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The role of STAT proteins in growth hormone signaling

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Growth hormone (GH) has long been known to be the body's primary regulator of body growth and a regulator of metabolism, yet the mechanisms by which GH regulates the transcription of specific genes required for these processes are just now being delineated. GH binding to its receptor recruits and activates the receptor-associated JAK2 that in turn phosphorylates tyrosines within itself and the GH receptor. These tyrosines form binding sites for a number of signaling proteins, including members of the family of signal transducers and activators of transcription (STAT). Among the known signaling molecules for GH, STAT proteins play a particularly prominent role in the regulation of gene transcription. This paper will review what is currently understood about which STAT proteins are regulated by GH, how they are regulated by GH, the GH-dependent genes they regulate, and discuss current theories about how GH-activated STAT signaling is regulated. Particular attention will be given to the novel role that STAT5 plays in sexually dimorphic gene expression in the liver as determined by the secretory pattern of GH and the role of STAT5 in body growth. *Oncogene* (2000) **19,** 2585–2597.

Keywords: growth hormone (GH); STAT proteins; tyrosines

Introduction

Despite the importance of growth hormone (GH) in the regulation of body growth and metabolism, the mechanisms by which GH regulates the transcription of specific genes required for these processes are just now being delineated. The GH receptor was the first member of the cytokine receptor family to be cloned (Leung *et al*., 1987) and to be recognized as associating with and activating a tyrosine kinase (Carter-Su *et al*., 1989; Endo *et al*., 1997; Foster *et al*., 1988; Wang *et al*., 1993). GH binding to its receptor is now known to recruit and activate the receptor-associated JAK2 that in turn phosphorylates tyrosines within itself and the GH receptor. These tyrosines form binding sites for a number of signaling proteins, including members of the family of signal transducers and activators of transcription (STAT). Other signaling proteins that are recruited to JAK2/GH receptor complexes and/or activated in response to GH include: (1) Shc proteins that are thought to lead to the activation of the Ras-MAP kinase pathway; (2) insulin receptor substrates that have been implicated in the activation of phosphatidylinositol-3′-kinase and the kinase AKT/PKB; (3) phospholipases that lead to formation of diacylglycerol and activation of protein kinase C; and (4) a variety of proteins that are involved in the regulation of the cytoskeleton, including focal adhesion kinase (reviewed Smit *et al*., 1999), paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck (Zhu *et al*., 1998a,b). Among these signaling molecules, STAT proteins play a particularly prominent role in the GH regulation of gene transcription. This paper will review the mechanism of GH activation of STATs, regulation of STAT activation/deactivation, and the physiological consequences of disrupted GH signaling via STATs.

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Mechanism of activation of STAT proteins by GH

An important pathway by which GH and other cytokines that activate members of the Janus kinase (JAK) family of tyrosine kinases have been shown to regulate gene transcription involves STAT proteins (Figure 1). STAT proteins, originally identified in IFN signaling pathways (Darnell *et al*., 1994), are latent, SH2-domain containing, cytoplasmic factors. Upon tyrosyl phosphorylation, often via a JAK kinase initiated cascade, cytoplasmic STAT proteins complex with other STAT proteins via an SH2 domain-phosphorylated tyrosine interaction, translocate to the nucleus, bind to DNA and activate transcription of target genes (Ihle, 1996). GH has been shown to activate STATs 1, 3, 5A and 5B (Figures 1 and Figure 2). GH-dependent tyrosyl phosphorylation of STATs 1, 3, 5A and 5B has been observed in 3T3-F442A fibroblasts, liver from hypophysectomized rats, cultured liver cells and in a variety of overexpression systems. Tyrosyl phosphorylation of STATs 5A and 5B has also been observed in human IM-9 cells and liver and skeletal muscle from normal rats (reviewed in Smit *et al*., 1999).

STAT1, also referred to as p91, was originally identified as a member of the IFN-stimulated gene factor 3 complex that initiates transcription of IFNα stimulated genes (Fu, 1992; Kessler *et al*., 1990; Shuai *et al*., 1992). STAT3 was originally identified as acute phase response factor (APRF) that mediates transcriptional regulation of a subset of acute phase response proteins in response to IL-6 (Raz *et al*., 1994; Wegenka *et al*., 1994; Zhong *et al*., 1994). STAT5, or mammary gland factor (MGF), was discovered initially as a PRL-induced transcription factor (Wakao *et al*., 1994). While a single STAT5 gene was initially identified in sheep, two forms of STAT5 (STAT5A and STAT5B), encoded by two different genes, have now been identified in mouse, human and rat cells (Hou *et al*., 1995; Kazansky *et al*., 1995; Lin *et al*., 1996; Liu *et al*., 1995; Mui *et al*., 1995; Ripperger *et al*., 1995; Silva *et al*., 1996). The genes encoding STAT5A and STAT5B are highly homologous, being $\sim$90% identical in coding sequence. They diverge primarily in their COOH-terminal transcription activation domains (Moriggl *et al*., 1996), exhibit differences in their

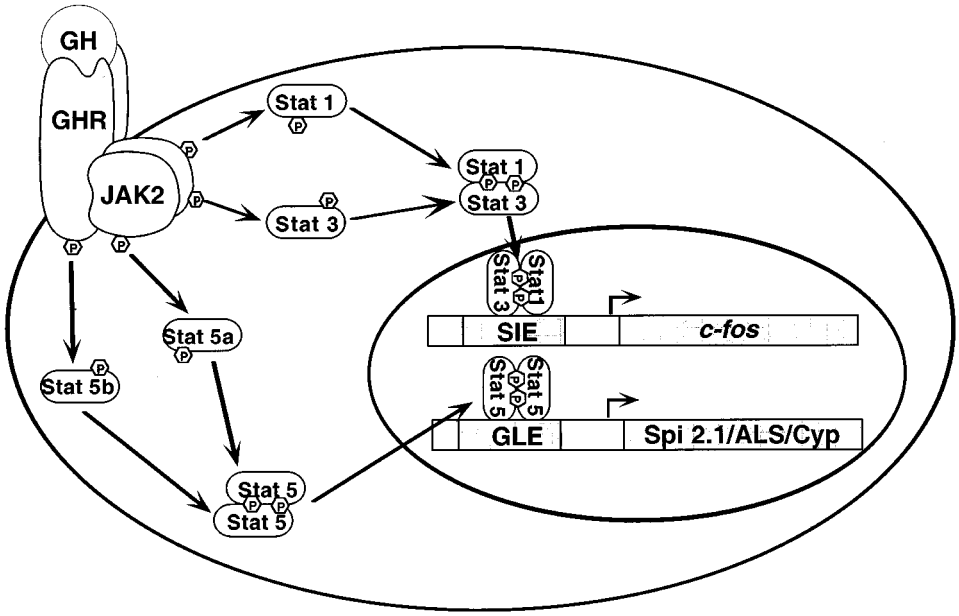

Figure 1 Growth hormone receptor (GHR) signaling via STAT proteins. P, phosphotyrosines; JAK, Janus kinase; SIE, Sis-inducible element; GLE, interferon-γ activated sequence-like element; Spi, serine protease inhibitor; ALS, acid labile subunit; Cyp, cytochrome P450. (Adapted with permission from Smit L, Meyer D, Argetsinger LS, Schwartz J and Carter-Su C. Handbook of Physiology, Oxford University Press: New York, NY. Vol 5. Eds: JL Kostyo and HM Goodman, pp 445–480, 1999)

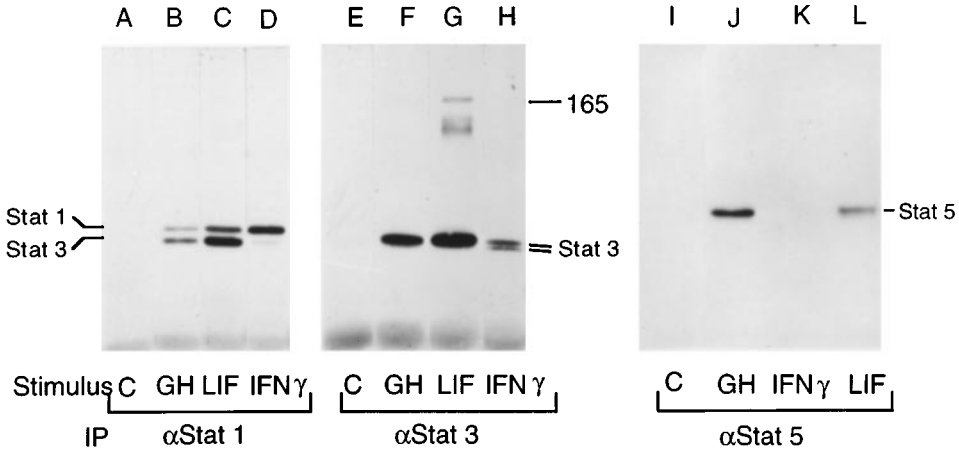

Figure 2 Growth hormone (GH), leukemia-inhibitory factor (LIF), and interferon-γ (IFN) induce tyrosyl phosphorylation of STAT1, STAT3, and STAT5 in 3T3-F442A fibroblasts. 3T3-F442A fibroblasts were incubated for 15 min in the absence of hormone or with 500 ng/ml human GH, 25 ng/ml human LIF or 10 ng/ml murine IFNγ. Whole cell lysates were immunoprecipitated with antibodies to STAT1 (αSTAT1), STAT3 (αSTAT3) or STAT5 (αSTAT5), as indicated. Immunoprecipitated proteins were subjected to Western blot analysis using anti-phosphotyrosine antibody. Migrations of STAT1, STAT3, and STAT5 are indicated. (Reprinted with permission from Carter-Su C, King APJ, Smit LS, VanderKuur JA, Argetsinger LS, Campbell GS. and Huo WJ *Anim. Sci.* **75** (Suppl. 2), 1–10, 1997)

DNA binding specificities (Boucheron *et al*., 1998; Verdier *et al*., 1998) and exhibit differences with respect to their tissue distribution (Liu *et al*., 1995; Mui *et al*., 1995). Thus, STAT5A and STAT5B are predicted to have both overlapping and distinct functions with respect to their role in GH signaling. Based upon the phenotype observed in mice with targeted disruption of their STAT5A *versus* STAT5B gene, STAT5A and STAT5B appear to have some overlapping functions. However, these same mice exhibit some differences in their phenotype and gene expression pattern, indicating that STAT5A and STAT5B also have distinct roles (Liu *et al*., 1997, 1998; Park *et al*., 1999; Teglund *et al*., 1998; Udy *et al*., 1997). The observation that a STAT5A-specific antibody immunoprecipitates some STAT5B in lysates from GH-treated 3T3-F442A fibroblasts and vice versa suggests that STAT5A and STAT5B form heterodimers in response to GH (Smit *et al*., 1997). Electrophoretic mobility shift assays using the STAT5 response element of the rat β-casein promoter and liver homogenates from mice with or without targeted disruption of the STAT5A or STAT5B gene also suggest the existence of STAT5A/STAT5B heterodimers (Park *et al*., 1999). Heterodimerization of STAT5A and STAT5B in murine mammary gland in response to prolactin has been reported and appears to vary during mammary gland differentiation, suggesting that STAT5A/5B heterodimers have distinct functions from the respective homodimers (Liu *et al*., 1996).

Understanding the mechanism by which STAT proteins become phosphorylated, are stimulated to bind to DNA, and regulate gene transcription is critical for understanding GH-regulated gene transcription by the STAT proteins. Analyses of GH signaling in a JAK2 deficient cell line and in several cell lines expressing truncated and mutated GH receptors indicate that GH-dependent activation of STATs 1, 3, 5A and 5B requires JAK2 activation (Hackett *et al*., 1995; Han *et al*., 1996; Smit *et al*., 1996, 1997; Wang and Wood, 1995). This is consistent with similar findings that activation of JAKs is required for STAT activation by other ligands that bind to cytokine family receptors (Muller *et al*., 1993; Watling *et al*., 1993). These findings suggest that JAKs are responsible for tyrosyl phosphorylating STAT proteins. Consistent with this hypothesis, constitutively active overexpressed JAK1 or JAK2 in COS cells stimulates STAT1 DNA binding activity (Silvennoinen *et al*., 1993) and co-expression of constitutively active JAK1, JAK2 or tyk2 with STAT3 in human kidney fibroblast 293 cells activates STAT3 (Raz *et al*., 1994). JAK1, JAK2 or tyk2 immunoprecipitates from Sf9 cells infected with virus expressing the respective JAK kinase similarly activate STAT1 (Quelle *et al*., 1995) and *in vitro*-transcribed and translated ovine STAT5 is able to bind DNA upon incubation with purified JAK2 and ATP (Gouilleux *et al*., 1994).

Although JAK2 activation appears to be sufficient for STAT phosphorylation and DNA binding activity when JAK2 and the STATs are overexpressed or in an *in vitro* setting, it does not appear to be sufficient for activation of STAT5 in intact cells. The current evidence suggests that STAT5 activation by GH requires phosphorylation of specific tyrosine residues within the GH receptor, thereby providing a high affinity binding site for the SH2 domain in the STAT proteins. Specific tyrosines in porcine GH receptor (Y534, Y566 and Y627, equivalent to Y534, Y566 and Y626 in rat GH receptor) have been identified as being required for GH-dependent tyrosyl phosphorylation of STAT5 and transcription of the *Spi 2.1* promoter (Hansen *et al*., 1996; Wang *et al*., 1996). An additional tyrosine (487) has also been suggested as being required for tyrosyl phosphorylation of STAT5 (Wang *et al*., 1996). In addition to these tyrosines in the C-terminal half of the cytoplasmic domain of GH receptor, tyrosines 333 and/or 338 in the N-terminal half of the cytoplasmic domain of rat GH receptor may also play a role in GH-dependent activation of STATs 5A and 5B (Smit *et al*., 1996, 1997). Association of STAT5 with GH receptor has been detected in 3T3-F442A fibroblasts and in L cells transfected with GH receptor and STAT5A cDNAs (Xu *et al*., 1996). Experiments with GST-GH receptor fusion proteins have suggested that sheep STAT5 and murine STAT5B associate with the C-terminal half of the cytoplasmic domain of GH receptor and the association is enhanced by phosphorylation of the GH receptor fusion protein (Sotiropoulos *et al*., 1996; Yi *et al*., 1996). However, in these experiments the GH receptor fusion proteins are phosphorylated in bacteria by a kinase other than JAK2 and it is not clear whether the phosphorylated tyrosines are the same as those phosphorylated by JAK2. These results suggest that GH-dependent activation of STAT5 proteins requires binding of the STAT to one or more phosphorylated tyrosines in the cytoplasmic domain of GH receptor. A similar requirement of multiple phosphorylated tyrosine(s) in the receptor for maximal activation of STAT5 has been demonstrated for EPO, PRL, IL-2, IL-5 and granulocyte-macrophage colony-stimulating factor (Damen *et al*., 1995; Fujii *et al*., 1995; Itoh *et al*., 1998; Pezet *et al*., 1997; Quelle *et al*., 1996; van Dijk *et al*., 1997).

As observed for GH-dependent activation of STAT5, phosphorylation of specific tyrosines within gp130 and LIF receptor subunits are required for ligand activation of STAT3 (Gerhartz *et al*., 1996; Stahl *et al*., 1995). Similarly, specific tyrosine(s) in gp130 and the IFNγ receptor are required for ligand-dependent activation of STAT1 (Gerhartz *et al*., 1996; Greenlund *et al*., 1994). However, significantly truncated forms of GH receptor which lack most or all phosphorylated tyrosines can mediate GH-induced tyrosyl phosphorylation of STATs 1 and 3 and binding of STATs 1 and 3 to the Sis inducible element (SIE) of c-*fos*. This suggests that specific phosphorylated tyrosines in GH receptor may not be essential for activation of STATs 1 and 3 by GH (Smit *et al*., 1996; Sotiropoulos *et al*., 1995, 1996; Wang *et al*., 1995; Yi *et al*., 1996). While not essential for GH-dependent activation of STATs 1 and 3, phosphorylated tyrosine(s) in the N-terminal half of the cytoplasmic domain of the rat GH receptor may contribute to maximal activation of STATs 1 and 3 in response to GH (Smit *et al*., 1996). A lack of a requirement of phosphorylated tyrosines in GH receptor for GH-dependent activation of STATs 1 and 3 would be consistent with the absence in GH receptor of the STAT3 (YXXQ) or STAT1 (YXPQ or YDXXH) association motifs found in gp130, LIFβ and/or IFNγ receptor subunits (Gerhartz *et al*., 1996; Greenlund *et al*., 1994; Stahl *et al*., 1995). In contrast, JAK2 contains two STAT3 association motifs and a STAT1-like association motif. Thus, for GH which is an extremely effective activator of JAK2 (Argetsinger *et al*., 1993, 1995), JAK2 may both provide a high affinity binding site for STAT1 and STAT3 and phosphorylate STATs 1 and 3. Alternatively, GH may utilize an intermediary protein in STAT activation, a region of the GH receptor other than a phosphorylated tyrosine, or a region of JAK2 other than a phosphorylated tyrosine. In support of activation of some STATs being a consequence of their binding directly to JAKs, the activation of STAT5 via gp130 receptor subunit does not need any phosphotyrosines on gp130 but instead STAT5 appears to bind directly to JAKs 1, 2 or 3 (Fujitani *et al*., 1997). It is also important to note that these conclusions are primarily based on mutagenesis studies which are always subject to the caveat that a mutation may simply alter the conformation of GH receptor.

Tyrosyl phosphorylation is required for activation of STAT DNA binding activity (Gouilleux *et al*., 1994; Shuai *et al*., 1993). However, recent studies have demonstrated that serine phosphorylation also plays a role in activation or regulation of STAT proteins. The observation that multiple tyrosyl phosphorylated STAT3 and STAT5 bands are observed in response to GH in Western blots (Campbell *et al*., 1995; Ram *et al*., 1996; Smit *et al*., 1996, 1997), despite the fact that a single tyrosyl phosphorylation site has been identified in these proteins, suggests that these STAT proteins may be

phosphorylated on serine residues as well as tyrosine residues. Treatment of GH-activated liver nuclear extracts with either the serine/threonine phosphate specific phosphatase, PP2A, or the tyrosine phosphate specific phosphatase, PTP-1B, results in conversion of slower migrating forms of STAT3 and STAT5 bands to faster migrating forms, suggesting that STAT3 and STAT5 undergo both tyrosyl and seryl and/or threonyl phosphorylation following GH treatment (Ram *et al*., 1996). The existence of multiple STAT5A and 5B bands in the absence of GH (Smit *et al*., 1997), suggests that STATs may be seryl and/or threonyl phosphorylated in the absence of ligand. Further evidence for the role of seryl phosphorylation in STAT activation derives from studies with other cytokines and factors. Seryl phosphorylation of STAT1, STAT3 and/or STAT5 in response to IFNγ, IL-6, IL-2 or insulin has been demonstrated directly by phosphoamino acid analysis (Beadling *et al*., 1996; Ceresa and Pessin, 1996; Eilers *et al*., 1995; Wen *et al*., 1995; Zhang *et al*., 1995), and it appears to be required for maximal activation of transcription of at least some target genes (Beadling *et al*., 1996; Wen *et al*., 1995). Whether it affects DNA binding is controversial. Indirect studies suggest that GH stimulates phosphorylation of STATs 1, 3 and 5 on serine or threonine in liver. This phosphorylation enhances the DNA binding of STAT1 and STAT3 and substantially alters the DNA binding of STAT5 (Ram *et al*., 1996). STATs 1, 3 and 5A contain a conserved consensus sequence for phosphorylation by MAP kinase and initial studies suggested that MAP kinase might be responsible for seryl phosphorylation of STAT1. A STAT1-derived peptide was able to serve as a substrate for MAP kinase (Wen *et al*., 1995) and expression of a dominant negative MAP kinase was reported to inhibit IFNγ-induced transcription by STAT1 (David *et al*., 1995). Experiments using the MEK inhibitor PD98059 and STAT5A with a mutated MAP kinase phosphorylation site support the hypothesis that MAP kinases phosphorylate STAT5A and that this phosphorylation is required for full GH-induced activation of STAT5A (Pircher *et al*., 1997). In contrast, if STAT5B is seryl and/or threonyl phosphorylated in response to GH, it is likely to be by a kinase other than MAP kinase since the conserved seryl residue found within the consensus site for phosphorylation by MAP kinase in other STAT family members is not present in STAT5B. Interestingly, the MAP kinase pathway is not required for lactogen-induced tyrosyl phosphorylation of STAT5A or STAT5B in mammary epithelial cells or transcriptional activation of a β-casein reporter construct, as evidenced by the lack of an effect of the MEK inhibitor, PD98059, on these events (Wartmann *et al*., 1996). In mammary epithelial cells PKCα and casein kinase II have been implicated in the regulation of STAT5 by prolactin (Marte *et al*., 1994; Schmitt-Ney *et al*., 1992). STATs 1, 3, 5A and 5B contain multiple consensus sites for phosphorylation by protein kinase C and casein kinase. Thus, multiple signaling pathways may converge on STAT proteins and contribute to transcriptional activation by GH.

Nuclear localization of STAT proteins

While significant work has accumulated regarding the mechanism of activation of STAT proteins by GH and other cytokines, the mechanisms regulating the cellular distribution of STAT proteins remain poorly understood. It is well documented that in response to GH, STAT proteins undergo a rapid, dramatic accumulation in the nucleus (Figure 3), yet they lack a conventional nuclear localization signal and are too big to diffuse through nuclear pores. Data is emerging on the mechanisms regulating STAT localization in response to ligands other than GH, that might eventually prove applicable to STAT localization in response to GH. For example, IFN-γ-stimulated STAT1 nuclear import requires the importin α/β and RAN/TC4 pathway (Sekimoto *et al*., 1996, 1997). However, STAT1 lacks a conventional nuclear localization sequence, the stretch of basic amino acids that bind importin α. Yet STAT1 dimers can associate with NPI-1, an importin-α homologue (Sekimoto *et al*., 1997). Nuclear localization of STAT1 in response to cytokines requires the amino-terminus of Stat1 (Strehlow and Schindler, 1998). Hence, it is possible that dimers of the amino-terminus of STAT1 create the structure required for binding to nuclear import

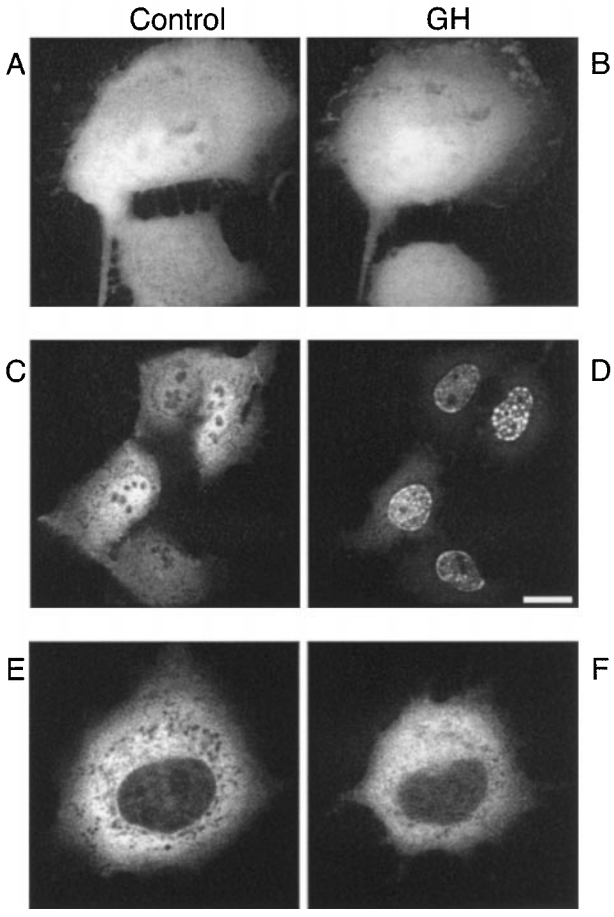

Figure 3 GH promotes nuclear localization of GFP-STAT5B. A–F, 2C4 cells were transfected with cDNA expression vectors (3 μg) for either GHR and GFP (**a** and **b**), GHR and GFP-STAT5B (**c** and **d**), or GHR and DNA binding domain mutant GFP-STAT5B$_{VVVI}$ (amino acids ^{466}VVVI^{469} to alanine; 6 μg, **e** and **f**). Cells were imaged by laser scanning confocal microscopy prior to (**a**, **c**, and **e**) and following (**b**, **d** and **f**) 40–60 min treatment with 500 ng/ml GH. Images **a**, **c**, and **e** are of the same cell as **b**, **d**, and **f**, respectively. Scale bar (**d**) represents 15 μm for **a–d** and 8 μm for **e** and **f**. (Reprinted in modified form with permission from Herrington J, Rui L, Luo G, Yu-Lee L and Carter-Su C. *J. Biol. Chem*., **274**, 5138–5145, 1999)

proteins. Alternatively, other proteins that associate with STATs may serve as chaperones for STAT entry into the nucleus. Ligand/receptor complexes have recently been suggested as candidates for this role (Johnson *et al*., 1998a,b). Interestingly, PRL-induced nuclear localization, but not tyrosyl phosphorylation, of STAT5 is inhibited by mutation of tyrosine 382 in the cytoplasmic domain of the PRL receptor (Ali and Ali, 1998). Green fluorescent protein (GFP)-STAT5B in conjunction with laser scanning confocal imaging revealed that a mutation that prevents binding of STAT5B to DNA (^{466}VVVI^{469}) abrogates GH-stimulated nuclear localization (Figure 3). This mutant fusion protein was tyrosyl phosphorylated and dimerized in response to GH (Herrington *et al*., 1999). This suggests that either high affinity binding to DNA contributes to nuclear accumulation of STAT5B or that this region is crucial for two functions, namely accumulation of STAT5B in the nucleus and DNA binding.

Activation of STAT phosphorylation by SH2-Bβ

Because activation of STAT proteins is highly dependent upon the activity of JAK and the number of phosphorylated tyrosines within cytokine receptor/JAK complexes, it is clear that proteins that regulate JAK activity would also regulate STAT activation. One such protein that regulates the activity of JAK2 is the SH2 domain containing protein SH2-Bβ (Figure 4). SH2-Bβ was initially identified as a JAK2-interacting protein that is tyrosyl phosphorylated in response to GH and other cytokines that activate JAK2 (Rui *et al*., 1997). While the structure of SH2-Bβ suggested that it was likely to serve as an adapter protein for JAK2, it has recently been shown to be a potent activator of JAK2 (Rui and Carter-Su, 1999). Consistent with this activation role, overexpression of wild-type SH2-Bβ has been shown to greatly enhance the ability of GH to stimulate the tyrosyl phosphorylation of STAT3 and STAT5B (Rui and Carter-Su, 1999) (Figure 5). In addition, mutating the critical lysine in the FLVR domain of SH2-Bβ, which prevents activation of JAK2 by SH2-Bβ, also inhibits the ability of STAT5B to migrate to the nucleus (Herrington *et al*., 2000).

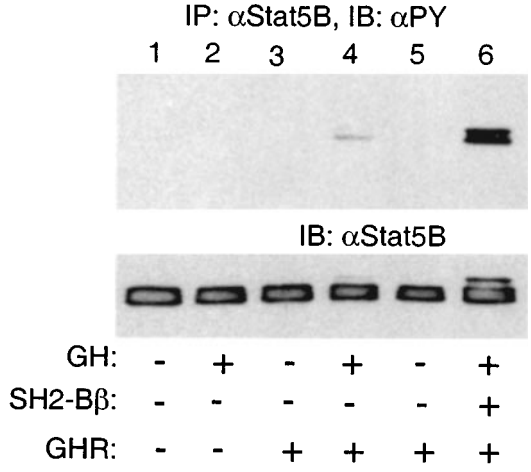

Figure 5 SH2-Bβ enhances GH-induced tyrosyl phosphorylation of STAT5B. COS cells were co-transfected with plasmids encoding GH receptor (GHR), STAT5B, and SH2-Bβ as indicated. Twenty-four hours after transfection, cells were deprived of serum overnight and treated with 50 ng/ml GH for 15 min. STAT5B was immunoprecipitated with αSTAT5B, immunoblotted with αPY (upper panel), and reprobed with αSTAT5B (lower panel). (Reprinted in modified form with permission from Rui L and Carter-Su C. *Proc. Natl. Acad. Sci. USA*, **96**, 7172–7177, 1999)

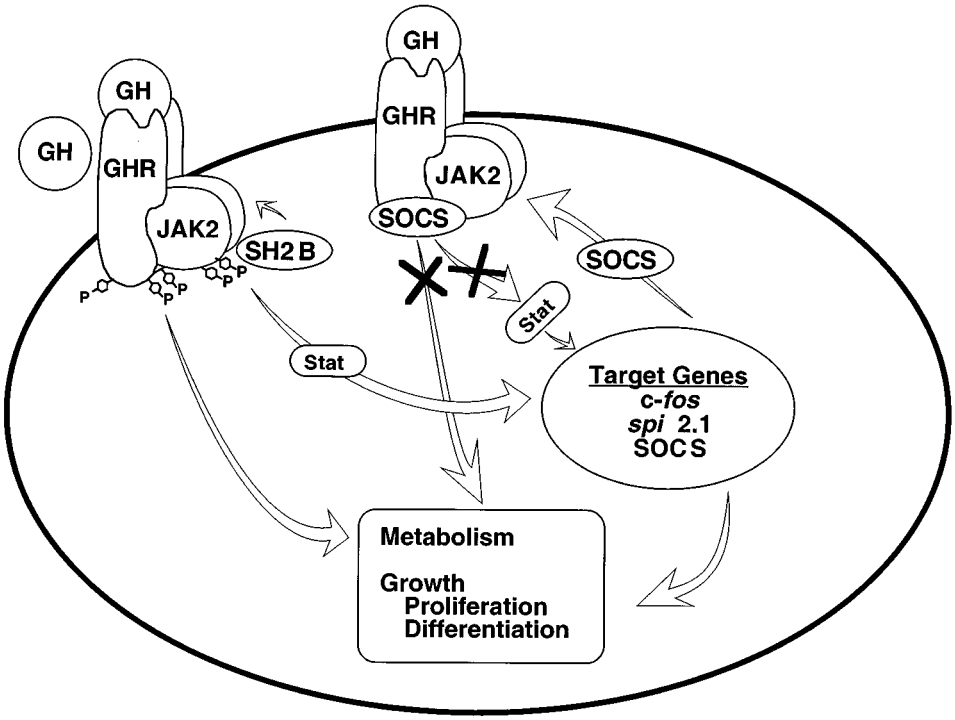

Figure 4 Regulation of STATs by SH2-B and SOCs family members. P, phosphotyrosines; JAK, Janus kinase; Spi, serine protease inhibitor; SOCS, suppressor of cytokine signaling

The role of tyrosine phosphatases in the termination of GH-activated STAT signaling

Termination of GH-activated STAT signaling is thought to involve at least two types of signaling events: dephosphorylation of tyrosines in GHR, JAK2 and STATs by phosphatases and expression of the suppressors of cytokine signaling (SOCS) family of proteins. Termination of GH-activated STAT signaling presumably involves activation and/or recruitment of a protein tyrosine phosphatase to GHR/JAK2 signaling complexes. This phosphatase would dephosphorylate GHR, JAK2, or the STATs themselves to down-regulate signaling. Since STATs are generally thought to require phosphorylated GHR for docking and subsequent activation by JAK2, dephosphorylation of GHR would be expected to terminate further STAT activation. Dephosphorylation of GHR may also signal its internalization and degradation (Gebert *et al*., 1999a,b). Dephosphorylation of the critical activating tyrosine within the kinase domain of JAK2 would be expected to deactivate JAK2 whereas dephosphorylation of the tyrosine in STAT critical for dimerization and high affinity DNA binding would also effectively terminate further signaling. Indeed, termination of activated STAT5B is thought to involve dephosphorylation of JAK2 and STAT5B (Gebert *et al*., 1997, 1999a).

The phosphatase(s) responsible for the above dephosphorylation events are unknown. Two candidate phosphatases that might serve as negative regulators of GH-activated signaling are the SH2 domain-containing phosphatases SHP-1 and SHP-2 (Feng *et al*., 1993). SHP-1 has been demonstrated to negatively regulate JAK/STAT signaling mediated by the various cytokine receptors in hematopoietic cells (David *et al*., 1996; Haque *et al*., 1998; Klingmuller *et al*., 1995; Yi *et al*., 1993). SHP-1 directly associates with JAK2 (Jiao *et al*., 1996) and SHP-1 has been suggested to play a role in the dephosphorylation of JAK2 in liver in response to GH (Hackett *et al*., 1997). Both SHP-1 and SHP-2 have been suggested as potential phosphatases for Stat5 (Ram and Waxman, 1997; Yu *et al*., 2000). SHP-2 associates with GHR in response to GH (Kim *et al*., 1998; Stofega *et al*., 1998) and mutation of tyrosine residues in GHR that serve as binding sites for SHP-2 prolong tyrosyl phosphorylation of GHR, JAK2 and STAT5B in response to GH (Stofega *et al*., 2000).

Other phosphatases are also likely involved in dephosphorylating GHR, JAK2 and STATs. For example, deactivation of JAK2 is slowed by the non-specific, serine/threonine kinase inhibitor H-7 and by the protein synthesis inhibitor, cyclohexamide (Fernandez *et al*., 1998; Gebert *et al*., 1999a). Hence, it has been suggested that deactivation of JAK2 may occur by the serine/threonine kinase-dependent expression of a tyrosine phosphatase (Gebert *et al*., 1999a).

The role of SOCS proteins in the termination of GH-activated STAT signaling

SOCS proteins constitute a negative feedback loop within the cell: their expression is induced by cytokines and hormones and they act to inhibit further signaling of the activated receptor complexes (Endo *et al*., 1997; Naka *et al*., 1997; Starr *et al*., 1997) (Figure 4). GH induces expression of SOCS-1, -2, -3 and CIS in rat liver to varying degrees and with different kinetics (Adams *et al*., 1998; Ram and Waxman, 1999; Tollet-Egnell *et al*., 1999). The signaling pathways involved in induction of SOCS expression in response to GH remain to be fully characterized, but are predicted to require STAT signaling (Naka *et al*., 1997). In the case of SOCS-2 and SOCS-3, STAT5B is thought to mediate GH induction of their expression in liver (Davey *et al*., 1999a).

More is known about how SOCS proteins inhibit GH-activated STAT signaling. SOCS-1 is best understood. SOCS-1 can interact directly with all four members of the JAK family (Endo *et al*., 1997; Naka *et al*., 1997). SOCS-1 inhibition of JAK2 activity requires interactions between the SH2 domain of SOCS-1 and the kinase activation loop of JAK2 (Yasukawa *et al*., 1999). It is thought that a region N-terminal to the SH2 domain acts as a inhibitory pseudosubstrate of JAK2 (Narazaki *et al*., 1998; Yasukawa *et al*., 1999). Consistent with a direct action of SOCS-1 on JAK2 activity, SOCS-1 inhibits tyrosyl phosphorylation of overexpressed JAK2 even when GH receptor is not present (Hansen *et al*., 1999; Ram and Waxman, 1999). Constitutively expressed SOCS-1 virtually abolishes GH-dependent STAT5B tyrosyl phosphorylation, DNA binding and STAT5-mediated gene expression (Adams *et al*., 1998; Hansen *et al*., 1999, Ram and Waxman, 1999). Interestingly SOCS-1 can also bind the cytoplasmic domain of GHR fused to GST, suggesting GHR may direct SOCS-1 to JAK2 (Hansen *et al*., 1999; Ram and Waxman, 1999).

SOCS-2 action on GH-induced STAT signaling is less clear. Constitutive overexpression of SOCS-2 has been reported to enhance, partially inhibit and have no effect on STAT5-driven gene expression (Adams *et al*., 1998; Hansen *et al*., 1999; Karlsson *et al*., 1999; Ram and Waxman, 1999). Recent studies have suggested that these disparities may be due to different levels of SOCS-2 protein and suggest that, at high levels, SOCS-2 may enhance GH signaling by relieving inhibition from more potent, endogenous SOCS molecules (Favre *et al*., 1999).

SOCS-3 is rapidly and prominently induced in liver by GH (Adams *et al*., 1998; Tollet-Egnell *et al*., 1999), suggesting it may have a prominent role in the termination of STAT signaling. In contrast to SOCS-1, SOCS-3 inhibits JAK2 by a mechanism requiring GHR (Hansen *et al*., 1999). The mechanism by which this occurs, however, is unclear. Studies using the cytoplasmic domain of GHR tyrosyl phosphorylated in bacteria by kinases other than JAK2 have shown that SOCS-3, as well as the other GH-regulated SOCSs, can bind directly to GHR. However, attempts at identifying the regions and specific tyrosines required in GHR using truncated and mutated GHR fusion proteins have yielded conflicting answers (Hansen *et al*., 1999; Ram and Waxman, 1999). These studies highlight the necessity of using GHR phosphorylated by JAK2 to determine the mechanism of JAK2 inhibition by SOCSs that require GHR.

CIS, like SOCS-3, is prominently induced in liver by GH (Adams *et al*., 1998; Tollet-Egnell *et al*., 1999; Ram and Waxman, 1999) and appears to also require GHR to inhibit JAK2 (Ram and Waxman, 1999). CIS

is known to associate with tyrosyl phosphorylated interleukin-3ß and erythropoietin receptors (Yoshimura *et al*., 1995). Consistent with an important physiological role of CIS in GH signaling, transgenic mice constitutively expressing CIS have reduced body weight and lower major urinary protein (MUP) levels in their urine (Matsumoto *et al*., 1999), characteristics of mice deficient in Stat5.

The role of STAT5 in GH-mediated gene expression

STAT5a and STAT5b have been strongly implicated in GH signaling through their participation in transcriptional activation of multiple GH-regulated genes. GH induces the binding of STAT5 proteins to IFNγ activated sequence (GAS)-like elements (GLE) in several different genes, including the GH-sensitive *spi 2.1, Insulin 1* and *p450 CYP3A 6 beta-hydroxylase* genes (Bergad *et al*., 1995; Galsgaard *et al*., 1996; Hansen *et al*., 1996; Subramanian *et al*., 1995; Wood *et al*., 1995). STAT5 mediates GH-dependent transcriptional activation of promoter-reporter constructs containing the STAT5-binding promoter elements of these genes (Bergad *et al*., 1995; Galsgaard *et al*., 1996; Hansen *et al*., 1996; Sotiropoulos *et al*., 1996; Subramanian *et al*., 1995; Wood *et al*., 1995).

STAT5 involvement in the expression of serine protease Inhibitor 2.1

The role of STAT5 in GH regulated gene transcription has been elucidated in particular detail by analysis of the gene encoding the serine protease inhibitor Spi 2.1. The expression of liver-specific *spi* 2.1 is tightly controlled by GH at the transcriptional level (LeCam *et al*., 1987; Paquereau *et al*., 1992). This GH-regulated gene was originally identified through the screening of rat hepatic cDNA libraries (LeCam *et al*., 1987; Yoon *et al*., 1987). Promoter analysis identified a sequence which could confer GH responsiveness to a heterologous promoter in primary hepatocytes (Yoon *et al*., 1990). Further analysis revealed two GH Response Elements (GHRE) (Paquereau *et al*., 1992). A nuclear factor, subsequently identified as STAT5 (Wood *et al*., 1995), was found to bind synergistically to two GLE sequences (GLE 1 and 2) (Bergad *et al*., 1995) in the GHRE2. Both GLE 1 and 2 bind STAT5A and 5B in a GH-dependent manner for transcriptional activation (Bergad *et al*., 1995; Sliva *et al*., 1994; Wood *et al*., 1995). A GH-responsive *spi* 2.1 GLE-containing reporter was used in the evaluation of those regions of the GH receptor involved in changes in gene expression. These studies demonstrate that the proline-rich region of the GH receptor required for JAK2 activation is required for *spi* 2.1 expression (Goujon *et al*., 1994) and that a GH receptor containing any one of five tyrosines in the cytoplasmic domain is sufficient to induce expression of the *spi* promoter-reporter gene (Enberg *et al*., 1994).

The physiological regulation of *spi* 2.1 expression by GH involves other transcription factors in addition to STAT5. In the *spi* 2.1 promoter, STAT5 has been shown to associate with the glucocorticoid receptor (GR) and YY1 (Bergad *et al*., 2000). In addition, five C/EBP sites have been identified in the *spi* 2.1 promoter (LeCam *et al*., 1994), one of which coincides with GLE1 and is essential for GH-dependent enhancer function (LeCam *et al*., 1994). The more downstream GHRE1 of *spi* 2.1 contains a GAGA sequence which has been shown to be essential for initiating GH-promoted *spi* 2.1 expression, possibly through chromatin remodeling (Simar-Blanchet *et al*., 1998). These studies indicate that GH-stimulated transcription of *spi* 2.1 is complex, and involves STAT5 as well as other proteins.

STAT5 involvement in GH regulation of genes associated with growth and metabolism

STAT5 has recently been shown to contribute to regulation of several components of the GH-insulin-like growth factor 1 (IGF-1) axis, long recognized as fundamental for growth-promoting actions of GH. In the circulation, IGF-1 forms a complex with the Acid Labile Subunit (ALS) and IGF-binding protein 3 (Boisclair *et al*., 1996; Dai *et al*., 1994). GH was found to stimulate ALS transcription in a hepatoma cell line (Ooi *et al*., 1997). A GH-responsive DNA sequence in ALS gene promoter (Boisclair *et al*., 1996) contains two ALS-GAS sites. STAT5A and STAT5B bind to the ALS-GAS1 in response to GH and mediate transcriptional activation of the ALS gene (Ooi *et al*., 1997). Although it is well-recognized that GH stimulates IGF-1 production and gene expression in liver and other cell types (Bichell *et al*., 1992; Isgaard *et al*., 1988; Mathews *et al*., 1986; Peter *et al*., 1993), identifying the GH-regulated transcription factors involved has been challenging. Establishment of novel GH-responsive cell lines has facilitated demonstration that STAT5 participates in GH-stimulated expression of IGF-1. STAT5 has been implicated in GH-dependent activation of the salmon IGF-1 promoter in Hep3B cells expressing the GH receptor (Meton *et al*., 1999). In these cells, the GH-dependent activation of the IGF-1 gene involves a synergistic action of STAT5 and HNF-1α when they are simultaneously expressed. Similarly, in C6 glioma cells overexpressing GH receptor and Jak2, GH stimulated reporter expression via the rat IGF-1 promoter. In these cells, GH also activated STATs 1, 3 and 5 (Benbassat *et al*., 1999), consistent with involvement of STATs in GH-regulated IGF-1 gene expression.

In addition to participating in regulation of genes associated with GH-regulated growth, STAT5 also contributes to genes associated with metabolic regulation by GH. GH excess is associated with hyperinsulinemia, and GH can stimulate expression of the insulin gene in the pancreatic β-cell line RIN-5AH (Billestrup and Martin, 1985). Such stimulation requires the C-terminal half of the cytoplasmic domain of the GH receptor and is associated with a GH-induced rise in intracellular calcium (Billestrup *et al*., 1995; Moldrup *et al*., 1991). A GH responsive GAS-like element in the insulin promoter can bind GH-induced STAT5 and is able to mediate GH-induced gene expression in RIN-5AH cells (Galsgaard *et al*., 1996).

GH-regulated binding of STAT5 to the GAS-like element in the promoter of the β-casein gene has also been observed in nuclear extracts from several different cell types (Gouilleux *et al*., 1995; Han *et al*., 1996; Ram *et al*., 1996; Silva *et al*., 1996; Smit *et al*., 1996, 1997;

Tourkine *et al*., 1995). An initial study reported that GH failed to induce transcription of a β-casein promoter-luciferase construct in COS cells transiently transfected with an ovine STAT5 cDNA expression vector (Gouilleux *et al*., 1995). In a subsequent report, GH was observed to stimulate transcriptional activation of a β-casein promoter luciferase reporter construct in CHO and 293 cells transfected with a GH receptor cDNA expression vector (Sotiropoulos *et al*., 1996), although prolactin, rather than GH regulates expression of this gene *in vivo*.

GH-regulated genes utilizing STATs 1 and 3

Initial evidence implicating STATs in GH action was based on observations that GH induced the phosphorylation and binding of STATs 1 and 3 to the Sis-inducible element of c-*fos* (Campbell *et al*., 1994; Gronowski and Rotwein, 1994; Gronowski *et al*., 1995; Meyer *et al*., 1994). Induction of the binding of STATs 1 and 3 occurred readily in several GH-responsive systems, including 3T3-F442A fibroblasts and liver from hypophysectomized rats treated with GH. Tandem copies of the SIE mediated transcription in response to GH in COS cells overexpressing GHR and STAT3 (Sotiropoulos *et al*., 1996). The physiological relevance of STATs 1 and 3 in GH-regulated transcription of c-*fos* is supported by mutational and deletion analysis of the c-*fos* enhancer. These studies revealed that the combined presence of the SIE, Serum Response Element (SRE), and an adjacent AP-1 site are required for full induction by GH; progressive deletions of each of these elements led to progressive decreases in reporter expression (Chen *et al*., 1995). The involvement of multiple regulatory elements in the physiological regulation of c-*fos* is also suggested by transgenic mouse studies in which mutations in specific regulatory regions (SIE, SRE, AP-1 and Cyclic AMP Response Element) were introduced into fos-promoter/LacZ constructs. Each of the multiple control elements was found to be required for normal c-*fos* expression *in vivo* (Robertson *et al*., 1995). The SRE also mediates transcription in response to GH, and GH can increase binding to C/EBP and AP-1 sites in c-*fos*. Whether these GH-regulated sequences, including the SIE, cooperate with each other in GH-promoted c-*fos* expression remains to be determined. Among other GH-regulated genes utilizing STATs 1 and 3, the promoter of the gene encoding Interferon Regulatory Factor (IRF-1) has been shown to bind STATs 1 and 3 induced in liver by GH treatment of hypophysectomized rats (Le Stunff and Rotwein, 1998). Thus, it is clear that STATs 1, 3 and 5 can participate in regulation of multiple genes in response to GH. Such regulation is likely to involve participation of other transcription factors as well as STATs, and to coordinate the expression of multiple GH-responsive genes in a variety of cell types.

Pulsatile GH secretion determines regulation of multiple genes via STAT5

GH secretion from the pituitary gland is pulsatile. In many species including rat, chicken and humans the pattern in males is characterized by higher peaks and deeper troughs than in females (Asplin *et al*., 1989; Eden *et al*., 1982; Johnson, 1988; Tannenbaum and Martin, 1976; Winer *et al*., 1990). The sexually dimorphic pattern of GH secretion produces distinct differences in the expression of certain genes. Notable among these are hepatic genes of the cytochrome P450 (CYP) family, particularly those in the gene family CYP2 and CYP3 which encode enzymes involved in the metabolism of endogenous steroids and lipophilic compounds (Nebert, 1991; Waxman *et al*., 1995). The male-specific rat liver P450s include androgen 16α and 2α hydroxylase CYP2C11, the steroid 6β-hydroxylase CYP3A2, testosterone 15α-hydroxylase CYP2A2, and the fatty acid ω-hydroxylase CYP4A2. The gene encoding hamster CYP 3A10/6β-hydroxylase has been shown to contain a GAS-like element which binds STAT5 in animals exhibiting a male pattern of pulsatile GH release (Subramanian *et al*., 1995), indicating the presence of a mechanism dependent on the pulsatile GH secretion pattern at the level of gene transcription and STAT5. The female-specific steroid sulfate 15β-hydroxylase CYP2C12 is expressed under conditions of continuous GH secretion (Legraverend *et al*., 1992; Nebert *et al*., 1991). Both 2C11 and 2C12 are regulated by GH at the transcriptional level (Sundseth *et al*., 1992).

STAT5B is responsive to pulsatile GH

Following a pulse of GH, activation of STAT5B in rat liver is fully restored when a second GH pulse is given 4 h later. In contrast, activation of STAT1 and STAT3 shows strong desensitization such that 4 h is not sufficient to restore full responsiveness to GH (Ram *et al*., 1996). Thus, STAT5B is unique among the GH-activated STATs in that it is appropriately sensitive to pulsatile GH, suggestive that many of the physiological actions of GH in males may be mediated in part by STAT5B. Studies of gene expression and body growth using STAT5B-deficient mice have supported this view (Davey *et al*., 1999c; Teglund *et al*., 1998; Udy *et al*., 1997).

Given that STAT5B appears to be important in transducing the actions of pulsatile GH, what are the properties of the GH signaling cascade that underlie this behavior? Studies on the activation and deactivation cycle of STAT5B in the immortalized rat hepatocyte-derived line, CWSV-1, have provided insight into this question. In general, the activated GHR/JAK2 complex is thought to possess a longer lifetime than activated STAT5B, such that the active GHR/JAK2 complex can initiate multiple STAT5B activation cycles prior to deactivation of GHR/JAK2 (Gebert *et al*., 1999a). Following a pulse of GH, deactivation of STAT5B as assessed by loss of DNA binding is rapid ($\sim$30 min) and is thought to involve dephosphorylation of STAT5B and deactivation of JAK2 (Gebert *et al*., 1997, 1999a).

Once STAT5B is deactivated, recovery of full responsiveness to GH requires approximately 2 more hours (Gebert *et al*., 1997). The mechanisms involved in resetting STAT5B sensitivity to GH are less clear. Recovery of full responsiveness does not appear to require new protein synthesis (Gebert *et al*., 1997). However, the serine/threonine kinase inhibitor H7 shortens the interpulse interval required for recovery of full responsiveness of STAT5B to GH. The

mechanism by which H7 shortens the required interpulse interval is unknown but may involve inhibition of GHR internalization and degradation (Gebert *et al*., 1999a).

Continuous GH exposure, characteristic of the female pattern of GH secretion, results in low level activation of JAK2 and STAT5B (approximately 10–20% of the maximum level induced by a GH pulse) (Gebert *et al*., 1999b). The lower level is thought to be due to increased phosphotyrosine phosphatase activity towards GHR/JAK2 and STAT5B. Unlike the case of GH pulse signaling to STAT5B, cycloheximide eliminates STAT5B activation with continuous GH exposure. Dephosphorylation of GHR leading to internalization and degradation has been suggested as a cause for the requirement for new protein synthesis (Gebert *et al*., 1999b).

The physiological importance of STAT5A and STAT5B as determined by knockout studies

STAT knockout mice have provided significant insight into the physiological role of the different STAT proteins. In the case of GH, mice deficient in STAT5A and/or STAT5B have provided the greatest insight into the role of STAT proteins in the actions of GH as will be discussed below. In contrast, STAT1 and STAT3 knockout studies have been less informative. The absence of STAT3 results in embryonic lethality (Takeda *et al*., 1997), thus it is not possible to use STAT3 knockout mice to gain insight into the role of STAT3 in GH actions. The STAT1 deficient mice are not small (Durbin *et al*., 1996; Meraz *et al*., 1996), suggesting that STAT1 may not be a major determinant of body growth. Whether it plays a role in other actions of GH has not been investigated.

When STAT5A-deficient mice were generated, STAT5A was found to be required for mammary gland development and lactogenesis, two processes mediated by PRL. No effect on body growth was noted (Liu *et al*., 1997). A second group has confirmed and extended these observations (Teglund *et al*., 1998). Body growth, serum IGF-1 levels, and expression of GH-regulated genes were not altered in STAT5A-deficient mice of either sex (Teglund *et al*., 1998). These results suggest that Stat5A plays a minor role in GH-regulated processes or that loss of STAT5A can easily be compensated by other factors, most notably STAT5B.

In striking contrast, mice deficient in STAT5B have pronounced impairment of body growth, especially in males (Teglund *et al*., 1998; Udy *et al*., 1997) (Figure 6). Male STAT5B-deficient mice grow at a rate similar to normal females (Udy *et al*., 1997). Serum IGF-1 levels are reduced in males but not females (Teglund *et al*., 1998). Expression of the testosterone 16α-hydroxylase CYP2D9, normally expressed higher in males, is reduced to female levels in STAT5B-deficient male mice (Teglund *et al*., 1998; Udy *et al*., 1997). In contrast, testosterone 15α-hydroxylase CYP2A4, normally repressed in males, is increased in STAT5B-deficient male mice to female levels (Teglund *et al*., 1998; Udy *et al*., 1997). Thus, STAT5B appears to be of major importance in transducing the sexual dimorphic pattern of GH secretion into sex-specific patterns of liver gene expression and body growth.

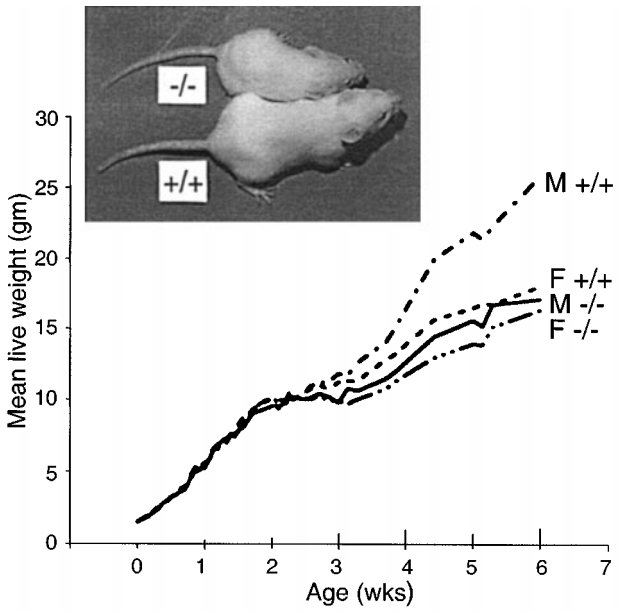

Figure 6 STAT5B is required for male pattern growth. Body weights of wild-type males were significantly different from STAT5B−/− males (M) and females (F) ($P<0.001$). No significant differences were seen between STAT5B−/− males, wild-type females, and STAT5B−/− females. (Reprinted in modified form with permission from Udy GB, Towers R, Snell RG, Wilkins RJ, Park S-H, Ram PA, Waxman DJ and Davey HW. *Proc. Natl. Acad. Sci. USA*, **94**, 7239–7244, 1997)

Studies comparing liver gene expression in hypophysectomized normal and STAT5B-deficient mice given GH pulse replacement support this idea (Davey *et al*., 1999b).

Given these data it is tempting to think of STAT5A as a female-specific mediator of PRL action and STAT5B as a male-specific mediator of GH action. Such categorization is likely an over-simplification for several reasons. STAT5B deficiency in females alters both liver gene expression and body growth, albeit modestly (Teglund *et al*., 1998; Udy *et al*., 1997). Deletion of STAT5A and STAT5B together supports the view for redundancy in function of these proteins in GH action. The most severe defects in liver gene expression and body growth, affecting both sexes, are found when mice lack both STAT5A and STAT5B (Teglund *et al*., 1998). Thus, both STAT5A and STAT5B, acting in concert, are apparently required for normal GH-dependent growth.

The establishment of mice deficient in the STAT5 proteins has led to the rapid advancement of our understanding of STAT5 signaling in GH physiology. In addition to body growth and liver gene transcription, STAT5B is reported to be involved in the lipolytic action of GH on adipose tissue (Fain *et al*., 1999). Yet other GH signaling molecules are likely to play similarly important roles. Hypophysectomized STAT5B-deficient male mice given GH pulse replacement suggest that STAT5B is required for pulsatile GH-driven growth (Davey *et al*., 1999b). However, male and female mice deficient in STAT5A and STAT5B either alone or in combination still grow, although significantly slower than normal. Whether this growth is GH-dependent awaits further studies. Also, STAT5 proteins appear not to be required for the insulin-like action of GH on glucose metabolism

and leptin release (Fain *et al*., 1999). Importantly, STATs have been shown to be regulated by GH in normal human fibroblasts and to be altered in cells from Laron Syndrome patients (Freeth *et al*., 1998).

Summary and conclusions

It is clear that STATs are key contributors to GH signaling and to the mechanisms by which GH activates genes that lead to its physiological actions. STATs 1, 3 and 5 are tightly regulated by GH-GHR-JAK2 interactions, and participate in the regulation of many genes, including genes associated with growth and metabolic effects of GH. Studies using knockout mice have substantiated a role for STATs 5A and 5B in GH-dependent growth regulation. It is notable that STAT5B appears to mediate the complex regulation of sexually dimorphic gene expression by 'interpreting' the GH secretion pattern in males *vs* females. Further understanding of the versatile roles of STATs in GH signaling and regulation of gene expression will add to our understanding of the multiple physiological actions of GH.

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