Interaction of Growth Hormone-activated STATs with SH2-containing Phosphotyrosine Phosphatase SHP-1 and Nuclear JAK2 Tyrosine Kinase

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Growth hormone (GH) rapidly stimulates tyrosine phosphorylation followed by serine/threonine phosphorylation of multiple cytoplasmic STAT transcription factors, including one, STAT5b, that is uniquely responsive to the temporal pattern of plasma GH stimulation in rat liver and is proposed to play a central role in the activation of male-expressed liver genes by GH pulses in vivo (Waxman, D. J., Ram, P. A., Park, S. H., and Choi, H. K. (1995) J. Biol. Chem. 270, 13262–13270). We now show that JAK2, the GH receptor-associated tyrosine kinase, is present both in the cytosol and in the nucleus in cultured liver cells and in rat liver in vivo and that GH-activated STAT3 but not STAT5b becomes associated with nuclear JAK2. GH is also shown to activate by 3–4-fold SHP-1, a phosphotyrosine phosphatase that contains two src homology 2 (SH2) domains. GH also induces nuclear translocation and binding of SHP-1 to tyrosine-phosphorylated STAT5b, suggesting that this GH-activated phosphatase may play a role in dephosphorylation leading to deactivation of nuclear STAT5b following the termination of a plasma GH pulse in male rat liver in vivo. No such association of SHP-1 with GH-activated STAT3 was detected, a finding that could help explain the marked desensitization of STAT3, but not STAT5b, to subsequent GH pulses following an initial GH activation event.

Growth hormone (GH) regulates the transcription of a variety of genes that mediate its diverse effects on body growth and metabolic function. GH actions at the cellular level include direct mitogenic effects (1), insulin-like metabolic effects, and gene regulatory actions (2). In liver, a major target of GH action, GH exerts both stimulatory and inhibitory effects on the expression of a wide range of gene products, including hormone receptors, secretory products, and enzymes such as cytochrome P450 (3). An important early cellular response to GH is the rapid tyrosine phosphorylation of GH receptor at the cell surface catalyzed by JAK2, a Janus family protein tyrosine kinase (4). This leads to the activation by tyrosine phosphorylation of intracellular signaling molecules termed STATs, SH2 domain-containing latent cytoplasmic transcription factors (5). The initial GH-activated STAT tyrosine phosphorylation event is followed by a STAT serine or threonine phosphorylation reaction that modulates the DNA-binding activity of the GH-activated STATs (6). STAT tyrosine phosphorylation is associated with STAT homodimerization and translocation to the nucleus (5, 7), enabling the hormone-activated STATs to interact with DNA enhancer elements that have been identified in several GH-responsive promoters (8–10).

In liver, three distinct STAT proteins respond to GH, STATs 1, 3, and 5b (6, 11–13), albeit with distinct dependence on GH concentration and with a differential sensitivity to the temporal pattern of plasma GH stimulation (6). Moreover, whereas STAT5b can be repeatedly phosphorylated by the regular, repeated pulses of GH that characterize adult male rodents, STAT3 and STAT1 become desensitized with respect to GH-induced tyrosine phosphorylation following a single GH pulse in vivo in the hypophysectomized rat liver model (6). These differences between the STATs may, in part, relate to their distinct mechanisms of activation; STAT5b appears to be activated by JAK2 kinase after docking to one or more phosphotyrosine residues along GH receptor’s cytoplasmic tail, whereas STAT3 activation is less reliant on interactions between the STAT and GH receptor’s intracellular domain and may involve direct binding of STAT3 to tyrosine-phosphorylated residues on JAK2 kinase (14–16).

Although several components of the GH signaling pathway have thus been identified, the precise mechanisms whereby GH-induced intracellular signals are terminated remain to be determined. These signal termination events are of particular biological importance in the case of GH, given the need for GH target cells to respond to intermittent plasma GH pulses, which stimulate a male pattern of long bone and whole body growth in male rodents (17, 18). GH pulses also induce a pattern of liver gene transcription in male rat liver that is distinct from the one induced in female liver in response to continuous plasma GH activation (3, 19, 20). Of the GH-responsive STATs, STAT5b is uniquely responsive to the pulsatile pattern of plasma GH found specifically in adult male rats and is proposed to be a key intracellular mediator of the physiological effects of GH pulses on male liver gene expression (12). This hypothesis is given strong support by our recent finding that targeted disruption of the STAT5b gene in mice leads to complete loss of male-specific liver gene expression, in addition to the feminization of whole body growth rates, a second important GH pulse-regulated biological response.

Recent studies in CWSV-1 cells, a GH-responsive liver cell
model (21), have revealed that phosphotyrosine phosphatases (PTPs) play an important role in GH signal termination (22). Rapid deactivation of GH-activated STAT5b following cessation of a GH pulse was shown to involve STAT5b phosphotyrosine dephosphorylation as an essential first step. This phosphatase reaction is required to reset the GH receptor/JAK2/STAT5b signaling apparatus so that it may respond to subsequent rounds of GH pulse activation (22). However, the PTPs involved in this key regulatory step have not been identified. PTPs that contain SH2 (phosphotyrosine-binding) domains have been proposed to play a role in signaling from activated receptors belonging to the cytokine receptor superfamily (23), of which GH receptor is a member (24). The PTP designated SHP-1 (25) negatively regulates signaling by several cytokine and growth factor receptors, apparently by promoting dephosphorylation of the receptor’s intracellular domain and/or the receptor-associated JAK tyrosine kinase (25–27). A second SH2 domain PTP, SHP-2, acts as a positive signal transducer in several cytokine and growth factor receptor pathways, including prolactin receptor (28–31). In the present study, we examine the role of PTPs in regulating GH pulse-dependent activation of STAT5b. We find that GH activates SHP-1, induces its translocation to the nucleus, and stimulates its association with GH-activated, tyrosine-phosphorylated STAT5b. We also show that a significant portion of JAK2 tyrosine kinase is nuclear, rather than cytoplasmic in liver cells, and that this nuclear JAK2 binds specifically to GH-activated, tyrosine-phosphorylated STAT5b.

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**EXPERIMENTAL PROCEDURES**

**Antibodies**—Anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) was used for Western blotting, and anti-phosphotyrosine antibody PY20, agarse conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoprecipitation studies. Mouse monoclonal anti-STAT antibodies (Transduction Labs, Lexington KY) were as follows: anti-STAT3 (S21320) was raised to amino acids 1–178 of human STAT3 (anti-STAT6 (S21320) was raised to amino acids 451–640 of sheep STAT5; and anti-STAT5b (C-17) is a STAT5b-specific (22) rabbit polyclonal antibody raised to a COOH-terminal peptide (residues 711–727) of mouse STAT5b (sc-835, Santa Cruz Biotechnology). Rabbit anti-JAK2 antiserum, raised against amino acids 758–776 of murine JAK2, was purchased from Upstate Biotechnology (06-255) or was provided by Dr. Christin Carter-Su, University of Michigan Medical School, Ann Arbor. Antibodies to PTPs were purchased from Transduction Labs; anti-SHP-1 was raised to the COOH-terminal region (residues 492–597) of human SHP-1 (anti-PTP-1C, P-17320); anti-SHP-2 was raised to residues 1–178 of human SHP-2 (anti-PTP-1D, P17420), and anti-PTP-1B was raised to residues 269–435 of human PTP-1B (P18020). Anti-GST antibody was from Santa Cruz (sc-198).

**Animal Studies and Liver Nuclear Extracts**—Fischer 344 rats, pituitary-intact or hypophysectomized at 8 weeks of age, were purchased from Taconic Farms, Inc. (Germantown, NY). Completeness of hypophysectomy was confirmed by lack of weight gain over a 2–3-week period. Hypophysectomized animals received a single intraperitoneal injection of rat GH (NIDDK, rGH-B-14-SIAFP) or vehicle control and then killed at times ranging from 5 min to 4 h, as indicated in each experiment. Livers were excised, and nuclear protein extracts were prepared as described (12). Cytosolic fractions were isolated from the same liver samples using methods described elsewhere (12).

**Cell Culture and Whole Cell Extracts**—CWSV-1 cells, kindly provided by Dr. Harriet Isom, Pennsylvania State University (21), were grown in 100-mm tissue culture dishes and cultured at 37 °C in a 10% CO2 atmosphere in complete RPCD medium, essentially as described (22). The cells were then rinsed three times with ice-cold PBS and scraped in lysis buffer (50 mM Tris-Cl, pH 8.0, 10% glycerol, 1% (v/v) Triton X-100, 150 mM NaCl, 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidene, and 10 μM/ml aprotinin). Cell lysates were centrifuged at 12,000 × g for 20 min, and the supernatants were aliquoted and immediately frozen in liquid nitrogen.

**Immunoprecipitation and Western Blotting Analysis**—Rat liver nuclear extracts (50 μg) or whole cell extracts (100–200 μg) were incubated in 0.1–0.2 μl of Immunoprecipitation Buffer (20 mM HEPES, pH 8.0, 1% Nonidet P-40, 10% glycerol, 2.5 mM EGTA, 2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.05 mM sodium orthovanadate, 0.5 mM NaF, 2 mM benzamidene, 0.7 μg/ml pepstatin, 0.2 μg/ml aprotinin, 0.7 μg/ml leupeptin, 50 μg/ml antipain, 0.7 μg/ml o-phenanthroline) at 4 °C with shaking for 2–4 h using antibodies to either JAK2 (1/1000 dilution of Carter-Su antibody), SHP-1 (0.75 μg), or SHP-2 (0.75 μg) or with antibody PY20, agarse conjugate (3 μg of 50% slurry). Immunocomplexes were bound to protein A-agarose beads (25 μg of 50% slurry) or were collected directly (PY20 agarse conjugate) during a 1–2-h incubation at 4 °C and then were washed three times with Immunoprecipitation Buffer. Proteins were eluted by boiling for 5 min in 30 μl of 2 × Laemml SDS sample buffer (3% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 2% glycerol, 200 mM Tris-Cl, pH 6.8, containing pyronin Y dye). The Immunoprecipitates were analyzed on SDS gels, electrotransferred to nitrocellulose, and then probed with anti-phosphotyrosine antibody 4G10 (1/3000), anti-STAT 1/3000; except for anti-STAT5b at 1/1000, or anti-SHP-2 (1/5000) antibodies at the indicated dilutions. To reprobe blots, the membranes were submersed in stripping buffer (2% sodium dodecyl sulfate, 100 mM 2-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.7) and incubated at 50 °C for 30 min while shaking. The membranes were washed with TST (10 mM Tris-Cl, pH 7.4, 0.1 mM NaCl, 0.1% Tween 20) and then were reprobed with the desired antibody.

**Phosphotyrosine Phosphatase Assays**—CWSV-1 cell extracts (100 μg of protein) were incubated with 3 μg of anti-SHP-1 antibody for 3–4 h at 4 °C in Immunoprecipitation Buffer in a total volume of 100 μl. Protein A-Sepharose (25 μl of a 50% suspension) was added to each sample, followed by incubation for 1 h at 4 °C with shaking. Samples were centrifuged and washed six times with 10 mM Tris-HCl, pH 7.4, buffer containing 100 μM dithiothreitol. The washed beads were resuspended in 25 μl of 10 mM Tris-HCl, pH 7.4, buffer and incubated with 100 μl of tyrosine phosphopeptide, NH2-RRLIEDAEpYAAARG-COOH (pY-phosphotyrosine) for 30 min at 25 °C using reagents obtained from Upstate Biotechnology (kit 17-125). Control samples included immunoprecipitated SHP-1 incubated without peptide and complete reaction mixtures prepared in the absence of anti-SHP-1 antibody. The reaction was terminated by the addition of 25 μl of malachite green solution and then incubated for 15 min to allow for color development. Phosphatase activity was measured at 620 nm in a microtiter plate reader in comparison to a free phosphate standard curve (0.1–1.0 nmol of P;). The consistency of SHP-1 immunoprecipitation in each assay sample was then analyzed by Western immunoblotting using anti-SHP-1 antibody, as described above.

**GST-GH Receptor Fusion Protein Binding Assays**—Bacterial extract containing a fusion protein between glutathione S-transferase (GST) and amino acids 389–620 or amino acids 540–620 of the human GH receptor, GST-GHR (389–620) and GST-GHR (540–620), were prepared in the presence or absence of the Elk tyrosine kinase as described (14) and were generously provided by Dr. Stuart Frank (University of Alabama, Birmingham). The 389–620 fusion protein contains the six COOH-terminal tyrosine residues of the GH receptor. Bacterial extract (9–13 μl) containing GST-GH receptor fusion protein bound to glutathione-agarose beads was incubated with extracts from untreated or GH-treated CWSV-1 cells (80 μg of protein) prepared in fusion lysis buffer (1% Triton X-100, 150 mM NaCl, 10% glycerol, 50 mM Tris-HCl, pH 8.0, 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin). After incubation for 90 min at 4 °C, the beads were washed five times with fusion lysis buffer and then eluted in SDS sample buffer. The eluate was resolved on a 10% SDS gel and immunoblotted as described above. The consistency of immunoprecipitation of each fusion protein was verified by reprobing the blot with anti-GST antibodies (0.2 μg/ml). Control experiments included bacterial expressed GST alone (+ Elk kinase) to control for nonspecific interactions of GST with the glutathione-agarose beads used for the affinity precipitation step.

**Immunofluorescence Studies**—CWSV-1 cells were seeded onto glass coverslips in 35-mm Corning dishes containing RPCD culture medium with 5% fetal calf serum and allowed to adhere for 3–4 h. Cells were incubated overnight in RPCD medium without calf serum in a 5% CO2 incubator at 37 °C. The following day, cells were treated at 37 °C with...
or without rat GH (500 ng/ml) for the indicated times. After incubation, cells were rinsed twice with PBS prior to fixation. Cells were fixed with methanol for 10 min at −20 °C. Fixed cells were blocked with 3% calf serum in PBS at room temperature and then incubated for 45 min at 37 °C in 0.04 ml of 3% calf serum containing anti-IgG antiserum (Upstate Biotechnology, 1:500 dilution), anti-SHP-1 (1.25 μg/ml), or anti-STAT5b antibody (0.2 μg/ml). In control experiments, antiseras were preincubated with their cognate peptide antigens (Santa Cruz Biotechnology, Inc.) prior to immunostaining (ratio of 5 μg of JAK2 control peptide/1 μl of antiserum, or 10 μg of STAT5b control peptide/1 μg of antibody) for 4 h at 4 °C. Following four washes with 3% calf serum, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1 μg/ml) in 3% calf serum was added and incubated for 1 h at 37 °C. Cells were washed four times with PBS. In some cases, nuclei were stained with 4',6-diamidino-2-phenylindole (1 μg/ml in PBS) for 5 min at room temperature followed by two washes and then mounted on glass slides. To visualize the fluorescence, a Zeiss Axiovert TV-100 CCD Microscope equipped with a 16-bit output CCD camera (TE/CCD, −40 °C) and epifluorescence mode was used with XP2 filters (excitation/emission maxima at 485/530 nm). Images were analyzed using ImageTool software (version 1.27) obtained from the University of Texas Health Science Center, San Antonio.4

RESULTS
Liver Expression and Subcellular Localization of PTPs—To identify specific PTPs that may be involved in dephosphorylation of GH-activated STAT proteins, we first examined the liver expression and intracellular distribution of three phosphotyrosine-specific phosphatases. Immunoblotting analysis of subcellular extracts prepared from rat liver revealed that SHP-1, molecular mass of ~68 kDa, was predominantly localized to the nuclear fraction in adult rats. By contrast, little or no SHP-1 was detectable in the cytosol (Fig. 1A, lanes 1 and 2 versus 5 and 6) or in a membrane fraction (data not shown). Interestingly, while only a small amount of immunoreactive SHP-1 was found in nuclear extracts prepared from hypophysectomized rat livers (lane 3), the concentration of SHP-1 in the nucleus increased markedly within 15 min after GH injection (lane 4). This increase was associated with a decrease in the cytosolic concentration of SHP-1 (lane 7 versus 8).5 In contrast, SHP-2, mass of ~72 kDa, was expressed both in the cytosol and nuclear fractions of adult male, female, and hypophysectomized rats, and its distribution was not substantially changed following GH replacement (Fig. 1B). SHP-2 was present at a much lower level in a liver membrane fraction (data not shown). A third phosphatase, PTP-1B, mass of ~50 kDa, was present at a level too low to measure reliably in the liver samples using our antibodies (data not shown).

The response of SHP-1 to GH appears similar to the response of liver STAT5b, which also undergoes cytosol to nuclear translocation7 in GH pulse-treated hypophysectomized rats (Fig. 1C) (12). However, in contrast to STAT5b, which can be found at much higher levels in pituitary-intact male compared with female rat liver nuclear extracts owing to the activation and nuclear translocation of STAT5b in response to male plasma GH pulses, SHP-1 was constitutively present in pituitary-intact rat liver nuclear extracts in both males and females (A versus C, lanes 1 and 2). A second GH-responsive STAT protein, STAT3, was constitutively present in both the nuclear and cytosolic fractions in a manner similar to SHP-2. In the case of STAT3 but not SHP-1 or SHP-2, however, a lower mobility band corresponding to tyrosine + serine/threonine-phosphorylated STAT3 (6) accumulated in the nucleus following GH treatment (Fig. 1D, lane 4). STAT6 is only detected in the cytosolic fraction, even after GH treatment (Fig. 1E, lanes 5–8), suggesting that this liver-expressed STAT is not GH-responsive. During the course of these studies we also observed that the GH-activated tyrosine kinase JAK2 was greatly enriched in the nuclear fraction as compared with either the cytosol (Fig. 1F) or a liver membrane fraction (data not shown). This enrichment is comparable to that seen for SHP-1 and STAT5 in the same samples (A and C). This latter observation was unexpected, since JAK2 is widely presumed to be either cytosolic or membrane-associated via its interactions with cell surface-bound growth factor receptors, such as GH receptor.

GH Activation of STAT-1—SH2-domain containing phosphotyrosine-specific phosphatases, such as SHP-1 and SHP-2, have been implicated in both positive and negative regulatory functions in cytokine and growth factor signaling. In the case of SHP-1, phosphatase activity can be regulated by an autoinhibitory interaction of the protein’s amino-terminal SH2 domain with its phosphatase active site in a phosphotyrosine-independent manner (32). Interaction of this SH2 domain with a phosphotyrosine-containing peptide, such as the one which includes amino acid 479 of the COOH-terminal cytoplasmic tail of

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4 Web site address: http://dddx.utshs.ca/
5 Cytosolic STAT5 is readily detectable at higher protein loadings for the samples shown in Fig. 1C, lanes 6 and 7 (cf. Ref. 12). Similarly, cytosolic SHP-1, for the sample shown in lane 7, and cytosolic JAK2, for the samples shown in lanes 5–8, can more readily be seen at higher protein loading or on longer exposures of the Western blot. Comparisons between the cytosolic and nuclear fractions shown in Fig. 1 need to take into account the much larger volume of cytoplasm in the cell and the fact that the nuclear samples analyzed are partially purified nuclear extracts.

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**Fig. 1. Detection of PTPs, STATs, and JAK2 kinase in liver nuclear and cytosolic fractions.** Liver nuclear extracts and cytosolic fractions were isolated from normal male (M) and female (F) rats and from hypophysectomized (HX) male rats treated with rat GH (GH) at 12.5 μg/100 g body weight and then killed 15 min later. Proteins were separated on SDS gels (20 μg protein/lane), transferred to a nitrocellulose membrane, and then probed sequentially with antibodies to the signaling molecules indicated in A–F. Shown are portions of the Western blot after detection on x-ray film by enhanced chemiluminescence. The specificity of each signal was confirmed using Jurkat cell extracts as a positive control and/or by the consistency of its electrophoretic mobility with the reported molecular mass values of the corresponding antigen (SHP-1, 68 kDa; SHP-2, 72 kDa; STAT5, 93 kDa; STAT3, 89 kDa; STAT6, 100 kDa; JAK2, 120 kDa). Cytosolic concentrations of SHP-1, STAT5, and JAK2 are too low to detect when analyzed at the same protein load as the nuclear samples (cf. requirement of ~5 times higher protein loading to detect liver cytosolic STAT5 (12)). The anti-JAK2 antibodies used for these studies are routinely seen to cross-react with one or two lower molecular mass bands (~100 kDa) both in our studies (cf. Figs. 6B and 8B) and in those of others (26).
GH Activation of Phosphotyrosine Phosphatase SHP-1

**Fig. 2. Effect of GH on SHP-1-dependent phosphotyrosine phosphatase activity in CWSV-1 cells.** A1, CWSV-1 cells were treated with 500 ng/ml rat GH for up to 120 min, after which the cells were lysed and incubated with monoclonal antibody specific for SHP-1 for 2–4 h at 4 °C. The anti-SHP-1 immunoprecipitates were then analyzed for phosphotyrosine phosphatase activity (nmol of Pi released per 30 min assay) as described under “Experimental Procedures.” In control samples shown in the last bar, SHP-1 immunoprecipitates from 45-min GH-treated cells were assayed with the phosphatase inhibitor pervanadate (PV) (40 μM) added to the final assay mixture. Data shown are mean ± S.D. values for n = 2 to 6 independent determinations and were obtained after subtraction of background phosphate values obtained from complete reaction mixtures from GH-treated cells incubated with malachite green in the absence of the phosphotyrosine peptide substrate. No activity was observed in samples prepared in the absence of SHP-1 antibody. STAT5b Western blot analysis of the SHP-1 immunoprecipitates demonstrated the presence of equivalent SHP-1 protein at 20 and 45 min after GH addition compared with unstimulated cells (Fig. 2B, lanes 1–5). This indicates that the increase in enzyme activity results from GH activation of pre-existing SHP-1 enzyme, rather than new SHP-1 protein synthesis. Similarly, the decline of SHP-1 activity to basal levels by 2 h (Fig. 2A1) is not the result of SHP-1 protein degradation (cf., Fig. 2B, lanes 6 and 7).

Repeat pulses of GH lead to repeat cycles of STAT5b activation and deactivation, followed by STAT5b reactivation (22). We therefore tested whether GH pulse treatment similarly leads to repeat cycles of SHP-1 activation and deactivation. CWSV-1 cells were treated with a pulse of GH, which stimulated an increase in SHP-1 activity that was maximal at 45 min and decayed back to basal levels by 60 min (Fig. 2C) (cf., Fig. 2A1). The cells were then washed to remove GH, incubated for 3 h to allow for maximal resetting of the GH/JAK/STAT5b pathway (22), and a second pulse of GH was then given. Enzymatic analysis of SHP-1 immunoprecipitates revealed a second cycle of SHP-1 activation and deactivation, essentially comparable to the first (Fig. 2C).

**GH-induced Association of Tyrosine-phosphorylated STAT5b with SHP-1—**The kinetics SHP-1 activation by GH in CWSV-1 cells (Fig. 2A) are similar to the kinetics of STAT5b tyrosine phosphorylation in these cells (Fig. 3A). This suggests that the activation of SHP-1 may result from a direct interaction of SHP-1’s amino-terminal SH2 domain with phosphotyrosine residue 694 of STAT5b, by analogy to the activation of SHP-1 upon binding to a COOH-terminal phosphotyrosine residue on the erythropoietin receptor (33). We therefore examined whether a binding interaction between SHP-1 and STAT5b could be detected. CWSV-1 cells were stimulated with GH for various periods, and whole cell extracts were immunoprecipitated with anti-phosphotyrosine antibody PY20 or with anti-SHP-1 antibody. STAT5b Western blot analysis of the anti-phosphotyrosine immunoprecipitates revealed a marked increase in the tyrosine phosphorylation of STAT5b, which was maximal at 45 min and returned to basal levels within 120 min.
Samples were analyzed on Western blots probed with anti-STAT5b antibody. Co-immunoprecipitation of STAT5b (Fig. 3A) or with anti-SHP-1 antibody (B). Samples were analyzed on Western blots probed with anti-STAT5b antibody. Activated STAT5b associates with SHP-1 in GH-stimulated cells (B1, lanes 5–7). Reprobing the blot shown in B1 with anti-SHP-1 indicates equal recovery of SHP-1 protein in each immunoprecipitate (B2, bottom panel). (Note electrophoretic artifact in B2, lane 2.)

(Fig. 3A), in agreement with our earlier studies (22). Immunoprecipitation with anti-SHP-1 antibody revealed a GH-dependent co-immunoprecipitation of STAT5b (Fig. 3B1). Of note, the association of STAT5b with SHP-1 (Fig. 3 B1) paralleled the tyrosine phosphorylation of STAT5b (Fig. 3A). Moreover, the major STAT5b protein revealed by these analyses comigrated with tyrosine + serine/threonine di-phosphorylated STAT5b (band 2, Ref. 22; data not shown). Thus, STAT5b that is not phosphorylated (e.g., STAT5b in CWSV-1 cell extracts earlier than 5 min after GH treatment) does not stably interact with SHP-1 (Fig. 3, lanes 2–4). Reprobing of the anti-SHP-1 immunoprecipitates with anti-STAT3 antibody indicated that STAT3 is not associated with SHP-1 in the GH-induced cells (data not shown). Moreover, in similar experiments employing GH-stimulated CWSV-1 cell extracts and anti-SHP-2 immunoprecipitation, no association of SHP-2 with either STAT5b or STAT3 could be detected. These latter findings highlight the specific nature of the SHP-1-STAT5b binding interaction.

Cytokine and growth factor-induced tyrosine phosphorylation of SHP-1 have been observed in several systems (29, 31, 34, 35). In the case of GH-treated CWSV-1 cells, however, no GH-induced protein mobility shift (characteristic of many phosphorylated proteins, e.g., Fig. 1D, lane 4 for STAT 3) was seen on SHP-1 Western blots (e.g., Fig. 2B and Fig. 3B2). In addition, SHP-1 could not be precipitated by anti-phosphotyrosine antibody PY20 in GH-activated cell extracts (data not shown). These findings suggest that SHP-1 does not become tyrosine-phosphorylated in liver cells following GH treatment.

The GH-induced association of STAT5b with SHP-1 was confirmed in studies carried out in rat liver in vivo. Fig. 4 shows an anti-SHP-1 immunoprecipitation analysis of liver nuclear extracts prepared following a single GH pulse given to hypophysectomized rats. GH stimulated the binding of STAT5b to nuclear SHP-1 at 45 min, but this interaction was fully reversed by 4 h (A and B, lanes 3–5). This latter time corresponds to a point when STAT5b is largely deactivated and depleted from the nucleus (12). GH-induced accumulation of SHP-1 in the nucleus was also seen both at 5 min and at 45 min (Fig. 4B, lanes 2 and 3), in agreement with our findings in Fig. 1A. Interestingly, SHP-1 was fully maintained in the nucleus 4 h after the GH pulse (Fig. 4B, lanes 4 and 5), despite the loss of STAT5b from the nucleus at this time (cf., Fig. 4A). Thus, retention of SHP-1 within the nucleus over a 4-h time period is not dependent on the continued presence of tyrosine-phosphorylated STAT5b. This conclusion is supported by our analysis of nuclear SHP-1 and its association with STAT5b in livers of intact male and female rats; SHP-1 was present in both male and female rat liver nuclear extracts, albeit at a somewhat lower level in the females (Fig. 4D), despite the absence of STAT5b in the female nuclear samples. Immunoprecipitation with antibodies to SHP-1 revealed an association of STAT5b with SHP-1 in the male but not the female nuclear samples (Fig. 4C). The STAT5b co-immunoprecipitated with SHP-1 in this experiment was resolved to give a doublet corresponding to the tyrosine-phosphorylated (lower band) and tyrosine + serine/threonine-diphosphorylated STAT5b (upper band) described in our earlier studies (6, 22). SHP-1 thus associates with both activated STAT5b forms. The absence of STAT5b in the SHP-1 immunoprecipitates from female liver nuclei is consistent with our earlier demonstration (12) that activated STAT5b is either absent or present at a very low level in female rat liver nuclear samples. As was found for GH-induced
CWSV-1 cells, no interaction between SHP-1 and activated STAT3 or between SHP-2 and either STAT3 or STAT5b could be detected in the nuclear liver samples (data not shown).

Probing for Interactions of SHP-1 with Tyrosine-Phosphorylated Cytoplasmic Tail of GH Receptor—We have previously reported that STAT5b associates with the COOH-terminal domain of GH receptor in transfected cells and that this association is markedly enhanced by tyrosine phosphorylation of the receptor's cytoplasmic domain (14). To test whether the co-immunoprecipitation of SHP-1 with tyrosine-phosphorylated STAT5b seen in the present study might involve an indirect scaffolding on these two signaling molecules on the GH receptor cytoplasmic tail, we carried out an affinity isolation of GH receptor-interacting proteins using a bacterially expressed, GST-GH receptor fusion protein (see lower mobility fusion protein band present in lanes 2, 4, and 6, detected by probing with anti-GST antibody; D). Glutathione-agarose affinity precipitated proteins were analyzed on a Western blot probe sequentially with antibodies to the four indicated proteins (A–D). Shown in lanes 7–9 are the corresponding untreated and GH-stimulated CWSV-1 cell extracts (20 μg/lane) analyzed directly, without the GST fusion protein affinity isolation step. The change in STAT5b mobility upon phosphorylation following GH treatment is masked in the exposure shown in B, lanes 7–9, where only a small protein mobility shift is seen from lanes 7–9.

FIG. 5. Association of STAT5b, but not SHP-1 or STAT3, with tyrosine-phosphorylated GH receptor COOH terminus. Whole cell extracts of CWSV-1 cells treated with GH (500 ng/ml) for 20 and 45 min (80 μg of protein) were incubated with glutathione-agarose beads containing bound GST-GH receptor fusion protein expressed in bacteria and containing COOH-terminal amino acids 540–620 of GH receptor, GST-GHR-(540–620). The fusion protein was expressed in E. coli in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of Elk tyrosine kinase, which phosphorylates the GST-GH receptor fusion protein (see lower mobility fusion protein band present in lanes 2, 4, and 6, detected by probing with anti-GST antibody). A, GST (lanes 1–6); B, STAT3b (lanes 7–9); C, SHP-1 (lanes 1–6); D, STAT5b (lanes 7–9). Shown in lanes 7–9 are the corresponding untreated and GH-stimulated CWSV-1 cell extracts (20 μg/lane) analyzed directly, without the GST fusion protein affinity isolation step. The change in STAT5b mobility upon phosphorylation following GH treatment is masked in the exposure shown in B, lanes 7–9, where only a small protein mobility shift is seen from lanes 7–9.

or not at all to the fusion protein (Fig. 5C), in agreement with our findings in Cos cell transfection experiments (14). Interestingly, the interaction of the tyrosine-phosphorylated GH receptor with STAT5b was independent of the phosphorylation state of STAT5b itself, as shown by parallel analyses of GH-activated and unactivated CWSV-1 cell extracts (lanes 4 and 6 versus lane 2). Moreover, the same result, binding of tyrosine-phosphorylated GH receptor fusion protein to STAT5b, but not SHP-1 or STAT3, was obtained when using a fusion protein that contained a more complete COOH-terminal segment of GH receptor, residues 389–620 (data not shown).

Nuclear Localization of JAK2: Tyrosine-phosphorylated STAT3 but Not STAT5b Binds to Nuclear JAK2—During the course of our studies of SHP-1 and its nuclear localization, we unexpectedly observed that JAK2 kinase, mass of ~120 kDa, is enriched in liver nuclear extracts (Fig. 1F). Since JAK2 is widely believed to be primarily cytosolic and/or associated with plasma membrane-bound cytokine and growth factor receptors, we examined the intracellular localization of JAK2 kinase using an independent technique, indirect immunofluorescence microscopy. These studies were carried out in CWSV-1 cells, where JAK2 kinase is transiently phosphorylated on tyrosine in response to GH treatment (Fig. 6). In untreated cells, JAK2 immunofluorescence was detected both in the cytosol and in the nucleus. JAK2 immunofluorescence was diffuse in the cytoplasm but was localized in discrete patches in the nucleus (Fig. 7A). The punctate and speckled distribution of nuclear JAK2 became further concentrated following GH treatment. In control experiments, JAK2 immunostaining could be substantially blocked using synthetic JAK2 peptide antigen to preabsorb the antiserum (see “Experimental Procedures”). No fluorescence was visible in control analyses using secondary antibody alone. We were also able to observe GH-stimulated nuclear translocation of SHP-1 in CWSV-1 cells (Fig. 7B), in agreement with the results of our liver cell nuclear fractionation studies (Figs. 1A and 4B). In contrast to JAK2, however, little or no SHP-1 immunoreactivity was detectable in CWSV-1 cell nuclei prior to GH stimulation (Fig. 7B). We also examined the effects of GH on STAT5b immunolocalization. In unstimulated cells, STAT5b immunoreactivity was primarily detected in the cytoplasm, whereas in cells stimulated with GH for 20 min, STAT5b became predominantly nuclear (Fig. 7C). Thus, GH treatment of CWSV-1 cells induces a major nuclear translocation of SHP-1 and STAT5b, while it somewhat increases the nuclear concentration of JAK2 kinase.

In view of the nuclear localization of JAK2 as well as these
factors, including erythropoietin (25), interferon 
regulator of cell signaling by several cytokines and growth 
pressed in hematopoietic and epithelial cells, acts as a negative 
containing protein-tyrosine phosphatase preferentially ex-
rose dephosphorylation reaction which serves as the initial 
CWSV-1, suggest that SHP-1 may catalyze the phosphoty-
liver cells 

activate, JAK2 kinase (25). This inhibition by SHP-1 of eryth-
poietin, SHP-1 inhibits the signaling cascade by docking to the 

receptor’s tyrosine-phosphorylated cytoplasmic tail, enabling 
SHP-1 to interact with and dephosphorylate, and thereby de-
activate, JAK2 kinase (25). This inhibition by SHP-1 of erythr-

other GH-activated signaling molecules, we next examined 
whether nuclear JAK2 interacts with GH-activated STAT pro-
tins. Nuclear extracts from untreated male and female rats and from hypophysectomized rats given GH for 15 or 45 min 
were immunoprecipitated with anti-JAK2 antibody, and the 
immunoprecipitates were then analyzed on Western blots 
probed with anti-STAT3, anti-STAT5b, and anti-JAK2 anti-
bodies. Fig. 8A shows the co-immunoprecipitation of tyrosine-
phosphorylated STAT3 with anti-JAK2 antibody. This interac-
tion between STAT3 and JAK2 was only seen in liver nuclear 
extracts from GH-treated rats, suggesting that it is dependent 
on the tyrosine phosphorylation state of one or perhaps both 
protein components. Indeed, JAK2-associated STAT3 corre-
sponded in mobility to the tyrosine-phosphorylated STAT3 
form (data not shown). In contrast to these results, no STAT5b 
could be detected in the JAK2 immunoprecipitates upon re-
probing with antibody to STAT5b (data not shown). Together, 
these findings indicate that while STAT5b associates with 
SHP-1, STAT3 associates with JAK2 in the nucleus after stim-
ulation with GH.

**DISCUSSION**

GH is presently shown to activate the phosphotyrosine-spe-
cific phosphatase SHP-1, to stimulate its nuclear translocation, 
and to induce the selective binding of SHP-1 to GH-activated, 

zyme-phosphorylated STAT5b. These studies, carried out in 
liver cells in vivo and in the liver-derived cell culture model 
CWSV-1, suggest that SHP-1 may catalyze the phosphoty-
rosine dephosphorylation reaction which serves as the initial 
step of STAT5b deactivation (22). SHP-1, an SH2 domain-
containing protein-tyrosine phosphatase preferentially ex-
pressed in hematopoietic and epithelial cells, acts as a negative 
regulator of cell signaling by several cytokines and growth 

factors, including erythropoietin (25), interferon α (36), and 
antigen receptor (26), although instances of positive regulatory 
effects have also been reported (37). In the case of erythropoi-
etin, SHP-1 inhibits the signaling cascade by docking to the 

receptor’s tyrosine-phosphorylated cytoplasmic tail, enabling 
SHP-1 to interact with and dephosphorylate, and thereby de-
activate, JAK2 kinase (25). This inhibition by SHP-1 of erythr-

opoietin signaling at the level of the cell surface receptor-JAK 
kine complex would appear to be distinct from the effects of 
SHP-1 on GH signaling, where we were unable to detect a 
direct GH receptor-SHP-1 binding interaction. Rather, SHP-1 
is proposed to down-regulate GH signals by dephosphorylation 
of STAT5b, a process that is critical for the rapid deactivation of 
STAT5b between plasma GH pulses in vivo and that enables 
STAT5b to recycle back to the cytoplasm in time to respond to 
an incoming GH pulse (12, 22). SHP-1 may thus participate in 
two fundamentally distinct signal desensitization processes. 

SHP-1-catalyzed dephosphorylation of cell surface receptors 
and/or JAK2 kinase blocks a catalytic cascade, whereby one 
growth factor receptor or JAK kinase dephosphorylation event 
prevents multiple downstream STAT5b activation events. By 
contrast, the dephosphorylation of GH-activated STAT5b by 
SHP-1 would correspond to a downstream inactivation event 
that is stoichiometric with respect to the STAT transcription 
factor. This deactivation of GH signaling at the level of STAT5b 
dephosphorylation does not, however, preclude the need for 
upstream step(s) to dephosphorylate GH receptor and to down-
regulate GH-activated JAK2, which dephosphorylates within 
45 min after GH addition in the case of liver cells (Fig. 6). These 
upstream deactivation events may be mediated by SHP-2, as 
suggested by the weak association that we have detected be-

between SHP-2 and the tyrosine- phosphorylated GH receptor 
COOH terminus.6

The mechanism by which SHP-1 binds to STAT5b is not yet 
known, but given the requirement for STAT5b tyrosine phos-
phorylation for binding to occur, this interaction is likely to 
involve a direct binding of phosphotyrosine residue 694 of 
STAT5b (38) by one of the SH2 domains of SHP-1. SH2 domain 
occupancy leads to activation of catalytic activity for both 
SHP-1 and SHP-2 (26, 32, 39), and thus the binding of acti-
vated STAT5b per se could directly stimulate the GH-depend-
ent increase in SHP-1 enzyme activity that we observed. Alter-
natively, other cellular molecules that undergo GH-induced 
tyrosine phosphorylation could be responsible for SHP-1 acti-
vation. SHP-1-STAT5b binding was seen with tyrosine-phos-
phorylated STAT5b and also with tyrosine + serine/threonine-
diphosphorylated STAT5b, indicating that the secondary 
serine/threonine phosphorylation reaction is not required for 

6 P. A. Ram and D. J. Waxman, unpublished experiments.
binding to SHP-1. Further studies are needed to ascertain whether STAT5b directly activates SHP-1, and if so, whether the two phosphorylated STAT5b forms both activate SHP-1 to the same extent. A possible differential effect of the two phosphorylated STAT5b forms is suggested by the partial inhibitory effect that the serine/threonine kinase inhibitor H7 had on GH-induced SHP-1 activation (Fig. 2A). This possibility is consistent with our earlier conclusion that a cellular serine/threonine kinase activity is required for rapid STAT5b deactivation (22).

Only a portion of the cellular pool of activated STAT5b appears to be bound by SHP-1, as judged by the incomplete association of STAT5b with SHP-1 (Fig. 3B and data not shown). This is not unexpected, insofar as the interaction of these two signaling molecules may be transient. Moreover, given the proposed catalytic role of SHP-1, whereby one molecule of SHP-1 could, in principle, dephosphorylate many STAT5b molecules, SHP-1 levels in the nucleus are likely to be sub-stoichiometric with respect to STAT5b. In this case, only a portion of the STAT5b pool would be stably associated with SHP-1 at any one time, allowing for the remaining STAT5b molecules to comprise a substrate pool.

GH stimulated a 3–4-fold increase in SHP-1 activity, as judged by phosphotyrosine phosphatase assay of SHP-1 immunoprecipitates from GH-activated CWSV-1 cells. This activity increase was reversed within 1–2 h and may reflect activation resulting from the binding of STAT5b’s phosphotyrosine residue to the amino-terminal SH2 domain of STAT5b. The observed activity increase could be a minimum estimate of the fold activation of SHP-1 in intact cells, as there may be some loss of STAT5b, or perhaps other bound activators during the immunoprecipitation step. In vitro activation assays using synthetic phosphotyrosine-containing peptides derived from cell surface receptors and other signaling molecules that bind to SHP-1 have revealed intrinsic fold activation values that range up to >25-fold (32, 33). However, this potential for a high degree of SHP-1 activation, evident in vitro, does not always translate into a corresponding major enhancement of SHP-1 activity in intact cells, as seen in the case of GH-treated GH receptor for the Fc region of IgG (26). The 3–4-fold activation observed for SHP-1 in GH-treated cells could similarly underestimate the intrinsic fold activation of this PTP by the STAT5b-derived phosphotyrosine peptide. Recruitment of STAT5b to both the NH2-terminal and the COOH-terminal SH2 domain of STAT-1 would be expected to moderate the extent of SHP-1 activation compared with NH2-terminal SH2 binding interactions alone (33).

STAT5b is only transiently active when stimulated by a GH pulse in liver in vivo (12). This allows for successive cycles of plasma GH pulsation to induce successive cycles of STAT5b activation. This periodic response of liver STAT5b to GH pulses does not depend on new protein synthesis (22), necessitating repeat cycles of STAT5b dephosphorylation followed by rephosphorylation. The translocation of SHP-1 to the nucleus following GH stimulation and the association of SHP-1 with tyrosinemodified STAT5b described in the present study are thus likely to be important, not only for recruitment of the phosphatase to nuclear STAT5b, but also for regulation of SHP-1’s phosphatase activity in a manner that allows for the repeated activation of STAT5b upon stimulation of hepatocytes by plasma GH pulses in vivo. Indeed, we have observed that repeat GH pulses also lead to repeat cycles of SHP-1 activation and deactivation (Fig. 2C).

In the hypophysectomized rat liver model, SHP-1 remained in the nucleus following GH treatment well after the deactivation and loss of nuclear STAT5b were complete (Fig. 4B). This observation is consistent with our observation that SHP-1 is constitutively present in the nucleus of male rat livers, both at the time of a plasma GH pulse and during the GH interpulse interval, when STAT5b has been dephosphorylated and is no longer present in the nucleus.6 SHP-1 is also present in female liver nuclei, albeit at a somewhat lower level than in the males (Figs. 1A and 4D), despite the fact that the STAT5b signaling pathway is down-regulated and is essentially inactive in these animals. Only in the case of chronic GH deficiency (hypophysectomized rat liver or CWSV-1 cells cultured in the absence of GH) did we observe low levels of nuclear SHP-1 and high levels of cytoplasmic SHP-1. We conclude that the GH-induced nuclear translocation of SHP-1 is not dependent on a concurrent nuclear translocation of STAT5b and thus may proceed by a mechanism that is distinct from the direct tyrosine phosphorylation mechanism that induces STAT5b nuclear translocation. Indeed, although cytokine/growth factor-induced tyrosine phosphorylation of SHP-1 and SHP-2 has been observed in other systems (e.g., Refs. 29, 31, 34, and 35), we were unable to detect tyrosine phosphorylation SHP-1 in our experiments. While it is conceivable that GH may induce SHP-1 tyrosine phosphorylation that could not be detected using our analytical methods, it seems more likely that SHP-1 nuclear translocation and its enzymatic activation do not involve SHP-1 tyrosine phosphorylation directly but rather proceed via a different mechanism. Activation of SHP-1 following GH treatment could simply involve its direct binding to tyrosine-phosphorylated signaling molecules, as exemplified by the SHP-1-STAT5b binding interaction observed in the present study. This possibility is an attractive one, since it provides a simple mechanism for deactivation of SHP-1 once its tyrosine-phosphorylated substrate has been dephosphorylated. Indeed, GH pulse treatment of cultured liver cells leads to a cycle of SHP-1 activation, followed by deactivation within 1–2 h (Fig. 2A), a time course that mirrors both the cycle of STAT5b activation and deactivation (Fig. 3A) and the binding of STAT5b to SHP-1 (Fig. 3B).

Previous studies have shown that the three GH-activated liver STATs, STAT1, 3, and 5b, differ in their responses to GH in several important ways. These include the striking dependence of STAT5b, but not STATs 1 and 3, on the temporal pattern of GH activation, the preferential activation of STAT5b, as compared with the other STATs, at physiological GH levels, and the marked desensitization of STATs 1 and 3, but not STAT5b, following a single GH pulse (6). These differences may, in part, result from differences in the mechanisms through which these STATs become activated, and subsequently inactivated, following GH treatment. Whereas STAT5b becomes tyrosine-phosphorylated as a consequence of its direct binding to the COOH-terminal cytoplasmic tail of GH receptor (Fig. 5B) (14–16), STAT3 is more reliant on interactions with JAK2 for activation. This model is now supported by the physical association of JAK2 with STAT3 but not STAT5b (Fig. 8) and by functional studies using JAK2 kinase-GH receptor chimeras (14). A further difference between the GH-activated STATs is apparent from the present SHP-1 binding studies, where SHP-1 binding was observed in the case of activated STAT5b, but not activated STAT3. Whereas SHP-1 seems likely to play an important role in the deactivation of STAT5b and its subsequent return to the cytosol, GH-activated STAT3 may be deactivated by a distinct mechanism. Ubiquitination leading to proteolytic degradation, recently described for interferon-γ activated STAT1 (40), is a possible alternative deactivation mechanism for STAT3. Proteolytic degradation of STATs 1 and 3 could help explain the marked desensitization that both STAT proteins undergo following an initial GH activation event in vivo (6).
Biochemical fractionation studies demonstrated that a significant amount of JAK2 is constitutively expressed in the nucleus of liver cells in vivo (Fig. 1F). Immunofluorescence studies further established that following GH stimulation, a portion of the cellular JAK2 translocates from the cytoplasm to the nucleus (Fig. 7A). While these studies were in progress, Lobie et al. (41) reported the constitutive nuclear expression of JAK2, but in their experiments GH-induced nuclear translocation was not observed. The function of nuclear JAK2 kinase is directly mediated some of the effects that GH has on nuclear signaling molecules. This possibility is supported by the earlier finding that both GH (42) and GH receptors (43) undergo nuclear translocation in GH-stimulated cells and by the observation that nuclear JAK2 becomes tyrosine-phosphorylated in GH-treated cells (41). Given our finding that STAT3, but not STAT5b, is constitutively present in liver nuclei in the inactive (non-tyrosine-phosphorylated) state (cf. Fig. 1D) (6), this model, involving GH activation of nuclear STAT3 via a direct JAK2 kinase interaction, versus GH activation of cytosolic STAT5b via a plasma membrane-associated GH receptor-JAK2 kinase complex could further explain the substantially higher GH dose requirement for STAT3 (and STAT1) activation seen in the liver in vivo (6), in view of the comparatively low efficiency with which GH is internalized to the nucleus of treated cells (42).

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