Increased SH2-B β content and membrane association in transgenic mice overexpressing GH

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Abstract

Transgenic mice overexpressing GH present a marked GH signaling desensitization, reflected by low basal phosphorylation levels of the tyrosine kinase JAK2, and signal transducer and activator of transcription-5 (STAT5) and a lack of response of these proteins to a high GH dose. To evaluate the mechanisms involved in the regulation of JAK2 activity by high GH levels *in vivo*, the content and subcellular distribution of SH2-B β were studied in GH-overexpressing transgenic mice. SH2-B is a member of a conserved family of adapter proteins characterized by the presence of a C-terminal SH2 domain, a central pleckstrin homology (PH) domain, and an N-terminal proline rich region. The isoform SH2-B β modulates JAK2 activity by binding to the phosphorylated enzyme, further increasing

its activity. However, it may also interact with non-phosphorylated inactive JAK2 via lower affinity binding sites, preventing abnormal activation of the kinase. SH2-B β may also function as an adapter protein, acting as a GH signaling mediator.

We now report that, in an animal model of GH excess in which JAK2 is not phosphorylated, although it is increased in the membrane-fraction, both the level of SH2-B β , and especially its association to membranes, are augmented (67% and 13-fold vs normal mice values respectively), suggesting SH2-B β could modulate JAK2 activity *in vivo*.

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Introduction

Growth hormone (GH) binding to its membrane receptor (GHR) induces a conformational change of the dimerized GHR (Rowlinson *et al.* 1998, Gent *et al.* 2003), resulting in activation of the constitutively associated tyrosine-kinase JAK2 and downstream signaling pathways, the signal transducer and activator of transcription-5 (STAT5) being one of the major GH-signaling mediators (Zhu *et al.* 2001). The control of JAK2 kinase activity is a crucial point of GH signaling regulation. The members of the suppressors of cytokine signaling (SOCS) family of proteins SOCS-1 and SOCS-3 inhibit JAK2 activity (Greenhalgh & Hilton 2001), while the SH2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) binds to phosphorylated tyrosines on the GH receptor and dephosphorylates JAK2, as well as STAT5 and GHR (Stofega *et al.* 2000).

JAK2 activity can also be positively regulated. SH2-B is a member of a conserved family of adapter proteins that includes APS and LnK, characterized by the presence of a C-terminal SH2 domain, a central pleckstrin homology (PH) domain, and an N-terminal proline rich region (Yokouchi *et al.* 1997). Four alternatively spliced isoforms

of SH2-B (α , β , γ and δ) have been reported in the mouse (Yousaf *et al.* 2001). SH2-B β was described as a substrate and a potent cytoplasmic activator of JAK2 (Rui *et al.* 1997, Rui & Carter-Su 1999). After GH stimulation, SH2-B β is recruited to membrane GHR-JAK2 complexes, binds through its SH2 domain to active tyrosyl-phosphorylated JAK2 and increases its kinase activity. SH2-B β is, in turn, phosphorylated by JAK2, suggesting it may act as a GH signaling molecule. Furthermore, SH2-B β was proposed to interact with non-phosphorylated inactive JAK2 via lower affinity binding sites, preventing spontaneous or abnormal JAK2 activation (Rui *et al.* 2000). This interaction could also augment the local concentration of SH2-B β around JAK2, increasing the efficiency of the GH-induced binding to phosphorylated JAK2, therefore leading to a more rapid and robust response to hormone stimuli (Rui *et al.* 2000).

Recently, we reported that the JAK2/STAT5 signaling pathway is markedly desensitized in transgenic mice overexpressing GH. These animals display increased GHR and JAK2 levels at the membrane compartment, but JAK2 and STAT5 do not become tyrosine-phosphorylated after a high GH stimulus, and basal phosphorylation levels of these mediators are similar to those of normal mice.

This lack of response to GH was ascribed to an important increase of CIS, a member of the SOCS family that competes with STAT5 for its docking site on the GHR (González *et al.* 2002, Miquet *et al.* 2004). While other SOCS proteins apparently were not related to the desensitization found in these animals, the tyrosine phosphatase SHP-2 could be implicated in JAK2 inhibition, as a marked increase in membrane-associated SHP-2 was observed in these transgenic mice (Miquet *et al.* 2004). However, the causes for the low phosphorylation levels of JAK2 in GH overexpressing transgenic mice are not completely elucidated. Since JAK2 is essential for the activation of downstream signaling, the aim of the present work was to evaluate another possible mechanism of regulation of JAK2 activity in a model of high and continuous GH levels *in vivo*. We thus evaluated the content and subcellular distribution of SH2-B β in transgenic mice overexpressing bovine GH.

Materials and Methods

Animals

PEPCK-bGH mice containing the bovine GH (bGH) gene fused to control sequences of the rat phosphoenolpyruvate carboxykinase (PEPCK) gene (McGrane *et al.* 1988) were derived from animals kindly provided by Dr Thomas E Wagner and Jeung S Yun (Ohio University, Athens, OH, USA). The hemizygous transgenic mice were produced by mating transgenic males with normal (C57BL/6 $\times$ C3H) F1 hybrid females purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Matings produced approximately equal proportion of transgenic and normal progeny; normal siblings were used as controls. Female adult animals (3–5 months old) were used. The mice were housed 3–5 per cage in a room with controlled light (12 h light/day) and temperature (22 ± 2 °C). The animals had free access to food (Lab Diet Formula 5001; PMI Inc., 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 with institutional regulations.

Chemicals

Ovine GH (oGH) was obtained through the National Hormone and Pituitary Program, NIDDK, NIH, USA. Kodak X-OMAT XAR 5 films, protein A-Sepharose and nitrocellulose membranes were obtained from Sigma Chemical Co. (St Louis, MO, USA). Antibodies anti-SH2-B β (α SH2-B β ; E-20, no. sc-10827), anti-STAT5b (α STAT5b; C-17, no. sc-835) and anti-tyrosine phosphorylated (α PY; PY-99, no. sc-7020) were purchased from Santa Cruz Biotechnology Laboratories (Santa Cruz, CA, USA), anti-JAK2 (α JAK2, no. 06–255) and

anti-phospho-STAT5a/b (α pSTAT5; Y694/Y699, no. 05–495) from Upstate Biotechnology (Lake Placid, NY, USA) and anti-phospho-JAK2 (α pJAK2; Tyr1007/1008, no. 3771S) from Cell Signaling Technology (Beverly, MA, USA). Anti-SH2-B antibody (α SH2-B) was kindly provided by Dr D D Ginty (The Johns Hopkins University School of Medicine, Baltimore, MD, USA; Qian *et al.* 1998). All other chemicals were of reagent grade.

Preparation of liver extracts and immunoprecipitation

The mice were starved overnight, then 5 μ g of oGH per g of body weight in 0.2 ml 0.9% NaCl were injected *i.p.* Additional normal and transgenic mice were injected with saline to evaluate basal conditions. Animals were killed 7.5 min after injection and the livers were removed. This period allows activation of JAK2 and STAT5 but does not affect the total content of the proteins studied (González *et al.* 2002, Miquet *et al.* 2004). The tissue was homogenized in buffer containing 1% Triton and protein and phosphatase inhibitors, as was previously described (Miquet *et al.* 2004). 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 of solubilized liver protein in a final volume of 1 ml were incubated at 4 °C overnight with 5 μ l of α SH2-B, after which 25 μ l of protein A-Sepharose (50%, *v/v*) were added and incubated with constant rocking for 2 h at 4 °C. Sepharose beads were then washed three times and the final pellet was resuspended in 50 μ l Laemmli buffer, boiled for 5 min, and stored at -20 °C until electrophoresis.

Preparation of liver microsomes

Liver was homogenized in buffer containing protease and phosphatase inhibitors; microsomes were obtained as previously described (Miquet *et al.* 2004). The samples were boiled in Laemmli buffer for 10 min and stored at -20 °C until electrophoresis.

Western-blotting

Samples were resolved by SDS-PAGE under reducing conditions (25 μ l of the supernatant obtained from the immunoprecipitation procedure or 60 μ g of microsomes resuspended in sample buffer) and transferred to nitrocellulose membranes, as previously described (Miquet *et al.* 2004). The antibody dilutions used for the immunoblotting were those recommended by the suppliers: α PY (1:500), α JAK2 (1:1000), α STAT5 (1:400), α pJAK2 (1:1000), α pSTAT5 (1 μ g/ml), α SH2-B (1:1000) or α SH2-B β (1:100). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) and band intensities quantified by optical densitometry (Densitometer model CS-930, Shimadzu, Japan).

Statistical analysis

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

Results

Transgenic mice used in this work display extremely high circulating bGH levels and a 90% increment in body weight compared with normal values (44.3 ± 1.2 g vs 23.0 ± 1.7 g respectively, $P < 0.001$, $n = 6$), in accordance with previous results (Miquet *et al.* 2004). We have described that the JAK2/STAT5 GH-signaling pathway is desensitized in GH-overexpressing mice (González *et al.* 2002, Miquet *et al.* 2004). In normal mice, GH stimulation dramatically increased tyrosine phosphorylation of STAT5 and JAK2 in liver homogenates and induced STAT5 recruitment to the membrane compartment. However, transgenic animals did not respond to GH stimulation, even when GHR and JAK2 membrane-associated basal levels were markedly increased (Miquet *et al.* 2004).

Tyrosine phosphorylation of membrane-associated JAK2 and STAT5 was determined, using specific anti-phospho-STAT5 and anti-phospho-JAK2 antibodies, to confirm and further extend our previous results. In contrast to striking stimulation of membrane-associated JAK2 and STAT5 phosphorylation in GH-treated normal mice, transgenic mice did not respond to this high GH dose and, despite the elevated GH levels they present, the basal phosphorylation of these proteins was comparable to that of normal mice (Fig. 1A and B).

To investigate SH2-B abundance, total solubilized liver protein was analyzed by Western-blotting with α SH2-B antibody, which recognizes all SH2-B isoforms. Transgenic mice showed a 2-fold increase in the levels of this protein compared with normal animals (Fig. 2A). Only one main band of approximately 95 kDa was detected, in accordance with data on molecular weight of endogenous mouse SH2-B β (Kong *et al.* 2002). The abundance of the β variant of SH2-B was then evaluated by immunoprecipitation with α SH2-B and further immunoblotting with an α SH2-B β antibody. SH2-B β content was 67% higher in transgenic over normal animals (Fig. 2B).

The presence of SH2-B β in the microsomal fraction was evaluated in order to estimate its association to membranes. An approximately 13-fold increase of SH2-B β was found in liver membrane fraction of transgenic mice

compared with normal mice values (Fig. 2C). In normal mice, a moderate increase in the membrane association of SH2-B β upon GH stimulus was detected, although this was not statistically significant (Fig. 2C). Tyrosine phosphorylation of SH2-B could not be detected in transgenic or normal mice, either before or after GH stimulus; coimmunoprecipitation of JAK2 with SH2-B β or GHR could not be achieved (data not shown).

Discussion

PEPCK-bGH transgenic mouse is an *in vivo* model of GH desensitization due to chronically elevated continuous GH levels. Transgenic animals used in this work exhibit a 90% increase of body weight, although GH levels are 70-fold increased, indicating that GH action is mainly suppressed (Miquet *et al.* 2004). In transgenic mice liver, an almost complete lack of tyrosine phosphorylation of JAK2 and STAT5 was observed, either under basal or GH-stimulating conditions, both in solubilized tissue (Miquet *et al.* 2004) or membrane fraction (Fig. 1A and B). As JAK2 activity is essential for the activation of downstream signaling molecules and, hence, a critical point of GH signaling regulation, the aim of the present work was to evaluate possible mechanisms of regulation of JAK2 activity. Studies in cells overexpressing SH2-B β or truncated forms of this protein suggested that an important mechanism by which the cell modulates JAK2 activity would involve the regulation of SH2-B β content or its ability to bind JAK2 (Rui & Carter-Su 1999). Thus, the content and subcellular distribution of SH2-B β were determined in liver of PEPCK-bGH transgenic mice and their normal littermates.

SH2-B β was first identified as a JAK2-binding protein that is tyrosine-phosphorylated in response to GH and interferon- γ (Rui *et al.* 1997). It binds preferentially through its SH2 domain to activated tyrosyl-phosphorylated JAK2 strongly increasing its kinase activity (Rui & Carter-Su 1999). Besides the SH2 domain, SH2-B β has one or more lower-affinity binding sites for JAK2 (Rui *et al.* 2000). This lower-affinity interaction is independent of JAK2 tyrosyl-phosphorylation and was proposed to prevent basal or abnormal activation of the kinase, therefore acting as an inhibitor of JAK2. Alternatively, this lower-affinity binding to inactive non-phosphorylated JAK2 would increase the local concentration of SH2-B β around the kinase in order to facilitate the binding of the SH2 domain of SH2-B β to JAK2 in response to hormone stimuli, increasing the efficiency of JAK2 activation and resulting in a more powerful response (Rui *et al.* 2000).

In the current study, SH2-B β content was determined in an animal model of GH signaling desensitization due to chronically high and continuous GH levels, in which JAK2 is not phosphorylated and, therefore, not activated.

Figure 1 JAK2 and STAT5 tyrosine-phosphorylation in hepatic microsomes. Normal mice (N) and PEPCK-bGH transgenic mice (T) were injected i.p. with ovine GH (5 μ g/g of body weight) (+) or saline (-), killed after 7.5 min and the livers removed. Microsomes were prepared, separated by SDS-PAGE and subjected to Western-blot analysis with α pJAK2 or α pSTAT5 antibodies. Blots shown are representative of different experiments. Quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for stimulated normal mice. Data are the means $\pm$ S.E.M. of five samples, each one representing a different animal, run in separate experiments. Statistical analyses were performed by ANOVA followed by the Tukey–Kramer test. * $P < 0.001$ vs non-stimulated normal mice. Membranes were re-blotted with antibodies anti-STAT5b or anti-JAK2 to confirm band identity (data not shown).

In these transgenic animals, SH2-B and SH2-B β exhibit a 99% and 67% increment in solubilized liver, respectively. SH2-B β association to membranes is 13-fold increased. These data are in agreement with the reported prevalence of SH2-B β in the membrane fraction in cells treated with GH (Herrington *et al.* 2000). SH2-B β may be recruited to membranes by different interaction motives: PH domains target proteins to the plasma membrane by binding to phospholipids, proline rich motives interact with SH3 containing signaling proteins, while SH2 domains bind to tyrosine-phosphorylated proteins (Pawson & Nash 2003). GH was proposed to induce SH2-B β recruitment to membrane GHR–JAK2 complexes (Rui *et al.* 1997). Both GHR and JAK2 are increased in the membrane fraction from transgenic mice liver (Miquet *et al.* 2004), suggesting the probable formation of GHR–JAK2–SH2-B β complexes at the plasma membrane. As in this model JAK2 is

not phosphorylated, SH2-B β would be interacting with inactive JAK2 by binding to sites independent of tyrosine phosphorylation, probably those defined as lower affinity binding sites (Rui *et al.* 2000). One of the presumed effects of this low-affinity binding is to prevent abnormal activation of JAK2, which could have a protective effect as membrane-associated JAK2 is markedly increased in transgenic mice (Miquet *et al.* 2004). In addition, this high SH2-B β membrane binding could be a facilitating mechanism for JAK2 activation, which is depressed in this model.

SH2-B knock out mice (SH2-B $^{-/-}$), another *in vivo* model, show a temporary delay in body weight gain, suggesting an impairment of GH or IGF-I signal transduction (Ohtsuka *et al.* 2002). However, these authors could not find evidence supporting the role of SH2-B as a JAK2 activator, and they proposed that SH2-B may not be

Figure 2 SH2-B and SH2-B β protein content. Extracts of solubilized liver of normal mice (N) and PEPCK-bGH transgenic mice (T) were separated by SDS-PAGE and subjected to Western-blot analysis (WB) with α SH2-B antibody (A), or immunoprecipitated (IP) with α SH2-B antibody, separated by SDS-PAGE and subjected to Western-blot analysis with α SH2-B β antibody to detect the content of this specific isoform (B). (C) Normal mice (N) and PEPCK-bGH transgenic mice (T) were injected i.p. with ovine GH (5 μ g/g of body weight) (+) or saline (-), killed after 7.5 min and the livers removed. Microsomes were prepared, separated by SDS-PAGE and subjected to Western-blot analysis with α SH2-B β antibody. Blots shown are representative of different experiments. Quantification was performed by scanning densitometry and expressed as a percentage of the mean value measured for non-stimulated transgenic mice. Data are the means $\pm$ S.E.M. of the indicated number of samples (n), each one representing a different animal, run in two (2A and 2B) or three (2C) separate experiments. Statistical analyses were performed by Student's *t*-test for Figures 2A and 2B and by ANOVA followed by the Tukey-Kramer test for Figure 2C. **P* < 0.001 vs non-stimulated normal mice SH2-B β content.

necessary for JAK2 activity, as JAK2/STAT5 signaling is not impaired in liver of these mice.

In addition to its action as a modulator of JAK2 activity, SH2-B β may play a role as a signaling molecule itself. In unstimulated cells it is present at the plasma membrane and in the cytosol, while in cells treated with GH or PDGF, SH2-B β colocalizes with actin filaments in membrane ruffles, recruiting Rac or other actin-regulators to activated GHR-JAK2 complexes (Herrington *et al.* 2000). The role of SH2-B β in GH-induced actin reorganization seems to be independent of its role as a JAK2 activity modulator (Herrington *et al.* 2000) and its ability to bind to Rac was reported not to depend on its tyrosine-phosphorylation state (Diakonova *et al.* 2002, O'Brien *et al.* 2003).

The high SH2-B β membrane binding observed in GH-transgenic mice could reflect an increment of SH2-B β activity as a signaling molecule. In normal GH-stimulated mice, membrane-associated SH2-B β content presented a slight increment, but this was not statistically different from non-stimulated normal mice values (Fig. 2C). SH2-B tyrosine-phosphorylation could not be detected in any of the studied conditions. This could be attributed to a lack of sensitivity, since tyrosine residues in SH2-B were proposed to be rapidly dephosphorylated

and thus difficult to detect in cells that do not overexpress this protein (Rui *et al.* 1999). In addition, SH2-B phosphorylation was not shown to be augmented with increased levels of protein expression (Ahmed & Pillay 2003). Thus, we could not obtain experimental evidence that SH2-B β acts as a GH signaling molecule in this animal model.

In summary, in transgenic mice exhibiting high and constant GH levels, in which JAK2 is not phosphorylated although it is augmented in the membrane-fraction, both total SH2-B β content and, especially, its association to membranes are increased, suggesting a role for SH2-B β as a negative modulator of the kinase JAK2 in this animal model.

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