Increased sensitivity to GH in liver of Ames dwarf (*Prop1^{df}/Prop1^{df}*) mice related to diminished CIS abundance

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Abstract

To investigate the influence of chronic GH deficiency on GH signaling *in vivo*, we have analyzed Janus kinase (JAK) 2/signal transducers and activators of transcription (STAT) 5 GH signaling pathway, and its regulation by the suppressors of the cytokine signaling SOCS and by the JAK2-interacting protein SH2-B β , in liver of Ames dwarf (*Prop1^{df}/Prop1^{df}*) mice, which are severely deficient in GH, prolactin and TSH, and of their normal littermates. *Prop1^{df}/Prop1^{df}* mice displayed unaltered GH receptor, JAK2 and STAT5a/b protein levels. No significant differences in the basal tyrosine-phosphorylation levels of JAK2 and STAT5a/b were found between both groups of animals. After *in vivo* administration of a high GH dose (5 μ g/g body weight (BW)), the tyrosine-phosphorylation levels of JAK2 and STAT5a/b increased significantly, reaching similar values in normal and dwarf mice.

However, after stimulation with lower GH doses (50 and 15 ng/g BW) the tyrosine-phosphorylation level of STAT5a/b was higher in dwarf mice. The protein content of CIS, a SOCS protein that inhibits STAT5 signaling, was approximately 80% lower in dwarf mice liver, while SOCS-2 and SOCS-3 levels were unaltered. The content of SH2-B β , a modulator of JAK2 activity, was reduced by approximately 30% in dwarf mice, although this was associated with normal JAK2 response to a high GH dose. In summary, *Prop1^{df}/Prop1^{df}* mice display increased hepatic sensitivity to GH, an effect that could be related to the lower abundance of CIS in this tissue. Furthermore, the lower CIS content found in this model of GH deficiency suggests that CIS protein levels are regulated by GH *in vivo*.

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Introduction

Growth hormone (GH) is considered the central endocrine regulator of growth, and it also exerts a wide range of metabolic effects (Waxman & Frank 2000). GH signaling is initiated by the hormone's binding to two GH receptor (GHR) molecules, inducing the transphosphorylation and activation of the receptor-associated tyrosine-kinase JAK2. The activated kinase then phosphorylates GHR on multiple intracellular tyrosine residues, which act as docking sites for the transcriptional activators STAT5a, STAT5b and other intracellular signaling molecules (Carter-Su *et al.* 2000, Zhu *et al.* 2001). Many studies have established STAT5 as a key intracellular mediator of GH action, and STAT5b as the principal regulator of the expression of several genes in rat liver (Choi & Waxman 1999, Herrington *et al.* 2000). The kinetics and magnitude of GH signal transduction are tightly regulated. An important negative regulatory mechanism of GH signaling is the SOCS/CIS (suppressors of cytokine signaling/cytokine-inducible SH2 containing

proteins) family, which is implicated in the regulation of the JAK/STAT pathway of cytokine response. GH induces the expression of CIS and SOCS-1, -2 and -3, apparently via STATs. SOCS proteins are induced by the same pathway they inhibit, therefore acting in a classical negative feedback loop (Greenhalgh & Alexander 2004). The adapter protein SH2-B β was described as a potent activator of phosphorylated JAK2, but it was also proposed to bind to the nonphosphorylated kinase, inhibiting its abnormal activation (Rui & Carter-Su 1999, Rui *et al.* 2000).

Recently, we described that the JAK2/STAT5 pathway of GH signaling is desensitized in two different lines of transgenic mice overexpressing GH, an effect that is mainly related to the markedly high CIS levels found in the liver of these mice (González *et al.* 2002, Miquet *et al.* 2004). CIS acts as a negative modulator by competing with STAT5 for common phosphotyrosine-binding sites on the GHR intracellular domain (Ram & Waxman 2000). SH2-B β protein content and membrane-association were also increased in GH-overexpressing transgenic mice,

which may be implicated in JAK2 desensitization (Miquet *et al.* 2005). In the present study, in order to investigate how the chronic lack of GH influences GH signaling pathway, we evaluated the first steps of GH signal transduction and its regulation by SOCS/CIS proteins and SH2-B β in an *in vivo* model of GH deficiency, the Ames dwarf mouse.

Ames dwarf mice are homozygous for a spontaneous mutation in the prophet of pituitary factor-1 (*Prop-1*) gene, leading to a primary deficiency of GH, prolactin (PRL) and TSH, and a secondary suppression of peripheral insulin-like growth factor (IGF)-I levels (Bartke 1964, 1979, Sornson *et al.* 1996, Bartke *et al.* 1999). They are also a genetic mammalian model of retarded aging and extended longevity (Brown-Borg *et al.* 1996, Bartke *et al.* 2004). They are of normal body size at birth, but postnatal growth is greatly reduced, and adult body size is 30–50% of normal values. GH treatment was reported to increase plasma IGF-I levels, body weight (BW) and other physiologic and biochemical parameters in dwarf mice, indicating that these animals respond to the hormone (Villanua *et al.* 1992, Chandrashekar & Bartke 1993, Brown-Borg & Rakoczy 2003). These features of Ames dwarf mice make them as adequate *in vivo* model to investigate how chronic GH deficiency influences GH signaling. In this work, we have determined the tyrosine-phosphorylation levels of the JAK2/STAT5 GH-signaling pathway components under basal conditions and after GH stimulation, as well as the abundance of the regulatory proteins SOCS/CIS and SH2-B β , in liver of Ames dwarf and normal mice, to assess GH signaling sensitivity under GH-deficit conditions.

Materials and Methods

Animals

We used female Ames dwarf mice (*Prop1^{df}/Prop1^{df}*) and normal (+/+ or +/*Prop1^{df}*) littermates, 6–9 months of age, produced in our breeding colony. Mice were bred in a closed colony with a heterogeneous genetic background. Normal littermates were used as controls; we are not aware of any evidence that animals heterozygous for Ames dwarfism differ from homozygous normal animals. Mice were housed in groups of 4–5 per plastic ‘shoe box type’ cages with wood chips in a room with controlled 12 h light:12 h dark cycle (lights on from 0600 to 1800 h) and a temperature of 22 $\pm$ 2 °C. Each cage was equipped with individual filter top (microisolator unit). Sentinel animals were housed in the same room to test for antibodies to all major murine pathogens. The results of the tests were uniformly negative. Animals were given free access to a nutritionally balanced diet (Rodent Laboratory Chow 5001; not autoclaved; 23.4% protein, 4.5% fat, 5.8% crude fiber; LabDiet, PMI Feeds, St Louis, MO, USA) and tap water. The appropriateness of the experimental procedure, the required number of animals used, and the

method of acquisition were in compliance with federal and local laws, and approved by the Southern Illinois University Animal Care and Use Committee.

Chemicals

Ovine GH (oGH) and mouse GH (mGH) were obtained through the National Hormone and Pituitary Program, NIDDK, NIH, USA. Recombinant human GH (hGH) was kindly provided by Biosidus SA, Buenos Aires, Argentina. BSA-fraction V, Kodak X-OMAT XAR 5 films, protein A-Sepharose, protein G-Sepharose and nitrocellulose membranes were obtained from Sigma. Antibodies anti-STAT5a (α STAT5a; L-20, catalog no. sc-1081), anti-STAT5b (α STAT5b; C-17, catalog no. sc-835), anti-CIS (α CIS; N-19, catalog no. sc-1529), anti-SOCS-1 (α SOCS-1; H-93, catalog no. sc-9021), anti-SOCS-2 (α SOCS-2; H-74, catalog no. sc-9022), anti-SOCS-3 (α SOCS-3; H-103, catalog no. sc-9023), anti-SH2-B β (α SH2-B β ; E-20, catalog no. sc-10827) and anti-phosphorylated tyrosine (α PY; PY-99, catalog no. sc-7020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-JAK2 antibody (α JAK2; catalog no. 06–255) and anti-phospho-STAT5a/b (Y694/Y699) antibody (α pSTAT5a/b; catalog no. 05–495) were obtained from Upstate Laboratories (Lake Placid, NY, USA). Anti-GHR antiserum (α GHR) was produced in our laboratory (Miquet *et al.* 2004), and anti-SH2-B antibody (α SH2-B) was kindly provided by Dr D D Ginty (Johns Hopkins University School of Medicine, Baltimore, MD, USA) (Qian *et al.* 1998). All other chemicals were of reagent grade.

Radioiodination of hormones

hGH and mGH were radiolabeled with limiting amounts of chloramine T, as previously described (Aguilar *et al.* 1992). Specific activity ranged from 70 to 120 μ Ci/ μ g.

Hormone RIA

mGH was measured as described in Sotelo *et al.* (1993).

Preparation of liver microsomes

Livers were homogenized in 50 mM Tris-HCl (pH 7.4) containing 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml bacitracine, at the ratio 1 ml/0.1 g. The homogenates were centrifuged at 12 000 g for 30 min, and the resulting supernatants were centrifuged at 100 000 g for 1 h. The pellets containing the membrane fraction were washed with 50 mM acetate/acetic acid buffer (pH 5.0) for 30 min at 4 °C to remove endogenous bound GH, and were centrifuged at 100 000 g for 1 h, and the resulting pellet was stored frozen until the following day. The microsomal pellets

were resuspended in 50 mM Tris/HCl and 2 mM PMSF buffer (pH 7.4). Protein concentration of the membrane fraction was determined by the Lowry procedure (Lowry *et al.* 1951).

Binding of 125 I-hGH to liver membranes

Somatogenic binding was determined as the difference between 125 I-hGH binding in the absence (total binding) and presence (lactogenic binding) of an excess (5 μ g) of unlabeled oGH, as described by Aguilar *et al.* 1992. Briefly, aliquots of the membrane preparation containing 100 μ g total protein were incubated with 3.5–5 ng 125 I-hGH in a total volume of 0.5 ml in the presence or absence of unlabeled oGH. Binding of the tracer was allowed to proceed for 15–18 h at 25 °C. The samples were centrifuged at 3000 g for 30 min at 4 °C, the supernatants were discarded and the pellet was counted in an automatic gamma counter (LKB-Wallac Clinigamma 1272, Turku, Finland).

The binding values were derived from matched experiments with normal and dwarf mice. All determinations were carried out in triplicate of a single membrane preparation for each individual animal, and the reported results represent means of these determinations.

Preparation of liver extracts and immunoprecipitation

The mice were fasted overnight, and then 5 mg oGH per kg BW in 0.2 ml 0.9% NaCl were injected i.p. To evaluate basal conditions, mice were injected with saline. Additional normal and dwarf mice received different doses of oGH: 12 000, 5000, 500, 150, 50, 15 and 0 ng oGH per g BW. The animals were killed 7.5 min after injection, and the livers were removed and homogenized at the ratio 1 ml/0.1 g in buffer composed of 1% Triton, 100 mM Hepes, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.035 trypsin inhibitory units/ml aprotinin (pH 7.4). Liver homogenates were centrifuged at 100 000 g at 4 °C for 50 min to remove insoluble material. Protein concentration of supernatants was determined by the Bradford assay (Bradford 1976). An aliquot of solubilized liver was diluted in Laemmli buffer, boiled for 5 min and stored at –20 °C until electrophoresis.

For immunoprecipitation, 10 mg solubilized total liver protein were incubated at 4 °C overnight with 15 μ l α GHR, 20 μ l α PY, 5 μ l α SH2-B, and 20 μ l α STAT5a, α STAT5b or α CIS. After incubation, 25 μ l protein A-Sepharose or G-Sepharose (50% v/v) were added to the mixture. The preparation was further incubated with constant rocking for 2 h at 4 °C and then centrifuged at 3000 g for 1 min at 4 °C. The supernatant was discarded and the precipitate was washed three times with 50 mM Tris, 10 mM vanadate and 1% Triton X-100 (pH 7.4).

The final pellet was resuspended in 50 μ l Laemmli buffer, boiled for 5 min, and stored at –20 °C until electrophoresis.

Western blotting

Samples were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. To reduce nonspecific antibody binding, membranes were incubated for 2 h at room temperature in T-TBS blocking buffer (10 mM Tris-HCl, 150 mM NaCl and 0.02% Tween 20 (pH 7.6)) containing either 3% BSA for phosphotyrosine detection or 5% nonfat powdered milk for protein detection. The membranes were then incubated overnight at 4 °C with α PY antibody (1:500), α GHR (1:500), α JAK2 (1:1000), α STAT5a (1:400), α STAT5b (1:400), α CIS (1:200), α SOCS-1 (1:200), α SOCS-2 (1:200), α SOCS-3 (1:200), α SH2-B β (1:200) or α pSTAT5a/b (1 μ g/ml). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL (Amersham) with preflashed Kodak XAR film. Band intensities were quantified by optical densitometry (Densitometer Model CS-930; Shimadzu, Kyoto, Japan) of the developed autoradiographies. For reblotting, membranes were stripped by incubation for 30 min at 50 °C in buffer containing 62.5 mM Tris-HCl, 2% SDS and 0.7% mercaptoethanol (pH 6.7). Blots were washed, reblocked and immunolabeled as described above.

Statistical analysis

Results are presented as means $\pm$ S.E.M. of the *n* number of samples indicated. Experiments analyzed all groups of animals in parallel, the number of separate experiments being indicated for each case. Statistical analyses were performed by ANOVA followed by the Tukey-Kramer test using the InStat statistical program by GraphPad Software (San Diego, CA, USA). Student's *t*-test was used when two groups were analyzed. Data were considered significantly different with *P* < 0.05.

Results

Animal characteristics

Data on BW and GH circulating levels are shown in Table 1. Female adult mice were used. Ames dwarf mice displayed an expected major reduction in BW of 55% of normal mice values (*P* < 0.0001). GH serum concentration in dwarf mice was below the detection limits for the assay used, in accordance with the reported deficiency of this hormone in Ames dwarf mice (Bartke 1964, 1979).

GH binding and GHR protein levels

GHR content was evaluated by immunoprecipitation and subsequent Western blotting of solubilized livers with

Table 1 Body weight and serum GH concentration of normal and Ames dwarf mice

Mice	Body weight (g)	mGH (ng/ml)
Normal	37.4 ± 1.3 (20)	3.9 ± 1.0 (6)
Dwarf	16.8 ± 0.7 (20)	ND (6)

mGH was determined by specific RIA. Values are mean ± S.E.M. (number of mice in parentheses).
a: $P<0.0001$ vs normal mice values.
ND: nondetectable.

α GHR antiserum. One main broad band with an apparent molecular mass of approximately 108 kDa was observed, in accordance with previous reports from our laboratory (González *et al.* 2002) and the expected molecular mass for the full-length form of the receptor in rodents (Frick *et al.* 1998). Densitometric analysis of autoradiographies showed no significant differences between normal and dwarf mice (Fig. 1A) (100 ± 7 for normal mice vs 117 ± 9 for dwarf mice; $n=10$ /group). When somatogenic binding to mice liver microsomes was determined, a reduction of 22% was observed in Ames dwarf mice compared with normal mice tissue, although this difference was not statistically significant (Fig. 1B) (100 ± 10 for normal mice vs 78 ± 7 for dwarf mice; $n=9$ /group).

JAK2, STAT5a and STAT5b tyrosine phosphorylation and protein levels

Tyrosine phosphorylation of JAK2 was evaluated by immunoprecipitation with antiphosphotyrosine antibody (α PY) and Western blotting with α JAK2 antibody. Basal tyrosine phosphorylation levels of JAK2 were similar in normal and dwarf mice (Fig. 2A). After GH administration (i.p. oGH 5 mg/kg BW for 7.5 min), JAK2 tyrosine phosphorylation increased 4.4-fold in normal animals and 5.4-fold in dwarf mice, in comparison with their respective basal values (Fig. 2A) ($P<0.001$; $n=6$ /group); JAK2 phosphorylation level for normal and dwarf mice after GH stimulus was not significantly different. This indicates that, under these experimental conditions, hepatic JAK2 responds similarly to GH stimulation in normal and dwarf mice. JAK2 protein content was evaluated by Western blotting of normal and dwarf mice liver extracts. Densitometry of autoradiographies showed that JAK2 concentration is not significantly different between dwarf and normal mice (Fig. 2D).

Tyrosine phosphorylation of STAT5 proteins was evaluated by immunoprecipitation with α STAT5a or α STAT5b antibodies and Western blotting with α PY antibody. Tyrosine phosphorylation of both STATs was dramatically increased upon GH stimulation (oGH 5 mg/kg BW) in normal and dwarf mice, reaching similar

Figure 1 (A) Liver extracts of normal (N) and dwarf (D) mice were prepared and equal amounts of solubilized tissue were immunoprecipitated (IP) with α GHR antibody, separated by SDS–PAGE and subjected to Western blotting (WB) with the same antibody. Quantification was performed by scanning densitometry and expressed as percentage of the mean value measured for normal mice. Data are the mean ± S.E.M. of 10 samples per group, each one representing a different animal, run in two separate experiments. (B) GH binding to its membrane receptor was estimated by specific binding of 125 I-hGH to liver membranes prepared from normal (N) and dwarf (D) mice. Unlabeled excess of oGH was used as a competitor, the difference between binding in the presence or absence of the competitor representing the somatogenic binding. Results are the means of triplicate determinations ± S.E.M. of nine samples per group, each one representing a different animal, and binding is expressed as percentage of somatogenic binding of normal mice.

Figure 2 (A–C) Normal (N) and dwarf (D) mice were injected i.p. with oGH 5 µg/g body weight (+) or saline (–) and killed after 7.5 min, and the liver was removed. Equal amounts of the solubilized tissue were immunoprecipitated (IP) and analyzed by Western blotting (WB) with the indicated antibodies. Protein phosphorylation was quantified by scanning densitometry and expressed as percentage of the mean value measured for GH-stimulated normal mice. Data are the mean $\pm$ S.E.M. of the indicated (*n*) number of samples per group, each one representing a different animal, run in five (A), two (B) and six (C) separate experiments. **P* vs nonstimulated normal mice; #*P* vs nonstimulated dwarf mice. (D–F) JAK2 protein content was analyzed by Western blotting of equal amounts of solubilized liver of normal (N) and dwarf (D) mice with αJAK2 antibody. For the evaluation of STAT5a and STAT5b protein content, the membranes used for the detection of tyrosine phosphorylation were stripped and reblotted with the same IP antibody. Quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for normal mice. Data are the mean $\pm$ S.E.M. of *n* samples per group, each one representing a different animal, run in two separate experiments (*n*=6 for JAK2; *n*=8 for STAT5a and STAT5b).

maximal values in both types of animals (Fig. 2B: $P < 0.001$; $n = 3$ /group; Fig. 2C: $P < 0.001$; $n = 9$ /group). No significant differences in the basal phosphotyrosine level of STAT5a or STAT5b between dwarf and normal mice were observed. The protein content of STAT5a and STAT5b in normal and dwarf mice liver was analyzed by stripping and reblotting the membranes with αSTAT5a or αSTAT5b antibodies. Densitometric analysis of autoradiographies showed that the concentration of both proteins is similar in dwarf and normal mice (Fig. 2E and F).

SOCS and CIS protein levels

CIS was immunoprecipitated from liver homogenates of control and dwarf mice with αCIS antibody and analyzed

by Western blotting with the same antibody. Dwarf mice displayed a markedly reduced CIS concentration compared with normal animals (Fig. 3A) (100 ± 18 vs 16 ± 7 ; $P < 0.001$; $n = 8$ /group). SOCS-1, SOCS-2 and SOCS-3 protein content was analyzed by Western blotting of liver homogenates of control and dwarf mice. Densitometry of autoradiographies showed that each protein is present in normal and dwarf mice in similar concentrations (Fig. 3B–D).

SH2-Bβ protein level

SH2-Bβ protein content was determined by immunoprecipitation with αSH2-B antibody and Western blotting with an antibody specific for the β isoform of this protein.

Figure 3 (A) Liver extracts of normal (N) and dwarf (D) mice were prepared, and equal amounts of the solubilized tissue were immunoprecipitated (IP) with α CIS antibody, separated by SDS-PAGE and analyzed by Western blot (WB) with the same antibody. Data are the mean $\pm$ S.E.M. of eight samples per group, each one representing a different animal, run in four separate experiments. * $P < 0.001$ vs normal mice. (B–D) SOCS-1, SOCS-2 and SOCS-3 protein content was analyzed by Western blotting of equal amounts of solubilized livers of normal (N) and dwarf (D) mice with the indicated antibodies. Data are the mean $\pm$ S.E.M. of six (B) or four (C and D) different individuals per group, run in the same experiment. In each case, quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for normal mice.

A reduction of approximately 30% was observed in dwarf mice (Fig. 4) (100 ± 4 vs 73 ± 9 ; $P = 0.026$; $n = 7/\text{group}$).

STAT5a/b tyrosine-phosphorylation in response to different GH doses

The response to different GH doses was evaluated by the determination of the tyrosine phosphorylation of STAT5. Liver extracts were subjected to Western blotting with an antibody against specific phosphotyrosines 694 and 699 of STAT5a and STAT5b respectively that are essential for the activation of these transcription factors. The phosphorylation of STAT5a/b in response to GH increased in parallel with the administered dose (Fig. 5A and B). In both dwarf and normal mice, a maximal response was

observed at a dose of GH of $5 \mu\text{g/g BW}$ (Fig. 5A and B). However, the phosphorylation levels of STAT5a/b in response to lower GH doses (50 and 15 ng/g BW) were significantly higher in liver of dwarf mice than in the normal group (Fig. 5C). Although it had been previously demonstrated that STAT5a and STAT5b protein levels do not vary between normal and dwarf mice (Fig. 2E and F), the homogeneity in STAT5 protein content was further confirmed by Western blotting with anti-STAT5 antibody (data not shown).

Discussion

Endocrine mutants offer a unique opportunity to examine the role of hormones and to elucidate mechanisms of their

Figure 4 Liver extracts of normal (N) and dwarf (D) mice were prepared, and SH2-B β protein content was evaluated by immunoprecipitation (IP) and Western blotting (WB) with the indicated antibodies. Quantification was performed by scanning densitometry and expressed as percentage of the mean value measured for normal mice. Data are the mean $\pm$ S.E.M. of seven different individuals per group, run in two separate experiments. * $P=0.026$ vs normal mice.

action at the molecular level. To study the effects of the lack of GH on its signal transduction, we used Ames dwarf mice as a model of GH deficiency, as these mice are characterized by primary pituitary deficiency consisting in the absence of, or extreme reduction in, anterior pituitary cells that produce GH, PRL and TSH. Elucidating GH signaling in these mutants is of particular interest because they present an increase of 40–65% in mean life span (Brown-Borg *et al.* 1996), as reduced activity of the somatotrophic axis is related to improved life expectancy (Bartke *et al.* 2004).

GH signaling is initiated by the hormone's binding to its membrane receptor. Apart from full-length GHR, GH can bind to various GHR-related membrane structures: membrane-associated GH-binding protein (MA-GHBP) in rodents or short truncated forms of GHR in other species (Frick *et al.* 1994, Dastot *et al.* 1996, Ross *et al.* 1997, Camarillo *et al.* 1998). As GH positively regulates its own receptor expression in liver (Herington *et al.* 1976, Baxter *et al.* 1982, Aguilar *et al.* 1992, González *et al.* 2001), it is important to evaluate GHR content as the first cellular constituent of GH signaling in this GH deficiency model. GHR was evaluated by Western blotting to visualize its molecular size, as well as its relative

abundance, and by 125 I-GH binding to hepatic membrane microsomes to determine its ability to bind GH. The present results in Ames dwarf mice show no significant differences from normal animals in full-length GHR content or GH membrane binding. These data agree with previous studies describing no difference from their respective normal controls in somatogenic binding in either GH-deficient dwarf 'little' mice or dwarf rats (Herington *et al.* 1983, Carmignac *et al.* 1992). hGH binding was reported to be lower in Snell dwarf mice, which are phenotypically identical to Ames dwarf mice, than in normal controls (Posner 1976). This difference was attributed to the simultaneous lack of GH and PRL these mice present, since hGH can bind to both somatogenic and lactogenic sites (Herington *et al.* 1983).

The JAK2/STAT5 pathway is a major mediator of GH actions (Udy *et al.* 1997, Teglund *et al.* 1998, Herrington *et al.* 2000). Thus, the content and tyrosine phosphorylation of JAK2, STAT5a and STAT5b under basal or GH-stimulated conditions were analyzed in liver, a major target organ of GH, in order to evaluate the integrity of GH signal transduction in this model of GH deficit. The protein content of the JAK2/STAT5 signaling pathway components was similar in dwarf and normal mice. Activation of this pathway was estimated by tyrosine phosphorylation of signaling proteins. No differences from normal mice values in the basal tyrosine phosphorylation levels of JAK2, STAT5a and STAT5b were detected in Ames dwarf mice, despite the lack of endogenous GH secretion in dwarf animals. This result is somewhat unexpected, but it could be due to insufficient sensitivity to detect the low values of basal phosphorylation or to other concomitant stimuli that activate this pathway. Since GH secretion in normal mice is pulsatile, only after an endogenous GH pulse would higher basal phosphorylation levels be observed. During GH troughs, basal phosphorylation levels would be minimal. After GH administration in high doses, the tyrosine phosphorylation of these signaling mediators dramatically increased, with no significant differences between normal and dwarf mice. This would indicate that the hepatic components of the JAK2/STAT5 GH signaling pathway in dwarf and normal animals present similar content and maximal capacity of being stimulated by a single GH injection.

Animal models with extreme hormone levels ranging from an excess to a complete deficiency were proposed as a strategy for elucidating the effects of hormone action (Berryman *et al.* 2004). By this approach, we have compared mice exhibiting high, normal or undetectable circulating levels of GH. We have used transgenic mice expressing human GH-releasing hormone (hGHRH) and transgenic mice overexpressing bovine GH (bGH) as models to study the effects of high and continuous GH levels on the JAK2/STAT5 GH-signaling pathway *in vivo*. We found an almost complete inhibition of GH-stimulated tyrosine phosphorylation of JAK2, STAT5a and

Figure 5 Liver extracts of normal (N) and dwarf (D) mice, stimulated i.p. for 7.5 min with the indicated doses of GH, were prepared. Equal amounts of the solubilized tissue were separated by SDS-PAGE and subjected to Western blotting analysis (WB) with α pSTAT5a/b antibody. Dose-response curves for normal (A) and dwarf (B) mice were performed, analyzing two subjects per dose in the same experiment. (C) Tyrosine phosphorylation of STAT5a/b in response to 50 and 15 ng/g body weight doses of GH was compared between normal and dwarf mice. Data are the mean $\pm$ S.E.M. of three samples per group, each one representing a different animal, run in the same experiment.

STAT5b in GH-overexpressing mice. This desensitization was mainly related to a dramatic increase of CIS protein content in transgenic animals (González *et al.* 2002, Miquet *et al.* 2004). CIS is an effective and specific negative regulator of GH-induced STAT5 activity. It interacts with tyrosine phosphorylated residues on the GHR cytoplasmic tail, thereby masking STAT5 recruitment sites and inhibiting its phosphorylation and activation (Ram & Waxman 2000, Inagaki-Ohara *et al.* 2003, Greenhalgh & Alexander 2004). The significant reduction in the level of CIS in Ames dwarf mice described in the

present paper and the dramatic increase previously found for GH-overexpressing mice correlate with the levels of GH displayed by these mice, suggesting a strong regulation of this protein by GH *in vivo* (Fig. 6A and B).

Although high CIS levels were related to GH desensitization in GH-overexpressing mice, as reflected by an almost complete inhibition of STAT5b activation, the significant reduction in CIS protein content observed in dwarf mice was not associated with a higher maximal capacity of phosphorylation in the main signaling pathway of GH, JAK2/STAT5. However, when doses that lead to

Figure 6 GH serum levels (A) and CIS protein content (B) of normal mice (N), GH-overexpressing transgenic mice (T) and Ames dwarf mice (D) (present work) are compared. A logarithmic scale is used for GH serum values due to different GH concentration ranges. Values for transgenic mice are taken from Miquet *et al.* (2004) and are included here for comparison.

a submaximal response were tested, dwarf mice presented a higher tyrosine phosphorylation of STAT5a/b in response to GH stimuli than normal animals, indicating hypersensitivity to GH at this level.

SOCS-1, -2 and -3 are other suppressors that regulate GH signaling. SOCS-1 directly interacts with phosphorylated JAK2, inhibiting its activity. SOCS-3 seems to be recruited to the GHR-JAK2 complex by binding to tyrosine phosphorylated activated receptor, inhibiting the activity of the associated kinase by preventing substrate access to the catalytic domain. Until now, there is no

conclusive evidence of any physiologic role of SOCS-1 and SOCS-3 in growth control (Greenhalgh & Alexander 2004). Although it was demonstrated that SOCS-2 binds to two phosphorylated tyrosines on the GHR, its mechanism of action (Greenhalgh *et al.* 2005), its actions on GH signaling have been disputed, including positive and inhibitory effects on STAT activation in different models (Favre *et al.* 1999, Greenhalgh *et al.* 2002, 2005). We determined SOCS-1, -2 and -3 in Ames dwarf mice in order to establish the impact of undetectable GH levels on these proteins. The content of these SOCS proteins in normal and dwarf mice is similar, suggesting that the expression of these suppressors is not affected by the lack of GH. We have previously reported that transgenic mice overexpressing GH show a reduction in the levels of SOCS-3, while no changes in SOCS-2 protein content were detected, compared with normal mice (González *et al.* 2002, Miquet *et al.* 2004). Taken together, these results suggest that, under chronic exposure, GH does not positively regulate the protein expression of these SOCS *in vivo*, as the high continuous levels in GH-overexpressing mice are not mirrored by a rise of the suppressors, nor is the lack of GH accompanied by a diminution of these proteins.

SH2-B β content is lower in dwarf mice than in normal animals. Interestingly, transgenic mice overexpressing GH displayed higher SH2-B β content, suggesting that GH levels may be implicated in the regulation of SH2-B β abundance. The reduction in SH2-B β levels in dwarf mice was not associated with altered JAK2 activation by a high GH dose.

Moreover, apart from GH deficiency, Ames dwarf mice exhibit a PRL and TSH deficiency that could also contribute to the findings obtained, especially since both GH and PRL mainly activate the JAK2/STAT5 signaling pathway and induce the same SOCS proteins.

In conclusion, dwarf mice present higher sensitivity to GH in liver as evaluated by the tyrosine phosphorylation of STAT5a/b. This could be due to the significant reduction in the content of CIS protein detected in this tissue. Furthermore, the low CIS abundance found in this model of GH deficiency suggests that CIS levels are regulated by GH *in vivo*.

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conflict of interest that would prejudice the impartiality of this scientific work.

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