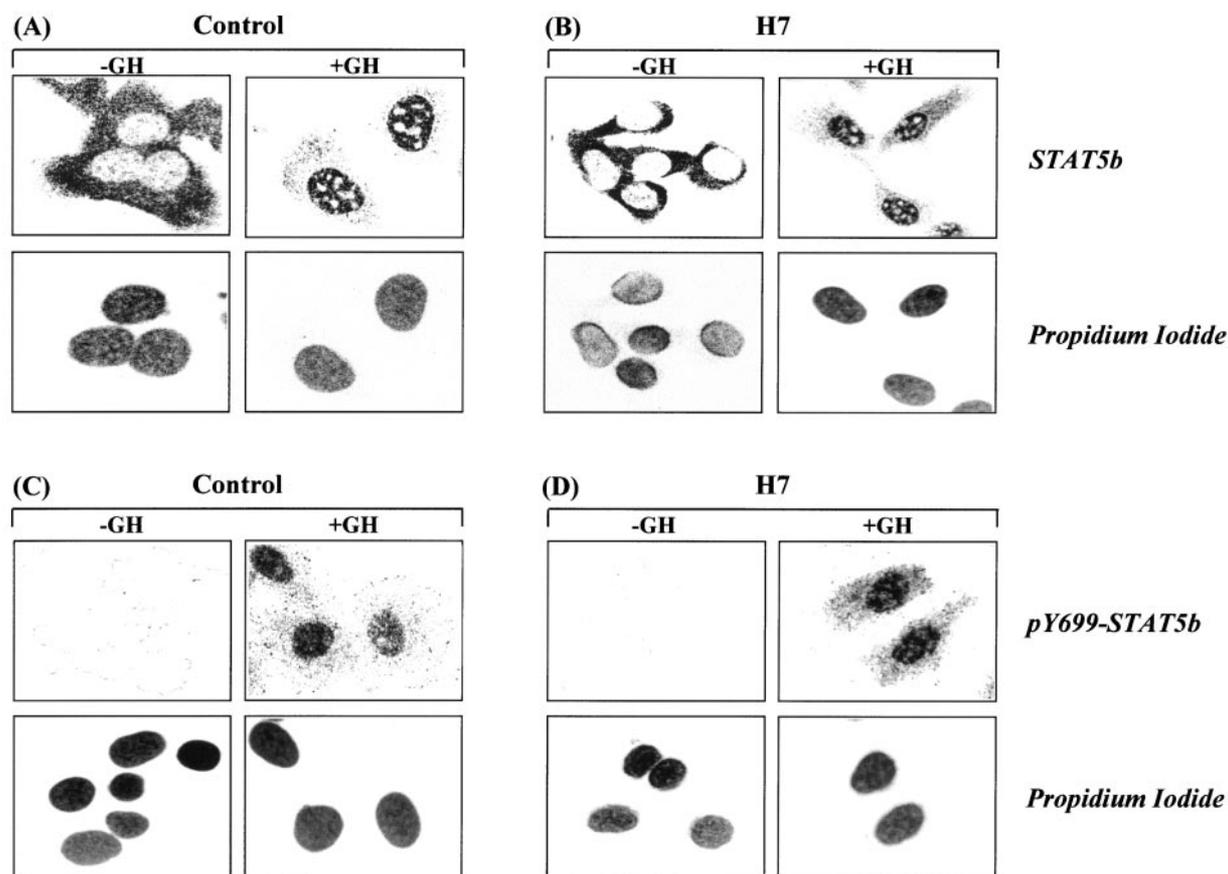


Fig. 6. Immunofluorescence Analysis of GH-Stimulated STAT5b: Effect of H7 on Nuclear Localization of STAT5b

Shown are confocal microscope images of STAT5b (*upper two sets of panels*) and anti-pY699-STAT5b indirect immunofluorescence (*lower two sets of panels*) indicating no effect of H7 treatment (B and D vs. A and C) on STAT5b nuclear translocation after GH stimulation. Control CWSV-1 cells or CWSV-1 cells pretreated with H7 for 1 h were untreated or were treated with GH for 30 min in the absence or presence of H7 as indicated. GH-treated cells were fixed and analyzed by immunofluorescence microscopy with anti-STAT5b antibody (A and B) or anti-pY699-STAT5b antibody (C and D). Propidium iodide was used to stain nuclei (*lower set of images in each panel*). STAT5b or pY699-STAT5b green fluorescence and propidium iodide red fluorescence images are presented as grayscale images prepared using Adobe Photoshop.

response that was also seen with STAT5b-S730A. In contrast to STAT5b Ser⁷³⁰, whose phosphorylation is inducible, STAT5a Ser⁷²⁵ and Ser⁷⁷⁹ can both be constitutively phosphorylated in a variety of cells and tissues, including developing mammary gland in the case of Ser⁷⁷⁹ (19, 20). It is unclear, however, whether both serine residues can be simultaneously phosphorylated in a given STAT5a molecule, leaving open the possibility that phosphorylation of Ser⁷²⁵ may inhibit, and thereby help regulate, Ser⁷⁷⁹ phosphorylation and the resultant phospho-Ser⁷⁷⁹-dependent transcriptional responses.

The stimulatory effects of the STAT5a-Ser⁷⁷⁹ and STAT5b-Ser⁷³⁰ mutations on GH-induced β -casein promoter activity seen in the present study contrast with the absence of a clear effect of these mutations in previous studies in PRL-stimulated cells (19, 20). This discrepancy does not reflect differences in the stimulating hormone, as we were able to duplicate our findings in experiments in which STAT5a and STAT5b

were activated via the PRL receptor. Rather, it may relate to differences in the time-dependent effects of the Ser⁷³⁰ mutation on β -casein promoter activity documented in the present study. Also of note, the two previous studies were carried out in COS-7 cells under conditions where only a 2- to 3-fold stimulation of β -casein promoter activity was achieved, which may limit or mask the modulatory effects of mutating the STAT5 serine phosphorylation sites. In contrast, the present studies were carried out in COS-1 cells under conditions where a 20- to 40-fold activation of the β -casein promoter was routinely achieved. Further study will be required to clarify this point.

The serine kinase inhibitor H7, which inhibits GH-stimulated STAT5b serine phosphorylation, strongly inhibited STAT5b-dependent reporter gene activity. A strong inhibitory action of H7 was also seen with STAT5b-S730A, independent of whether activity was assayed with the *ntcp* or β -casein promoter, indicating

that the transcriptional inhibition effected by H7 is unrelated to the resultant changes in STAT5b Ser⁷³⁰ phosphorylation. Mechanistic studies revealed that H7 does not interfere with GH-stimulated STAT5b tyrosine phosphorylation, nuclear translocation, or DNA-binding activity, strongly suggesting that H7 exerts a specific inhibitory action at the level of STAT5 *trans*-activation. This effect may thus be distinct from the prolonged signaling by the GH receptor-JAK2 complex after H7 treatment that we have previously described in GH-stimulated liver cells (24). Conceivably, H7 may inhibit STAT5-dependent transcription by altering the phosphorylation of a STAT5b-interacting coactivator that is required for the STAT transcriptional response. Alternatively, the inhibition by H7 of STAT5 transcriptional activity may be mechanistically linked to the prolonged signaling by GH receptor-JAK2 to STAT5b by way of a block in STAT5-stimulated transcription of feedback inhibitory regulators of GH receptor-JAK2 signaling, such as SOCS/CIS proteins (33, 34). Further investigation is needed to address this issue.

The kinase(s) that catalyze the constitutive phosphorylation of STAT5a on Ser⁷²⁵ and Ser⁷⁷⁹ and the signaling pathways that lead to the inducible phosphorylation of STAT5b on Ser⁷³⁰ in response to GH or PRL stimulation remain to be identified. Inhibitor studies suggest a role for a MAPK-like activity in the constitutive phosphorylation of STAT5a on Ser⁷²⁵, but not for the PRL-inducible phosphorylation of STAT5b on the corresponding Ser⁷³⁰ (19). Interestingly, in cells in which the constitutive phosphorylation of STAT5a Ser⁷²⁵ is blocked by the MAPK kinase inhibitor PD98059, PRL can induce phosphorylation at that site (19), demonstrating that Ser⁷²⁵ is intrinsically responsive to PRL stimulation, in a manner that is analogous to the inducible phosphorylation of Ser⁷³⁰ in the case of STAT5b. The additional site of STAT5a phosphorylation, at residue Ser⁷⁷⁹, is within the COOH-terminal 20 amino acids of STAT5a, where the two STAT5 proteins are highly divergent in sequence. This residue is thus absent from STAT5b. *In vitro* phosphorylation of STAT5a by MAPK is strongly inhibited by mutation of Ser⁷⁷⁹, as is the interaction of STAT5a with the MAPK ERK1 and ERK2 (21), suggesting a role for MAPK in this phosphorylation reaction as well. In other studies, carried out in a different cell model, phosphorylation of STAT5a at Ser⁷⁷⁹ was not blocked by inhibitors of MAPK or PI3K (20). Interestingly, Ser⁷⁷⁹ occurs within a sequence (RLSPPA) that corresponds to a consensus motif for phosphorylation by PKA, but not by nine other serine protein kinases, as revealed by computer analysis using the web-based PhosphoBase program (35). Accordingly, further investigation of the role of PKA/cAMP-dependent signaling pathways in the phosphorylation of STAT5a at this COOH-terminal site may be warranted.

STAT5a and STAT5b play distinct physiological roles in mediating hormonal responses to PRL (STAT5a) and GH (STAT5b) in the mammary gland and

liver, respectively (36, 37). Although this differential endocrine function may largely reflect the distinct tissue distributions of the two STAT5 forms, there is increasing evidence that the biological properties of STAT5a and STAT5b, although very similar, are distinguishable in several important ways. STAT5a and STAT5b not only display biochemical differences in apparent DNA binding specificity (38, 39) and propensity to bind to DNA as tetramers (STAT5a > STAT5b) (29, 40), but they exhibit potentially important differences in their regulation by serine phosphorylation. Thus, the phosphorylation of STAT5a vs. STAT5b on Ser^{725/730} is not only subject to differential regulation (constitutive phosphorylation of STAT5a vs. inducible phosphorylation of STAT5b), but leads to modulatory effects on gene transcription only in the case of STAT5b. In the case of STAT5a, such a modulatory effect requires phosphorylation on Ser⁷⁷⁹, a residue unique to this STAT5 form.

MATERIALS AND METHODS

Plasmids and Preparation of STAT5 Mutant Constructs

STAT5 plasmids containing site-specific mutations of serine to alanine (S725A and/or S779A for STAT5a, S730A for STAT5b) or tyrosine to phenylalanine (Y694F for STAT5a, Y699F for STAT5b) were prepared from double-stranded plasmid DNA using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and oligonucleotide primers designed to introduce each mutation, as described previously (19). Site-specific mutations were verified by DNA sequence analysis. Expression plasmids for mouse STAT5a and STAT5b (Dr. L. Hennighausen, NIH, Bethesda, MD), rat GH receptor (Dr. N. Billestrup, Hagedon Research Institute, Gentofte, Denmark), and human PRL receptor (Dr. P. Kelly, INSERM, Paris, France) were obtained from the indicated sources. Luciferase reporter constructs containing either four copies of a STAT5 binding site derived from the promoter of the rat *ntcp* gene (4×NTCP-Luc) or the β -casein gene promoter (nucleotides –344 to –1; pZZ1-Luc) were respectively provided by Drs. M. Vore (University of Kentucky, Lexington, KY) and B. Groner (Institute for Experimental Cancer Research, Freiburg, Germany).

Cell Culture and Transfections

COS-1 and HepG2 cells were maintained in DMEM containing 10% FBS, 50 U/ml penicillin, and 50 μ g/ml streptomycin. For transient transfections, cells were seeded in 24-well plates at a density of 1.3×10^5 HepG2 cells/well or 5×10^4 COS-1 cells/well. Fugene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used as described in the manufacturer's protocol, at a ratio of 1.3:1 of Fugene 6/DNA (vol/wt). Each well received a total of 600 ng DNA, including 150–200 ng luciferase reporter plasmid, 50 ng GH receptor, and 100–200 ng STAT5 expression plasmid. pRL-tk-Luc plasmid (*Renilla* luciferase; 50 ng DNA) was included as an internal control for transfection efficiency. Twenty-four hours after transfection, the cells were treated with rat GH (200 ng/ml) or rat PRL (10 nM) for an additional 18–24 h unless specified otherwise. H7 (200 μ M) was included as indicated. Total cell extracts were prepared using $1 \times$ lysis buffer (Promega Corp., Madison, WI) for measuring luciferase activities. For Western blot and EMSA analysis,

total cell lysates were centrifuged for 30 min at $15,000 \times g$. Firefly and *Renilla* luciferase activities were measured using a Dual Reporter Assay System (Promega Corp.) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Data shown in the individual figures are relative values based on normalized luciferase activity (*i.e.* firefly/*Renilla* luciferase activities; mean \pm SD for three replicates).

Growth and passage of CWSV-1 cells was carried out as described previously (14). For serine phosphorylation studies, CWSV-1 cells were stimulated with GH at 200 ng/ml in the presence or absence of H7 (200 μ M) for varying periods of time. Total cell extracts were prepared in lysis buffer containing 20 mM HEPES (pH 7.9); 1% Triton X-100; 1 mM each of EDTA, EGTA, Na_3VO_4 , $\text{Na}_2\text{P}_2\text{O}_7$, and dithiothreitol; 0.5 mM phenylmethylsulfonylfluoride; and 1 μ g/ml each of pepstatin, antipain, and leupeptin. Total cell extracts were passed through a 27-gauge needle seven times, adjusted to 150 mM NaCl, and centrifuged at $15,000 \times g$ for 30 min at 4 C. Protein concentrations were determined using the Dc detergent protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA).

EMSA Analysis

Total cell extracts (5 μ g) were assayed for STAT5 DNA-binding activity using a β -casein STAT5 response element probe (14). Gels were exposed to PhosphorImager plates overnight, followed by quantitation of radioactivity intensity using a Molecular Dynamics, Inc. PhosphorImager and ImageQuant software (Sunnyvale, CA).

Western Blotting and Immunoprecipitation

Total cell extracts (20–30 μ g) were electrophoresed on 7.5% Laemmli SDS gels, electrotransferred to nitrocellulose membranes, and then probed with anti-STAT5b antibodies (catalogue no. sc-835, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Blocking and probing conditions were described previously (41). Probing with anti-pY699-STAT5b antibody (Cell Signaling Technology, Beverly, MA) was performed with a 1-h incubation of the blot at room temperature in TST buffer [10 mM Tris-HCl (pH 7.6), 0.1% Tween 20, and 100 mM NaCl] containing 5% nonfat dry milk, followed by incubation with anti-pY699 antibody (1:1000 dilution) in 5% BSA-TST buffer overnight at 4 C. Washings were carried out as specified by the manufacturer. For STAT5 immunoprecipitation, CWSV-1 cells grown on 100-mm dishes were solubilized in 1 ml lysis buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM $\text{Na}_2\text{P}_2\text{O}_7$, 50 mM NaF, 1 mM Na_3VO_4 , 1% Triton X-100, and 1 mM phenylmethylsulfonylfluoride] in the presence of phosphatase inhibitors (14). Clarified total cell extracts were incubated for 2 h on ice with 2 μ l polyclonal rabbit anti-STAT5b antiserum (antibody raised against a peptide corresponding to amino acid residues 776–786 of mouse STAT5b was obtained from Dr. L. Hennighausen, NIH) (42). Immune complexes were captured with protein A-Sepharose beads (Pharmacia Biotech, Piscataway, NJ), electrophoresed on 7.5% Laemmli-SDS gels, and then transferred onto nitrocellulose membranes (Millipore Corp., Bedford, MA). Membranes were blocked for 1 h at 37 C with 5% nonfat dry milk in TST buffer. Incubations with site-specific antiphosphoserine STAT5 antibody (anti-pS730) (19) were carried out for 16 h at a dilution of 1:5000 in the cold-room. Anti-pS730-STAT5 antibody was raised against the phosphopeptide DQAP[pS]PAVC, corresponding to amino acid residues 726–734 of human STAT5b (19). Antibody binding was visualized on x-ray film by enhanced chemiluminescence using the ECL kit from Amersham Pharmacia Biotech (Arlington Heights, IL; anti-STAT5b and anti-pS730-STAT5b) or the SuperSignal ECL kit from Pierce Chemical Co. (Rockford, IL; anti-pY699-STAT5b).

Immunofluorescence Studies

CWSV-1 cells were seeded at about 60% confluence onto four-well chamber slides (catalogue no. 62409-294, VWR Scientific Products, Boston, MA) in RPCD medium (14) containing 3% FBS and allowed to adhere overnight. The medium was then replaced with serum-free RPCD medium. The following day, the cells were pretreated with H7 (200 μ M) for 1 h as indicated, then treated with GH (200 ng/ml) and H7 for 30 min. Cells were rinsed with ice-cold PBS and fixed with 100% MeOH for 20 min at -20 C. Fixed cells were blocked with 3% charcoal-stripped calf serum in PBS for 1 h at room temperature and then incubated with anti-STAT5b antibody (1:500 dilution; Santa Cruz Biotechnology, Inc.) in blocking solution overnight at room temperature. For anti-pY699-STAT5b immunostaining, fixed cells were blocked with 5.5% charcoal-stripped calf serum in TBST buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100] for 1 h at room temperature and then incubated for 24 h at 4 C with anti-pY699-STAT5b antibody (1:500 dilution; Cell Signaling Technology, Beverly, MA) in TBS buffer [50 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 3% BSA. The samples were then washed (three times, 5 min/wash) with PBS containing 3% calf serum for anti-STAT5b and with TBST for anti-pY699-STAT5b antibody. Cells were then incubated for 1 h at 37 C with fluorescein isothiocyanate-conjugated goat antirabbit IgG antibody (1 μ g/ml; Molecular Probes, Inc., Eugene, OR). Cells were counterstained with 50 ng/ml propidium iodide (Sigma) to localize nuclei. For confocal analysis, immunofluorescent cells were scanned with an BX-50 confocal laser scanning microscope (Olympus Corp., New Hyde Park, NY) equipped with a $\times 60$ objective (Carl Zeiss, New York, NY).

Acknowledgments

The authors thank Drs. L. Hennighausen, N. Billestrup, P. Kelly, M. Vore, B. Groner, and F. Lemaigre for providing plasmid DNAs and antibodies.

Received May 9, 2001. Accepted August 28, 2001.

Address requests for reprints to: Dr. David J. Waxman, Department of Biology, Boston University, Boston, Massachusetts 02215. E-mail: djw@bio.bu.edu.

This work was supported in part by NIH Grant DK-33765 (to D.J.W.).

REFERENCES

1. Darnell JEJ 1997 STATs and gene regulation. *Science* 277:1630–1635
2. Waxman DJ, Frank SJ 2000 Growth hormone action: signaling via a JAK/STAT-coupled receptor. In: Conn PM, Means A, eds. *Principles of molecular regulation*. Totowa: Humana Press; 55–83
3. Aoki N, Matsuda T 2000 A cytosolic protein-tyrosine phosphatase PTP1B specifically dephosphorylates and deactivates prolactin-activated STAT5a and STAT5b. *J Biol Chem* 275:39718–39726
4. Wen Z, Zhong Z, Darnell Jr JE 1995 Maximal activation of transcription by Stat 1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* 82:241–250
5. Boulton TG, Zhong Z, Wen Z, Darnell Jr JE, Stahl N, Yancopoulos GD 1995 STAT3 activation by cytokines utilizing gp130 and related transducers involves a secondary modification requiring an H7-sensitive kinase. *Proc Natl Acad Sci USA* 92:6915–6919
6. Ceresa BP, Pessin JE 1996 Insulin stimulates the serine phosphorylation of the signal transducer and activator of

- transcription (STAT3) isoform. *J Biol Chem* 271:12121-12124
7. Decker T, Kovarik P 2000 Serine phosphorylation of STATs. *Oncogene* 19:2628-2637
 8. Luttkicken C, Coffey P, Yuan J, Schwartz C, Caldenhoven E, Schindler C, Kruijjer W, Heinrich PC, Horn F 1995 Interleukin-6-induced serine phosphorylation of transcription factor APRF: evidence for a role in interleukin-6 target gene induction. *FEBS Lett* 360:137-143
 9. Zhang JJ, Zhao Y, Chait BT, Lathem WW, Ritz M, Knippers R, Darnell JE 1998 Ser⁷²⁷-dependent recruitment of MCM5 by Stat1 α in IFN- γ -induced transcriptional activation. *EMBO J* 17:6963-6971
 10. Kim H, Baumann H 1997 The carboxyl-terminal region of STAT3 controls gene induction by the mouse haptoglobin promoter. *J Biol Chem* 272:14571-14579
 11. Chung J, Uchida E, Grammer TC, Blenis J 1997 STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol Cell Biol* 17:6508-6516
 12. Grimley PM, Dong F, Rui H 1999 Stat5a and Stat5b: fraternal twins of signal transduction and transcriptional activation. *Cytokine Growth Factor Rev* 10:131-157
 13. Ram PA, Park SH, Choi HK, Waxman DJ 1996 Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. Differential kinetics of hormone desensitization and growth hormone stimulation of both tyrosine phosphorylation and serine/threonine phosphorylation. *J Biol Chem* 271:5929-5940
 14. Gebert CA, Park SH, Waxman DJ 1997 Regulation of STAT5b activation by the temporal pattern of growth hormone stimulation. *Mol Endocrinol* 11:400-414
 15. Dinerstein-Cali H, Ferrag F, Kayser C, Kelly PA, Postel-Vinay M 2000 Growth hormone (GH) induces the formation of protein complexes involving stat5, erk2, shc and serine phosphorylated proteins. *Mol Cell Endocrinol* 166:89-99
 16. Kirken RA, Malabarba MG, Xu J, Liu X, Farrar WL, Hennighausen L, Larner AC, Grimley PM, Rui H 1997 Prolactin stimulates serine/tyrosine phosphorylation and formation of heterocomplexes of multiple Stat5 isoforms in Nb2 lymphocytes. *J Biol Chem* 272:14098-14103
 17. Wartmann M, Cella N, Hofer P, Groner B, Liu X, Hennighausen L, Hynes NE 1996 Lactogenic hormone activation of Stat5 and transcription of the β -casein gene in mammary epithelial cells is independent of p42 ERK2 mitogen-activated protein kinase activity. *J Biol Chem* 271:31863-31868
 18. Beadling C, Ng J, Babbage JW, Cantrell DA 1996 Interleukin-2 activation of STAT5 requires the convergent action of tyrosine kinases and a serine/threonine kinase pathway distinct from the Raf1/ERK2 MAP kinase pathway. *EMBO J* 15:1902-1913
 19. Yamashita H, Xu J, Erwin RA, Farrar WL, Kirken RA, Rui H 1998 Differential control of the phosphorylation state of proline-juxtaposed serine residues Ser725 of Stat5a and Ser730 of Stat5b in prolactin-sensitive cells. *J Biol Chem* 273:30218-30224
 20. Beuvink I, Hess D, Flotow H, Hofsteenge J, Groner B, Hynes NE 2000 Stat5a serine phosphorylation. Serine 779 is constitutively phosphorylated in the mammary gland, and serine 725 phosphorylation influences prolactin-stimulated *in vitro* DNA binding activity. *J Biol Chem* 275:10247-10255
 21. Pircher TJ, Petersen H, Gustafsson JA, Haldosen LA 1999 Extracellular signal-regulated kinase (ERK) interacts with signal transducer and activator of transcription (STAT) 5a. *Mol Endocrinol* 13:555-565
 22. Pircher TJ, Flores-Morales A, Mui AL, Saltiel AR, Nordstedt G, Gustafsson JA, Haldosen LA 1997 Mitogen-activated protein kinase kinase inhibition decreases growth hormone stimulated transcription mediated by STAT5. *Mol Cell Endocrinol* 133:169-176
 23. Davey HW, Wilkins RJ, Waxman DJ 1999 STAT5 signaling in sexually dimorphic gene expression and growth patterns. *Am J Hum Genet* 65:959-965
 24. Gebert CA, Park SH, Waxman DJ 1999 Termination of growth hormone pulse-induced STAT5b signaling. *Mol Endocrinol* 13:38-56
 25. Ganguly TC, O'Brien ML, Karpen SJ, Hyde JF, Suchy FJ, Vore M 1997 Regulation of the rat liver sodium-dependent bile acid cotransporter gene by prolactin. Mediation of transcriptional activation by Stat5. *J Clin Invest* 99:2906-2914
 26. Delesque-Touchard N, Park SH, Waxman DJ 2000 Synergistic action of hepatocyte nuclear factors 3 and 6 on CYP2C12 gene expression and suppression by growth hormone-activated STAT5b. Proposed model for female specific expression of CYP2C12 in adult rat liver. *J Biol Chem* 275:34173-34182
 27. Gouilleux F, Wakao H, Mundt M, Groner B 1994 Prolactin induces phosphorylation of Tyr⁶⁹⁴ of Stat 5 (MGF), a prerequisite for DNA binding and induction of transcription. *EMBO J* 13:4361-4369
 28. Bergad PL, Shih HM, Towle HC, Schwarzenberg SJ, Berry SA 1995 Growth hormone induction of hepatic serine protease inhibitor 2.1 transcription is mediated by a Stat5-related factor binding synergistically to two γ -activated sites. *J Biol Chem* 270:24903-24910
 29. Soldaini E, John S, Moro S, Bollenbacher J, Schindler U, Leonard WJ 2000 DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. *Mol Cell Biol* 20:389-401
 30. Zhu M, John S, Berg M, Leonard WJ 1999 Functional association of Nmi with Stat5 and Stat1 in IL-2- and IFN γ -mediated signaling. *Cell* 96:121-130
 31. Pfitzner E, Jahne R, Wissler M, Stoecklin E, Groner B 1998 p300/CREB-binding protein enhances the prolactin-mediated transcriptional induction through direct interaction with the transactivation domain of Stat5, but does not participate in the Stat5-mediated suppression of the glucocorticoid response. *Mol Endocrinol* 12:1582-1593
 32. Gowri PM, Ganguly TC, Cao J, Devalaraja MN, Groner B, Vore M 2001 Conversion of threonine 757 to valine enhances stat5a transactivation potential. *J Biol Chem* 276:10485-10491
 33. Ram PA, Waxman DJ 1999 SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *J Biol Chem* 274:35553-35561
 34. Hansen JA, Lindberg K, Hilton DJ, Nielsen JH, Billestrup N 1999 Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. *Mol Endocrinol* 13:1832-1843
 35. Kreegipuu A, Blom N, Brunak S 1999 PhosphoBase, a database of phosphorylation sites: release 2.0. *Nucleic Acids Res* 27:237-239
 36. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev* 11:179-186
 37. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, Ram PA, Waxman DJ, Davey HW 1997 Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. *Proc Natl Acad Sci USA* 94:7239-7244
 38. Boucheron C, Dumon S, Santos SC, Moriggi R, Hennighausen L, Gisselbrecht S, Gouilleux F 1998 A single amino acid in the DNA binding regions of STAT5A and STAT5B confers distinct DNA binding specificities. *J Biol Chem* 273:33936-33941
 39. Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P 2001 DNA binding specificity of different STAT proteins. Comparison of *in vitro* specificity with natural target sites. *J Biol Chem* 276:6675-6688

40. Verdier F, Rabionet R, Gouilleux F, Beisenherz-Huss C, Varlet P, Muller O, Mayeux P, Lacombe C, Gisselbrecht S, Chretien S 1998 A sequence of the CIS gene promoter interacts preferentially with two associated STAT5A dimers: a distinct biochemical difference between STAT5A and STAT5B. *Mol Cell Biol* 18:5852–5860
41. Park SH, Liu X, Hennighausen L, Davey HW, Waxman DJ 1999 Distinctive roles of STAT5a and STAT5b in sexual dimorphism of hepatic P450 gene expression. Impact of Stat5a gene disruption. *J Biol Chem* 274: 7421–7430
42. Liu X, Robinson GW, Gouilleux F, Groner B, Hennighausen L 1995 Cloning and expression of Stat 5 and an additional homologue (Stat 5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc Natl Acad Sci USA* 92:8831–8835

2002 Prolactin Gordon Research Conference

The next Gordon Research Conference on Prolactin will be held in Ventura, California, from Jan. 27–Feb. 1, 2002. As has been the case for the past several years, we will also address similar issues for growth hormone. There are two highlighted presentations: The meeting begins with a debate on Sunday evening entitled, “Do prolactin and growth hormone cause cancer?” M. G. Rosenfeld will end the meeting with the Keynote Address on Thursday evening, speaking on “Genetic control of pituitary development.” Morning and evening sessions during the week will cover various aspects of PRL and GH secretion, receptors, and actions, including mouse models, microarray analysis and proteomics, PRL and GH physiology, pituitary development and regulation, agonists, antagonists and assays, effects in peripheral tissues, behavior and lifestyles, and PRL and GH signaling. There will also be eight Platform Presentations by graduate students, postdoctoral fellows, or junior faculty, who will present late-breaking, up-to-the-minute results from their submitted abstracts. The remaining abstracts will be presented in two poster sessions. For more information on the full program or GRC registration, check the Web site at <http://www.grc.org>.

Paul Kelly, Chair, 2002 Prolactin GRC
Kelly@necker.fr