STAT5b Is Required for GH-Induced Liver Igf-I Gene Expression

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Although the increased expression of Igf-I in liver in response to GH is well characterized, the intracellular signaling pathways that mediate this effect have not been identified. Intracellular signaling molecules belonging to the Janus kinase-signal transducer and activator of transcription 5b (JAK2-STAT5b) pathway are activated by GH and have previously been shown to be required for sexually dimorphic body growth and the expression of liver cytochrome P450 proteins known to be regulated by the gender-specific temporal patterns of pituitary GH secretion. Here, we evaluate the role of STAT5b in GH activation of Igf-I by monitoring the induction of Igf-I mRNA in livers of wild-type and Stat5b−− mice stimulated with exogenous pulses of GH. GH induced the expression of liver Igf-I mRNA in hypophysectomized male wild-type, but not in hypophysectomized male Stat5b−− mice, although the Stat5b−− mice exhibit both normal liver GH receptor expression and strong GH induction of Cytokine-inducible SH2 protein (Cis), which is believed to contribute to the down-regulation of GH-induced liver STAT5b signaling. Thus, STAT5b plays an important and specific role in liver Igf-I gene expression. (Endocrinology 142: 3836–3841, 2001)

THE EFFECTS OF GH on postnatal growth are mediated, at least in part, by the 70 amino acid peptide IGF-I, which is produced both in the liver (approximately 75% of total circulating IGF-I) and in peripheral tissues. Gene deletion studies have established that liver-derived IGF-I is dispensable for normal body growth, suggesting that IGF-I produced locally in peripheral tissues mediates the major effects of GH on postnatal growth (1–7) and that liver-derived IGF-I functions as a component of insulin action in peripheral tissues (8). However, the elevated plasma GH levels seen in liver IGF-I-deficient mice (8) may support growth via increased peripheral tissue expression of IGF-I, raising the possibility that liver-derived IGF-I may indeed help mediate the growth effects of GH under normal physiological conditions.

GH-stimulated dimerization of GH receptors on the cell surface of target tissues results in the activation of multiple intracellular signaling molecules, including MAP kinase, insulin receptor substrates, phosphoinositol 3′-phosphate kinase, diacylglycerol, PKC, intracellular calcium, and STAT signaling pathways (9–11). These intracellular factors mediate GH-stimulated changes in gene expression, and have diverse effects on growth and metabolism (11–13).

Although GH treatment of hypophysectomized rats induces Igf-I gene transcription within 30 min (14), the signaling pathways used by GH to activate Igf-I expression are unknown (11). One possibility is that GH-stimulated Igf-I expression is regulated by the JAK-STAT5b pathway, which we have shown to be required for sexually dimorphic body growth and also for the expression of the major urinary proteins and several cytochrome P450 enzymes in murine liver (15, 16). These well characterized gender differences in body growth and gene expression are particularly striking in rodents and result from the distinct temporal patterns of pituitary GH secretion that occur in males and females (17).

The availability of mice that are deficient in STAT5b has provided a simple in vivo model to test the hypothesis that GH-induced Igf-I expression is dependent on STAT5b signaling. In the present study, wild-type (WT) and Stat5b−− male mice were hypophysectomized to eliminate endogenous GH, and then GH was replaced by injections given twice daily. Expression of Igf-I mRNA was stimulated by GH in livers of WT, but not Stat5b−− mice, providing strong evidence for the involvement of STAT5b in GH-induced liver Igf-I gene expression.

Materials and Methods

Animals

Male 129 X BALB/c WT and Stat5b−− mice were hypophysectomized by the parapharyngeal route (18) at 4–17 wk of age and maintained on a 12-h light, 12-h dark schedule, with free access to food and drinking water (supplemented with 5% glucose wt/vol) for 8 wk. Bovine GH (2 µg per g body weight; American Cyanamid Co., Princeton, NJ) was then injected intraperitoneally at 12-h intervals for 7 d. This protocol has previously been shown to reestablish male-specific gene expression patterns in GH-deficient mice (16, 19). No thyroxine was given before or during this experiment, although some mice had been treated for 1 wk for an independent experiment to measure major urinary protein expression (16), at least 2 wk before the GH treatment. There was no glucocorticoid replacement at any time. Liver tissue from 3-month-old intact male and female WT and Stat5b−− mice was also analyzed. Mice were killed by CO2 asphyxiation (at similar times after GH injection for both WT and Stat5b−− mice), and livers were collected and frozen in liquid nitrogen.

Abbreviations: Hx, Hypophysectomized; KO, knockout; WT, wild-type.
The experimental procedures were approved by the Ruakura Animal Ethics Committee acting in accordance with the guidelines of the New Zealand Animal Ethics Advisory Committee.

**RNA isolation, Northern analysis, and quantitative PCR**

Total RNA was extracted using TRIZOL (Life Technologies, Inc.). The RNA (15 μg) was fractionated on a 1% formaldehyde-agarose gel, then transferred and fixed to Hybond N+ (Amersham Pharmacia Biotech). cDNA probes were labeled with [-α-32P]dCTP (Rediprime II labeling kit, Amersham Pharmacia Biotech). For Igf-1 (ovine Igf-1 cDNA, provided by Dr. T. E. Adams, CSIRO, Parkville, Australia), the murine cDNA probes were labeled with [-α-32P]dCTP (Rediprime II labeling kit, Amersham Pharmacia Biotech). For Ghr (ovine Ghr cDNA, provided by Dr. E. A. Wong, Virginia Polytechnic Institute and State University), the hybridization was at 60 C for 7 h (20), and the membrane was washed at 60 C to 0.5 × SSC, 0.1% SDS. For probes of expression for GH receptor (ovine Ghr cDNA provided by Dr. T. E. Adams, CSIRO, Parkville, Australia), Cis (mouse cDNA provided by Dr. D. J. Hilton, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and βactin (21), the Super Hyb kit was used (Molecular Research Center, Inc., Cincinnati, OH). Membranes were exposed to X-OMAT AR film with an intensifying screen at −80 C.

In addition to Northern analyses, we used the ABI Prism 7700 Sequence Detection System (Applied Biosystems) to quantify relative levels of gene expression. TaqMan probes and primers were designed for murine Igf-1, GH receptor (Ghr) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA transcripts, and were used to measure the relative amounts of these mRNAs in liver tissue by real-time quantitative RT-PCR. To detect Igf-1 transcripts, the probe and primers were designed to detect exons 3 and 4, which are common to all Igf-I mRNAs (22, 23) (m[probe] 5′-CTC CAG CAT TCG GAG GCC ACC TC 3′; forward primer 5′-CTT CAA AAG GCC ACC AGT TGT G 3′; reverse primer 5′-GCT CCG GAA GCA ACA CTC ATG CA 3′). For GH receptor mRNA, the primers and probe were designed to detect exons 7 and 8 (m[probe]- 5′-CAC ATG CTT CCA ATA ATG TCT TCG TAC GAA G 3′; forward primer 5′-TTC AGC GAA GTC TCT CGT GTG ATG 3′; reverse primer 5′-AGA ACC ATG GAA ACT GGA TAT CTT CTG 3′). GH binding protein mRNA, which is derived from splicing of an alternative 8A exon (24, 25) is not amplified using these primers, and genomic DNA sequences are not amplified efficiently because the primers span intron sequences. Reverse transcriptase (Superscript II, Life Technologies, Inc.) was used to synthesize cDNA before PCR. Control reactions without reverse transcriptase were included in the assays in order to confirm that DNA, including pseudogene sequences, did not contribute to the quantification results. Gapdh was used to normalize differences in the amounts of template RNA used in the TaqMan assays (m[probe] probe 5′-TGC AAG GGC TCA TGA CCA CAG TCC A-3′; Forward primer 5′-TGG ACC ACC AAT TGC TTA GC 3′; Reverse primer 5′-GTC TGC TGG GTG GCA ATG 3′). Universal PCR Master Mix (PE Applied Biosystems) was used in these studies. The concentration of primers and probe were 300 nt and 200 nt, respectively. The PCR program was 50 C for 2 min; 95 C for 10 min; then 40 cycles of 95 C for 15 sec and 60 C for 1 min.

The relative amounts of mRNA were calculated from the threshold cycle numbers, and are presented relative to the amounts of mRNA in one sample that is arbitrarily set at one. The threshold cycle is the first PCR cycle for which there is a statistically significant increase in fluorescence resulting from the 5′ nuclease activity of the AmpliTaq Gold DNA polymerase. The fluorescence increases exponentially with the amount of PCR product and the threshold cycle reflects the amount of target RNA or DNA in the sample (i.e. more target results in a lower threshold cycle). The threshold cycle for Gapdh was used to standardize the amount of sample RNA in the reaction i.e. the difference in the threshold cycles for Igf-1 or Ghr and Gapdh mRNA was used to calculate the relative amounts of Igf-1 or Ghr mRNAs in each sample.

**Serum IGF-I assay**

Mouse serum IGF-I concentrations were measured after acid-ethanol cryoprecipitation using a RIA, as previously described (26).

**Statistical analysis**

Serum IGF-I concentrations were analyzed by ANOVA of the log transformed concentrations, using Minitab Release 12 (Minitab Inc., State College, PA). The results are presented as the backtransformed geometric means.

TaqMan mRNA data are presented as the means and S.D.s. The PCR threshold cycle differences were transformed using 2^−[(mean cycle)] to determine the relative levels of mRNA expression (because there is a 2-fold exponential increase in the amount of DNA product/fluorescence following each PCR cycle). Levels of significance were calculated by ANOVA using Minitab Release 12. t tests were also used to make comparisons between two groups where appropriate.

**Results**

**Completeness of hypophysectomy**

Several criteria were used to confirm that the pituitaries were successfully removed. These included a marked reduction in the excretion of major urinary proteins in urine (measured 2 and 8 wk following surgery), low serum PRL concentrations, the absence of the pituitaries on post mortem inspection of the base of the skull, and the absence of body growth (18) (data not shown). Following hypophysectomy, the body weights of the mice did not increase; even young mice that weighed about 15 g remained at this weight. A few of the older mice (>30 g) lost weight immediately following hypophysectomy, but there was no apparent difference between WT and Stat5b/−/− mice.

**Liver Igf-I mRNA is induced by GH in hypophysectomized WT but not hypophysectomized Stat5b/−/− mice.**

The levels of Igf-I mRNA in the livers of intact male and female WT and Stat5b/−/− mice, and in male hypophysectomized WT and Stat5b/−/− mice either with or without GH replacement were determined using real-time quantitative PCR (Fig. 1A) and Northern blot analysis (Fig. 1B). STAT5b deficiency, in itself, resulted in a 50% reduction (P < 0.01, t test) in the average Igf-I mRNA in the liver (Fig. 1A). Following hypophysectomy, the levels of Igf-I dropped dramatically, in both WT and Stat5b/−/− male mice, to 8% and 1.3% of the levels found in intact WT mice. GH injection twice daily for 7 d induced the expression of Igf-I mRNA in hypophysectomized WT mice to levels that were similar to the intact WT mice. By contrast, in hypophysectomized Stat5b/−/− mice there was no significant increase in liver Igf-I mRNA in response to GH (Fig. 1A).

These findings were confirmed and extended by Northern blot analysis, which revealed multiple Igf-I mRNA transcripts (ranging in size from about 0.8–8 kb), as is typical of Igf-I expression in mammals. Igf-I mRNA levels were barely detectable on northern blots of liver RNA from untreated hypophysectomized mice. GH injection, twice daily for 7 d, strongly increased the abundance of Igf-I mRNA in the livers of WT mice but not in Stat5b/−/− mice (Fig. 1B), in agreement with the real-time PCR data shown in panel A.

**Serum IGF-I levels follow similar trends to liver mRNA levels.**

Stat5b/−/− mice have about 30% lower levels of serum Igf-I compared with WT mice (15). As expected, hypophysectomy greatly reduced serum Igf-I levels in both WT and Stat5b/−/− mice (Table 1). Pulsatile GH replacement for 7 d stimulated a dramatic 13-fold increase in serum Igf-I in hypophysectomized WT mice (P < 0.001). By contrast, in hypophysec-
Table 1. Average serum IGF-I concentrations in male wild type and Stat5b−/− mice (from Refs. 15 and 28 as indicated), and in hypophysectomized male wild-type and Stat5b−/− mice that were untreated (−GH) or treated for 7 days with GH (+GH) (geometric means derived from backtransformation of log transformed means)

<table>
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<tr>
<th></th>
<th>GH</th>
<th>IGF-I (ng/ml)</th>
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<tr>
<td>Intact</td>
<td></td>
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<tr>
<td>Male wild-type</td>
<td>−</td>
<td>302 ± 19 (Ref. 15)</td>
</tr>
<tr>
<td>MaleStat5b−/−</td>
<td>+</td>
<td>340 ± 6 (Ref. 28)</td>
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<tr>
<td>Hypophysectomized</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male wild-type</td>
<td>−</td>
<td>18±</td>
</tr>
<tr>
<td>MaleStat5b−/−</td>
<td>+</td>
<td>238±</td>
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<td>−</td>
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<td>+</td>
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The hypophysectomized mice are the same as used in the Northern blot (Fig. 1B), except that IGF-I values for two mice, one wild-type −GH and one Stat5b−/−+GH, are missing. The minimum significant ratios for means of the hypophysectomized mice, at the 5% and 1% levels of significance, were 4.3 and 9.2, respectively. Significant differences between the means are indicated by letters. "a" differs from "b", "c" and "d" (P < 0.01); "c" differs from "d" (P < 0.05).

GH induced the expression of Cis mRNA in hypophysectomized liver

In the liver, Cytokine-inducible SH2 protein (Cis) mRNA levels increased in response to GH treatment in intact WT mice and to a lesser extent in Stat5b−/− mice (27). GH-stimulated Cis expression is therefore indicative of a functional GH receptor and intracellular signaling pathway. In the present study, Cis mRNA levels, measured by Northern analysis, increased dramatically in hypophysectomized mice given replacement pulses of GH (Fig. 3). This effect was seen in both WT and Stat5b−/− mice, providing evidence that the livers of both genotypes of hypophysectomized mice are intrinsically responsive to GH stimulation. The expression of Cis following GH pulse treatment in WT mice was 3-fold greater than in Stat5b−/− mice. Phosphorimage signals normalized to β-actin yielded the following relative mRNA values: WT-GH, 9 ± 4; WT + GH, 203 ± 42; Stat5b−/−-GH, 5 ± 2; Stat5b−/− + GH, 61 ± 16. This difference in Cis expression between WT and Stat5b−/− mice, in response to GH, is similar to our previous findings in intact mice (27).

Discussion

Our previous characterization of Stat5b−/− mice (15, 16) confirmed earlier reports (9, 10) on the involvement of STAT5b in intracellular GH signaling. These mice are unable to recognize and respond to the male-specific pulsatile pattern of GH present in blood (16). As a result, the expression of male- and female-specific P450 proteins in the liver is altered, and Stat5b−/− male mice grow at a slower, female-
pressed relative to the level in untreated hypophysectomized male levels reported are normalized to identify the indicated mRNAs in six to nine mice per group. The mRNA Materials and Methods

Stat/H9262 RNA (15

ers Stat/hypophysectomized male WT (WT) and

approximately 30% in addition, we found that serum IGF-I levels were reduced by like growth rate beginning at approximately 3 wk of age. In

FIG. 2. A, Relative levels of Ghr mRNA in livers of WT (WT) and Stat5b−/− (KO) mice and in hypophysectomized (Hx) male mice that were untreated (−) or treated for 7 d with GH (+) as described in Materials and Methods. TaqMan real-time RT-PCR was used to quantify the indicated mRNAs in six to nine mice per group. The mRNA levels reported are normalized to Gapdh mRNA levels and are expressed relative to the level in untreated hypophysectomized male Stat5b−/− mice. Differences between the means are indicated by letters. a differs from b (P < 0.01). B, GH receptor mRNA in individual hypophysectomized male WT (WT) and Stat5b−/− (KO) mice. Total RNA (15 µg) was electrophoresed on a 1% denaturing agarose gel, transferred to nylon membrane and hybridized with radiolabeled Ghr cDNA. The membrane was exposed to X-OMAT-AR film. C, Northern blot from B, stripped and reprobed with a human β actin cDNA probe.

Fig. 3. Cis mRNA in individual hypophysectomized male WT and Stat5b−/− (KO) mice. Total RNA (15 µg) was electrophoresed on a 1% denaturing agarose gel, transferred to nylon membrane and hybridized with radiolabeled Cis cDNA. The membrane was exposed to X-OMAT-AR film.

also found reduced circulating IGF-I levels in Stat5b−/− male mice, but in contrast to our results there was no reduction in IGF-I levels in females (28). However, when both Stat5 genes were disrupted (Stat5a−/−/Stat5b−/− mice) IGF-I levels were reduced in both females and males (28). Body growth in the Stat5a−/−/Stat5b−/− females was also reduced, with the differences becoming apparent at puberty, whereas disruption of either Stat5a or Stat5b alone did not affect female body weight profiles (28).

GH rapidly and transiently stimulates transcription of Igf-I in liver in vivo (14, 29) and activates a number of intracellular signaling pathways (11, 13) including those mediated by STATs 1, 3, 5a, and 5b (9, 10, 30–32). However, the relative importance of these events for GH-induced Igf-I gene expression was not previously resolved by these, or other, experiments (33, 34). Serum IGF-I levels are dramatically reduced in hypophysectomized rats, to about 5% of those found in intact rats, and these low basal levels have facilitated studies of the kinetics of GH-induced Igf-I expression. Using this model, a single injection of GH increases Igf-I transcription within 30 min (14). In the present study, we also used hypophysectomy to eliminate endogenous GH and circulating IGF-I. The induction of liver Igf-I mRNA in WT, but not in Stat5b−/− mice following GH replacement provides a simple demonstration of the requirement for STAT5b in the GH-induced expression of Igf-I in hypophysectomized mice. This requirement of STAT5b for GH pulse stimulation of Igf-I (this study) and male-specific liver cytochrome P450s seen earlier (16) does not reflect a generalized loss of GH signaling, as evidenced by the GH-inducible expression of Cis mRNA in the same mice (Fig. 3).

The present demonstration that STAT5b plays a role in liver Igf-I expression raises several questions. The average level of liver Igf-I mRNA in intact Stat5b−/− mice was 50% of that in WT mice, which is in agreement with our finding that Stat5b−/− mice have serum IGF-I levels that are approximately 30% lower than WT mice (15). This result would not be predicted, however, if the sole signaling pathway for expressing Igf-I was via GH activation of STAT5b. The dramatic reduction in serum IGF-I levels seen in hypophysectomized Stat5b−/− mice (Table 1) indicates that factors secreted by or regulated by the pituitary gland play an important role in maintaining serum IGF-I in a STAT5b-independent manner. That GH is primarily responsible for Igf-I expression is indicated by the near complete restoration of serum IGF-I in response to GH treatment of WT hypophysectomized mice. One possible explanation for these observations is that compensatory mechanisms, involving pituitary-derived factors other than GH, operate in intact but not hypophysectomized Stat5b−/− mice, resulting in substantial expression of Igf-I in liver, and consequently maintenance of plasma levels of IGF-I despite the absence of STAT5b. There is also indirect evidence that pituitary GH secretion may be elevated in Stat5b−/− mice (15, 35), which may stimulate Igf-I expression in peripheral tissues by a STAT5b-independent pathway perhaps involving STAT5a.

A further question is whether GH acts directly to activate Igf-I gene expression via the JAK-STAT5b pathway, or whether GH-activated STAT5b operates via an indirect mechanism to induce Igf-I gene expression. GH has an im-
mediate effect on Igf-I gene transcription in WT hypophysectomized rats (14). However, whereas there may be a direct action of GH on Igf-I transcription in the present experiments, the STAT5b requirement for Igf-I expression may nevertheless be an indirect one, i.e. STAT5b may not directly activate Igf-I gene transcription by directly binding to the promoter or interacting in transcription complexes. STAT5 DNA-binding consensus elements have not been identified in rodent Igf-I 5′ flanking DNA sequences, but the analysis has not been extensive. Moreover, STAT5 tetrmers have the ability to bind to adjacent weak consensus elements that are not revealed in a search for standard STAT5 consensus sequences (36). Further investigation, including a more detailed analysis of Igf-I gene regulatory elements, and a determination of whether a single pulse of GH stimulates a rapid increase in Igf-I mRNA in hypophysectomized WT but not Stat5b−/− mice, will be required to establish the precise role of the STAT5b pathway in GH-induced liver Igf-I gene expression.

Also of interest is whether STAT5b is specifically required for Igf-I gene expression, or whether STAT5a, which shares 92% amino acid identity with STAT5b, acts equivalently in this regard. Greater than 90% of the Stat5 mRNA in liver is Stat5b (37), and thus Stat5b−/− mice are estimated to have dramatically lower levels of Stat5 per se than WT mice. Liver STAT5a protein levels are not altered by Stat5b gene disruption or by hypophysectomy of the Stat5b−/− mice (16) and because the pattern of STAT5a activation in the liver is similar to that for Stat5b (37, 38), the reduced amounts of total STAT5 protein, rather than the loss of Stat5b per se, could explain the lack of GH-induced Igf-I mRNA in the livers of hypophysectomized Stat5b−/− mice.

GH treatment of hypophysectomized Stat5b−/− mice stimulated a modest increase in serum Igf-I levels (P < 0.05), which was greater than the increase in liver Igf-I mRNA levels. This proportionally greater increase in serum Igf-I may reflect changes in extrahepatic Igf-I production, as well as changes in other GH-regulated factors that modulate plasma Igf-I, such as IGFBP3 and the acid labile subunit ALS (39), that may, at least in part, be independent of STAT5b. Alternatively, as in intact Stat5b−/− mice, a portion of serum Igf-I may be derived from GH-JAK2 signaling mediated by Stat5a, which is activated in hypophysectomized Stat5b−/− mice by GH treatment (16). This latter observation, together with the induction of Cis expression in the livers of GH-treated hypophysectomized Stat5b−/− mice noted above, provides clear evidence for a functional GH receptor/JAK2 pathway in these mice. Accordingly, defects in these components can be ruled out as the basis for the unresponsiveness of the Igf-I gene (Fig. 1) and the sexually dimorphic liver cytochrome P450s (16), to GH pulse stimulation in Stat5b−/− mice.

In conclusion, our data provides strong evidence for STAT5b playing an important role in liver Igf-I expression.

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References


35. Luckman SM, McGuinness I, Thomas GR, Robinson ICAF, Udy GR, Davey HW 1998 Signal transducer and activator of transcription, STAT5b, and growth hormone (GH) feedback on somatostatin (SOM) neurons. Society for Neuroscience (Abstract)


38. Choi HK, Waxman DJ 1999 Continuous GH, but not prolactin, maintains low-level activation of STAT5a and STAT5b in female rat liver. Endocrinology 140:5126–5135