Mice carrying a hepatocyte-specific inactivation of the glucocorticoid receptor (GR) gene show a dramatic reduction in body size. Growth hormone signaling mediated by the Stat5 transcription factors is impaired. We show that Stat5 proteins physically interact with GR and GR activity depends on the binding of its ligand, which is released on physiological, circadian, and stress stimuli, and thus GR participates in coordinating the organism’s responses to the environment. GR can both activate and repress transcription of target genes via DNA binding to glucocorticoid responsive elements (GREs) or cross-talk with other transcription factors [Beato et al. 1995; Tronche et al. 1998]. Because the liver is a major target organ for GCs in control of glycogen metabolism and gluconeogenesis, we wished to address the function of hepatic GR by genetic means. As shown previously, loss of the receptor leads to lethality [Cole et al. 1995; Tronche et al. 1998]. We therefore have generated cell/tissue-specific and function-selective mutations using the Cre/loxP system [Reichardt et al. 1998; Tronche et al. 1999]. To define the role of GR in hepatocytes, we expressed the recombinase in hepatocytes under the control of the albumin promoter and the albumin and α-fetoprotein enhancers to generate mutant mice with selective inactivation of GR in these cells only (GRAlfpCre mice; Kellendonk et al. 2000). This approach allowed us to partially circumvent the perinatal lethality of GR null mutants. Surprisingly, adult mice with the hepatocyte-specific loss of GR have a severe reduction in body weight. Analysis of these mice revealed not only a novel function of GR in growth control, but also an unprecedented mode of activity.

Results and Discussion

Approximately 50% of the homozygous mutants died within 48 h after birth, likely due to the metabolic consequences of the hepatocyte-specific GR knock-out [data not shown]. No increase in mortality was observed at later stages. Mice lacking GR in hepatocytes were indistinguishable from their littermates until 3–4 wk of age, but later displayed a severe growth deficit that was more pronounced in adult males than in females (reduction by 32% and 26% respectively; Fig. 1A). The growth deficit was not caused by altered fat deposition, because the body mass index was similar in both genotypes (0.25 ± 0.015 and 0.25 ± 0.007, n = 8), but rather was due to decreased length of bones and the size of internal organs (Fig. 1B).

The characteristics of the growth deficit in mutant animals, including the similar body sizes at birth between mutant and control animals and the sexual dimorphism in adults, suggest impaired growth hormone (GH) signaling via the Jak/Stat pathway (Gouilleux et al. 1995; Tannenbaum et al. 2001; Kofoed et al. 2003). In vertebrates, body growth depends on GH produced in the anterior pituitary, which binds to the GH receptor (GHR) in peripheral organs, finally initiating a phosphorylation cascade that involves Janus Kinase2 (Jak2) and Stat5a/b proteins (signal transducer and activator of transcription 5). Dimerization of Stat5 and translocation to the nucleus results in the transcriptional activation of GH-regulated genes. The suppressor of cytokine signaling-2 (SOCS-2) is thought to negatively regulate this cascade via a feedback loop [Metcalf et al. 2000]. Remarkably, the short stature of GRAlfpCre mice is very similar to that of Stat5b or Stat5ab mutant mice [Udy et al. 1997; Teglund et al. 1998; see also Fig. 4A, below]. Growth impairment has also been previously observed for GH as well as GHR-deficient mice [Donahue and Beamer 1993; Zhou et al. 1997], whereas inactivation of SOCS-2 leads to gigantism [Metcalf et al. 2000].

In a first analysis, the morning serum levels of GH and GCs were found unchanged in mutant animals (Table 1),
GRAlfpCre mice show a growth deficit becoming discernible at 4–5 wk of age. The body weight of individuals from several litters was followed over a period of 10 wk. The growth curve of mutant (closed circle) and control (open square) female animals is shown in the left panel, and the corresponding growth curve of mutant (closed circle) and control (open square) males is shown in the right panel (n = 5 to 9 per group, error bars indicate standard deviation). Control animals carrying the AlfpCre transgene but wild-type GR alleles displayed a normal growth rate (data not shown). (B) The body, carcass, organ weights, and length of bones were measured in mutant and control animals of both genders (female n = 5 and n = 5; male n = 4 and n = 8, respectively). The proportional differences as well as the level of significance (Student’s t-test) are shown. (*) P < 0.05; (**) P < 0.01; (****) P < 0.001.

which, together with the fact that no differences between genotypes were detected in the levels of the GHR mRNA (Fig. 2A), suggests that the defect in GH function might reside in the hepatic intracellular GH signaling cascade. We therefore quantified the mRNA abundance of GH-regulated genes in the liver of mutant and control animals and found that mRNA expression of IGF-I (Thomas et al. 1994; Woelfle et al. 2003), ALS (Ooi et al. 1994; Kofoed et al. 2003) was drastically reduced (Fig. 2A). A similar decrease was observed in the major urinary protein (MUP) mRNAs whose expression is known to depend on different strength of male and female GH pulses, which get transmitted via Stat5b in a sexual dimorphic rhythm (Davey et al. 1999; Tannenbaum et al. 2001). As expected, the mRNA levels of IGF-binding protein-3 (IGFBP-3), a GH-induced Stat5 target gene expressed only in nonparenchymal liver cells and strongly reduced in a patient carrying a missense mutation of Stat5b, remained unchanged [Fig. 2A; Domene et al. 1993; Chin et al. 1994; Kofoid et al. 2003]. Therefore, IGFBP-3 serves as an internal control that restricts the origin of the growth deficit to hepatocytes only. Consistent with growth hormone resistance and the decrease in IGF-I mRNA in GRAlfpCre mice, circulating IGF-I levels were significantly lower in mutant animals (see Table 1). In GRAlfpCre mice, circulating levels of ALS, crucial for the bioactivity of IGF-I with which it interacts, were reduced by >40% (P < 0.05; data not shown; Yakar et al. 2002).

In order to identify at which level the absence of GR affects GH signaling in hepatocytes, we studied Stat5 proteins. Neither the levels of Stat5 proteins nor their phosphorylation status were significantly different in protein extracts from livers of untreated wild-type and mutant animals [data not shown]. To define Stat5 activation, we followed Stat5 DNA-binding activity in wild-type mice after GH treatment, given alone or combined with dexamethasone, a GR agonist, for 60 min [Fig. 2B]. As expected, a strong GH-dependent increase of Stat5 DNA-binding activity was observed. In the liver of GRAlfpCre mutant mice, neither DNA-binding activity nor Stat5 tyrosine phosphorylation were reduced; rather, extracts from mutant mice had a higher level of Stat5 protein expression with which it interacts, were reduced by >40% (P < 0.05; data not shown; Yakar et al. 2002).

**Figure 1.** Growth deficiency of GRAlfpCre mice. (A) Both genders of GRAlfpCre mice show a growth deficit becoming discernible at 4–5 wk of age. The body weight of individuals from several litters was followed over a period of 10 wk. The growth curve of mutant (closed circle) and control (open square) male animals is shown in the left panel, and the corresponding growth curve of mutant (closed circle) and control (open square) female animals is shown in the right panel (n = 5 to 9 per group, error bars indicate standard deviation). Control animals carrying the AlfpCre transgene but wild-type GR alleles displayed a normal growth rate (data not shown). (B) The body, carcass, organ weights, and length of bones were measured in mutant and control animals of both genders (female n = 5 and n = 5; male n = 4 and n = 8, respectively). The proportional differences as well as the level of significance (Student’s t-test) are shown. (*) P < 0.05; (**) P < 0.01; (****) P < 0.001.

**Table 1.** Serum levels of GH, corticosterone, and IGF-I in wild-type and GRAlfpCre mice

|                        | Control       | GRAlfpCre     |
|------------------------|---------------|---------------|
| Growth hormone (ng/mL) | 1.1 ± 0.3, n = 6 | 1.1 ± 0.1, n = 5 |
| Corticosterone (ng/mL) | 21 ± 10, n = 5  | 20 ± 12, n = 4  |
| IGF-I (ng/mL)          | 699 ± 148, n = 8 | 506 ± 52, n = 6, p < 0.01 |

Morning levels of GH, corticosterone, and IGF-I levels were determined by radioimmunoassay.
phosphorylation [Fig. 2D] and a concomitant increase in DNA-binding activity [Fig. 3C]. These results suggest that GR is involved in growth control by implementing the GH-dependent transcriptional activity changes in Stat5.

GR/Stat5 interaction was first documented in cell lines and suggested to be required for efficient milk protein synthesis in mammary epithelial cells following lactogenic hormone treatment [Stocklin et al. 1996, 1997; Cellé et al. 1998]. Interestingly, the GR was found to copurify with Stat5 on DNA affinity columns, using Stat5 responsive DNA elements from the Spi-2.1 promoter and nuclear extracts from rat liver [Bergadé et al. 2000]. In addition, an interaction between Stat5 and GR in rat liver was observed by communoprecipitation [Bergadé et al. 2000]. We confirmed this observation. As shown by reciprocal communoprecipitation [Fig. 3A], Stat5 and GR interact or coresize in a complex in mouse liver protein extracts and can be pulled down in either direction. GR interacts with both phosphorylated and unphosphorylated forms of Stat5.

To further investigate Stat5 and GR interaction, we performed chromatin immunoprecipitation [ChIP] to determine whether in vivo GR is present in protein complexes bound at the promoter of GH-regulated genes. Using a similar approach in cell culture, the investigators in a recent study showed the presence of GR on the α2-macroglobulin enhancosome when Stat5 is bound, even though no GRE is present in the enhancosome [Lerner et al. 2003]. We chose to investigate transcriptional DNA regulatory regions from two genes contributing to growth regulation, the ALS promoter and the first intron of IGF-I [Ooi et al. 1998; Yakar et al. 2002; Woelflé et al. 2003]. Both genes were reported to be sensitive to GH signaling via Stat5-binding sites but to lack GREs [Fig. 3B]. We injected wild-type mice with PBS or with GH and Dex. We injected GRAlfpCre mice similarly with GH and Dex as a control. An important enrichment for both ALS and IGF-I DNA sequences was observed in treated animals using an antibody directed against Stat5 [Fig. 3C]. Using an antibody directed against GR, we obtained a significant enrichment in treated control mice, demonstrating that GR is present at the level of Stat5-binding sites. The specificity of the reaction was confirmed by the lack of enrichment when GRAlfpCre mice were used [Fig. 3C]. In addition, the simultaneous presence of Stat5 and GR on ALS and IGF-I promoters was confirmed by successive ChIP experiments using the anti-Stat5 and then the anti-GR antibodies [Fig. 3D].

To strengthen the fact that Stat5–GR interaction is indeed essential for GH signaling, we analyzed growth and expression of GH target genes in mice that were carrying a point mutation in the GR gene (GRdim). This point mutation impairs GR homodimerization and consequently the activity of GR through binding to its cognate DNA-responsive elements. Interestingly, the mutant GR protein is able to repress AP1 and NF-κB activity [Reichardt et al. 1998, 2001b]. Communoprecipitation of Stat5ab and GR was maintained in liver protein extracts from GRdim/dim mice [data not shown; Reichardt et al. 2001a]. As shown in Figure 4A, GRdim/dim mice did not have a growth defect and displayed normal mRNA expression levels for IGF-I, Spi-2.1, and ALS [Fig. 4B]. These data demonstrate that the activity of GR in GH signaling is independent of GR DNA binding and is most probably mediated by protein–protein interaction with Stat5. When the growth of GRAlfpCre mice is compared with the growth of Stat5ab−/− mice, GRdim/dim mice, and triple mutant mice [Fig. 4A], it is obvious that loss of DNA-binding-dependent activities of the GR in addition to loss of Stat5ab does not further impair growth. These observations strengthen the idea that Stat5 and GR act interdependently to efficiently sustain the transcription of genes essential for postnatal body growth. Altogether, these observations suggest that GR acts as a coactivator of Stat5 to promote the expression of target genes on GH stimulation in hepatocytes.

To follow the consequence of GR inactivation, we studied the expression of genes in livers of wild-type and GRAlfpCre mutant mice using Affymetrix DNA chips [Table 2]. Under basal conditions, in fed and nonstressed animals, killed at the beginning of the day phase when GC levels are low, we found 26 genes [21 known genes and 5 ESTs] whose expression was reduced more than twofold in mutant mice. Among the known genes, a large fraction [24%] are under the control of GH through Stat5 activation, illustrating the importance of GR for the control of liver gene expression by GH. Besides the mRNA for IGF-I, ALS, Spi-2.1, and MUP, we also found...
reduction in SOCS-2 mRNA, a negative regulator of Stat5 signaling whose inactivation leads to gigantism [Metcalf et al. 2000]. This result strongly suggests that SOCS-2 is a GR target and likely indicates that for expression of SOCS-2 by Stat5, the GR is required, similar to IGF-I, ALS, and Spi-2.1 mRNA. In addition, this result explains the increased phosphorylation and DNA-binding activity of Stat5, on GH treatment, observed in protein extracts from liver of mutant mice when compared with control animals. We also found a decrease in the level of EGFR mRNA, in line with the observation that EGFR is up-regulated by IGF-I [Bor et al. 2000].

Surprisingly, our results demonstrate that GR function in hepatocytes is essential for body growth. The results emphasize the central role of the liver in the control of body growth, an important conclusion in the context of the somatomedin hypothesis. Several decades ago, this hypothesis postulated an essential role to the liver in control of postnatal body growth [Salmon and Daughaday 1957]. The evolving concepts of this hypothesis [Le Roith et al. 2001] and the observed Stat5–GR interaction here extends the somatomedin hypothesis to insights in signaling pathway cross-talk in the liver. The growth defect described here is most probably due to impaired expression of GH-dependent target genes. The normal plasma level of GH and the normal expression of GHR mRNA suggest that GH signaling is impaired downstream of its receptor. The reduced expression of Stat5 target genes in GR\textsuperscript{AlfpCre}, despite an enhanced activation of Stat5, and their intact expression in GR\textsuperscript{dim/dim} mice demonstrate that Stat5 action requires GR as an essential coactivator. GR or Stat5 alone is unable to sus-
Materials and methods

Animals
Mice with hepatocyte-specific inactivation of GR (GRAlfpCre) were generated by crossing mice in which both alleles of the third exon of GR were flanked by two loxP sites (GRAlfpCre; Tronche et al. 1999) with mice expressing the Cre recombinase under control of the albumin/heteprotein control sequences (Kellendonk et al. 2000). GRAlfpCre were maintained on a mixed genetic background (C57BL/6, 129SvEv and FVB/N). GRloxP, Stat5ab mutant, and intercrosses were maintained on a mixed genetic background (C57BL/6 and 129SvEv) and were genotyped as previously described (Reichardt et al. 1998; Teglund et al. 1998). For some experiments, mice were injected intraperitoneally with 100 μg of dexamethasone phosphate disodium salt (Sigma) and 2 μg recombinant hGH (Serono) and killed 60 or 150 min later. Serum and liver were shock-frozen in liquid nitrogen and stored for further use at −80°C.

To determine body growth, we weighed animals every week. Only animals from litters containing both mutant and control animals were included in this analysis. The body, carcass, and organ weights of adult animals were measured. The length of bones were measured on X-ray radiographies of animals. Histological analysis of paraffin sections did not reveal any alteration in the liver of mutant animals.

Blood measurements
Serum corticosterone, IGF-I, and GH were measured by commercially available kits (ICN Biochemicals, Crystal Chem, and DSL laboratories) and used according to the instructions given by the supplier. To measure morning basal levels of corticosterone, we killed animals by decapitation with 3 h after the beginning of the day phase. Levels of ALS were analyzed in serum samples from four animals of each genotype. Serum was separated by 12% SDS PAGE and blotted to PVDF membranes. ALS was identified by Western immunoblotting (ALS antibody 7H3-b; dilution 1:400, kindly provided by Dr. C. Strasser, Munich, Germany) and detected by the ECL system (Amersham Pharmacia) according to standard protocols.

Northern blotting
Total RNA from liver tissues of day-24 males (GRAlfpCre and control mice) was isolated using RNeasy kits with DNase-I digestion on column (Qiangen). RNA quantity was checked using the Bioanalyzer 2100 Lab-on-a-chip system (Agilent Technologies). Ten micrograms of total RNA was amplified according to the standard protocol given by the Affymetrix instructions. The transcription were profiled on Affymetrix murine U74Av2 arrays according to the manufacturer’s instruction. Raw data were analyzed using the MAS 5.0 version of the Affymetrix software package. Final data were calculated from three independent pools, genes with a reduced expression ratio >2 were considered.

Quantitative PCR
Primers were designed using PrimerExpress 2.0 software (Applied Biosystems). Quantification of precipitated DNA and input DNA fragments was carried out on an ABI PRISM 7000 sequence detection system using SYBR green in triplicates. Relative fold in vivo enrichment of DNA fragments was calculated using the following formula: ChIP/input normalized/input normalized. We used three independent normalizers for IGF-I and ALS, which are located downstream of the corresponding gene’s 3′-prime end.

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Glucocorticoid receptor function in hepatocytes is essential to promote postnatal body growth

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