Dysregulation of the Insulin/IGF Binding Protein-1 Axis in Transgenic Mice Is Associated With Hyperinsulinemia and Glucose Intolerance

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The insulin/IGF binding protein-1 (IGFBP-1) axis is important in coordinating insulin- and IGF-mediated regulation of glucose metabolism and glycemia. Dysregulation of the axis may play a role in the pathophysiology of disorders of insulin deficiency and resistance. We have investigated this hypothesis by generating transgenic mice that overexpress hIGFBP-1. To study the axis in its true physiological context, we used a human (h) IGFBP-1 cosmid clone so that transgene expression is responsive to normal hormonal stimuli. hIGFBP-1 mRNA is expressed in a tissue-specific fashion, and measurement of serum protein levels by specific immunoassay indicates normal physiological regulation in response to fasting/feeding and appropriate post-translational modification as indicated by the detection of phosphorylated and nonphosphorylated isoforms of the protein. The hypoglycemic response to exogenous IGF-I is attenuated in transgenic mice. Transgenic mice exhibit an enhanced insulin secretory response to a glucose challenge, although basal and stimulated blood glucose levels are similar to controls. There is a sexual dimorphism in phenotypic expression: male transgenic mice had higher stimulated glucose and insulin levels than did females. Transgenic mice exhibit fasting hyperglycemia and hyperinsulinemia and glucose intolerance in later life, indicating an age-related decline in glucocompetence. These findings demonstrate the importance of the normal inverse relationship between serum insulin and IGFBP-1 levels in glucoregulation and that sustained dysregulation of the insulin/IGF-I/IGFBP-1 axis is associated with impaired glucose tolerance and abnormalities of insulin action. *Diabetes* 49:457–465, 2000

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hIGFBP-1, human IGF binding protein; HNF, hepatocyte nuclear factor; IGFBP, IGF binding protein; IRMA, immunoradiometric assay; MODY, maturity-onset diabetes of the young; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; rIGFBP, rat IGF binding protein; RT, reverse transcription.

The IGFs (IGF-I and -II) have insulin-like actions on glucose metabolism in vivo, stimulating peripheral glucose uptake and utilization and reducing hepatic glucose production (1). These observations have led to speculation that the IGFs play a role in regulating carbohydrate metabolism and glycemia. The actions of the IGFs are modulated by the IGF binding proteins (IGFBPs), a family of high-affinity binding proteins that determine IGF bioavailability (2). One of the major functions of serum IGFBPs is regulation of the hypoglycemic potential of circulating IGFs, which are ~1,000-fold greater than insulin levels (3). IGFBP-1 is believed to play an important role in the acute regulation of serum free IGFs.

Insulin has multiple reciprocal interactions with the IGFs and their binding proteins. Insulin is a positive regulator of IGF-I synthesis in vivo and in vitro, acting at the level of hepatic gene transcription (4,5). In vivo studies of IGF-I administration have consistently shown an inhibitory effect on insulin secretion (6,7). Furthermore, IGF-I increases tissue sensitivity to insulin in patients with type 1 or type 2 diabetes (8,9). Serum IGFBP-1 levels fluctuate in response to nutritional status, largely as a result of negative regulation of hepatic IGFBP-1 gene expression by insulin (10,11). Epinephrine, glucagon, and corticosteroids are positive physiological regulators of IGFBP-1, although their effects are secondary to the inhibitory action of insulin. The hormonal regulation of IGFBP-1 synthesis is characteristic of counterregulatory hormones, leading to suggestions of a possible role in glucose counterregulation (12).

Dysregulation of the insulin/IGFBP-1 axis is a consistent feature of the human disease states of insulin deficiency and resistance. IGFBP-1 levels are elevated in type 1 diabetic patients and in some forms of acquired insulin resistance, such as type 2 diabetes (13,14). The elevated IGFBP-1 levels in these disease states reflect the reduced inhibitory effect of insulin on IGFBP-1 synthesis. The increase in IGFBP-1 levels may not be merely a consequence of insulin deficiency/resistance but might play a role in the pathophysiology of these conditions by virtue of its inhibitory effects on IGF activity. Some of the metabolic derangements in type 1 diabetic patients may be due to attenuation of the insulin-like actions of the IGFs (15). Furthermore, it has been suggested that increased levels of IGF inhibitory IGFBPs may play a role in growth impairment in prepubertal children with poorly con-

trolled type 1 diabetes and also in late-onset complications of type 1 diabetes, such as retinopathy and nephropathy (16).

To explore the role of the insulin/IGFBP-1 axis, we have experimentally manipulated the axis so that the normal inverse relationship between insulin and IGFBP-1 is disturbed. This has been achieved by overexpressing human (h) IGFBP-1 in transgenic mice. A transgenic model of IGFBP-1 overexpression has been previously generated, but the use of a heterologous promoter to control transgene expression means that its usefulness in elucidating the physiological function of the insulin/IGFBP-1 axis is limited (17). We have improved on this model by developing transgenic mice using a cosmid clone encompassing the entire hIGFBP-1 structural gene and its regulatory sequences that is responsive to normal hormonal stimuli, thereby allowing us to study the axis in its true physiological context.

RESEARCH DESIGN AND METHODS

Generation of transgenic mice. A cosmid clone, chBP1:2, encompassing the hIGFBP-1 structural gene, has been reported previously (18). A 35-kb NotI fragment of chBP1:2 was gel purified, quantitated, and then diluted to give a final concentration of 1–2 µg/ml. The DNA was microinjected into the male pronucleus of fertilized C57Bl/CBA oocytes. Transgenic mice were initially identified by Southern blot analysis of DNA isolated from tail biopsies taken at weaning. EcoRI digests of mouse DNA were transferred to Hybond N$^+$ hybridization membrane by standard procedures (19) and probed with a [α-^{32}P]dCTP-labeled hIGFBP-1 cDNA (20). Founder animals positive for the transgene were mated with CBA/Ca mice to produce heterozygous transgenic F$_1$ animals. F$_1$ and F$_2$ transgenic males were bred back to CBA/Ca females to yield F$_3$ animals, which were used in these studies. Control mice used in the experiments described were age- and sex-matched nontransgenic littermates.

RNA isolation and analysis. Total RNA was extracted from frozen tissue using RNAzolB according to the manufacturer's instructions (Biogenesis, Poole, U.K.). For Northern analysis, 20 µg of total RNA was electrophoresed on a 1.2% denaturing formaldehyde gel, transferred to Hybond N$^+$, and probed with a [α-^{32}P]dCTP-labeled hIGFBP-1 cDNA. Gene expression was also analyzed by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA (2.5 µg) was reverse-transcribed using Superscript II and random hexamers following the manufacturer's guidelines (Life Technologies, Paisley, U.K.). cDNA was amplified by PCR with the primer pair HBP1 F (5′-AACCTCTGCACGCCCTCACC-3′) and HBP1 R (5′AGGGATCCTCT TCCCATTCCAAGGGTAGAC-3′), which specifically amplify hIGFBP-1 cDNA.

Competitive RT-PCR

Construction of the internal cRNA standard for competitive RT-PCR. The internal cRNA standard for quantitative RT-PCR was prepared by amplifying human liver cDNA with the primer pair CPT7HBP1 F (5′-CGGGATCCGGATCCTA ATACGACTCACTATAGGGAGAAACCTCTGCACGCCCTCACC-3′) and CPHBP1 R (5′-AGGGATCCTCTTCCCATTCCAAGGGTAGACTCTTGTTGCAGTTTGGC AG-3′). The internal competitor is designed to be amplified by the same primer pair as the target but it generates a product that is 52 bp smaller that can be distinguished from the target by agarose gel electrophoresis. The amplified product was transcribed in vitro using T7 polymerase (Stratagene, Cambridge, U.K.), and the transcript was run on a 1.5% denaturing formaldehyde gel to confirm its size and integrity and quantitated by spectrophotometry.

cDNA synthesis and PCR amplification. Total liver RNA was isolated from fasted and ad libitum–fed transgenic mice. An equal mass of RNA from individual transgenic animals in each group was pooled, and 2.5 µg of it was reverse transcribed in the presence of twofold dilutions of the internal standard cRNA. cDNA was amplified using the primer pair HBP1 F and HBP1 R. PCR products were analyzed by electrophoresis on 2% agarose gels. The band intensity was determined by densitometry (GDS 7000 and Gel Pro Analyser software; UVP, Cambridge, U.K.), and the mean ratio of the intensity of the target (A$_t$) to competitor (A$_c$) was calculated for each amount of the internal standard added.

Western ligand blot analysis. Samples of sera from transgenic and wild-type animals containing an equal mass of protein were diluted with sample buffer and denatured at 100°C for 5 min before electrophoresis on a 12% SDS-polyacrylamide gel. The gel was electroblotted to nitrocellulose (Hybond C; Amersham, Amersham, U.K.). After incubation with ^{125}I-labeled IGF-I and –II, the filter was washed to remove unbound ligand, and the IGFBPs were visualized by autoradiography.

Assays for hIGFBP-1, insulin, and IGF-1. Total and nonphosphorylated hIGFBP-1 in sera of transgenic mice was measured by immunoradiometric assay (IRMA) (Diagnostic Systems Laboratories, Webster, TX) (21). Both assays show absolute specificity for hIGFBP-1. Murine insulin and IGF-I were measured by radioimmunoassay (Biogenesis, Poole, U.K.). For comparison of fasting and nonfasting sera, samples were collected from overnight fasted mice. These mice were then allowed access to food for 4 h, after which a second sample was taken.

Intraperitoneal glucose tolerance tests. Mice were fasted overnight before testing. Blood samples were collected before and 30 min after administration of an intraperitoneal injection of glucose (1 mg/g body weight in sterile saline). Samples for measurement of hIGFBP-1, insulin, and IGF-I were centrifuged for 10 min at 2,500 rpm, and the plasma was stored at –20°C. The time course of glucose uptake was determined by measurement of whole blood glucose before and 30, 60, and 120 min after a similar glucose challenge. Whole blood glucose was determined in duplicate at each time point using a a portable blood glucose analyzer (Hemocue, Sheffield, U.K.).

IGF-I administration. Recombinant human IGF-I (0.2 µg/g body weight) was administered subcutaneously to overnight fasted mice, and whole blood glucose was measured as described above.

Statistical analysis. Results are presented as means ± SE. Comparisons were made by analysis of variance or Student's t test after log normalization of nonparametric data. Where $P < 0.05$, analysis of variance was completed using Fisher's least significant difference test. Correlations were made by simple regression analysis.

RESULTS

Generation of hIGFBP-1 transgenic mice. Three of seven mice (designated c1:2-1, c1:2-2, and c1:2-11) born after microinjection of the NotI fragment of chBP1:2 were positive for the transgene. Southern blot analysis of tail DNA from all three founders indicates that the cosmid integrated without any gross rearrangements and was present in multiple copies (Fig. 1B). The copy number was estimated to range from 8 to 20 by slot blot analysis in the three lines (data not shown).
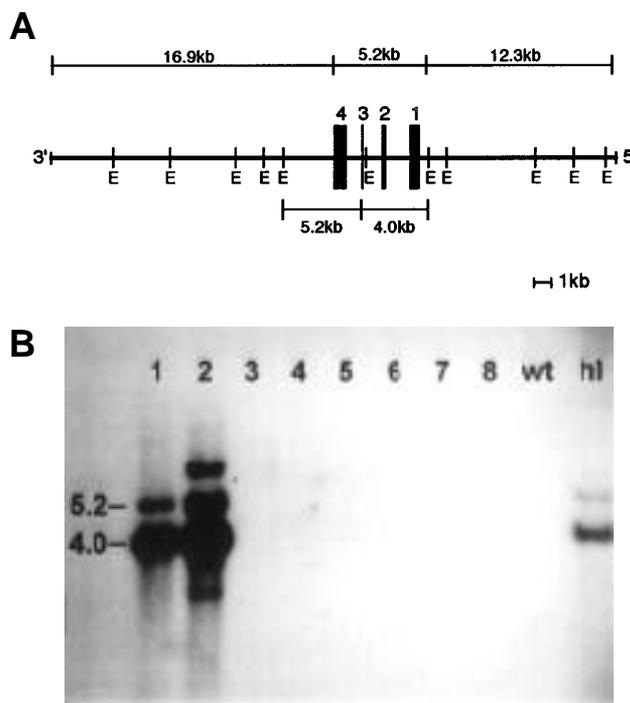

FIG. 1. Identification of hIGFBP-1 transgenic mice by Southern blot analysis. A: Schematic diagram of the hIGFBP-1 cosmid clone chBP1:2. Exons (1–4) are represented by solid bars and the positions of EcoRI (E) sites are indicated. B: Southern blot analysis of EcoRI digested DNA from two founder mice showing the characteristic 5.2- and 4-kb cross-hybridizing bands of the hIGFBP-1 gene (**lanes 1 and 2**). **Lanes** 3–8 contain DNA from nontransgenic animals. Wild-type mouse (wt) and human liver DNA (hl) were loaded as controls.
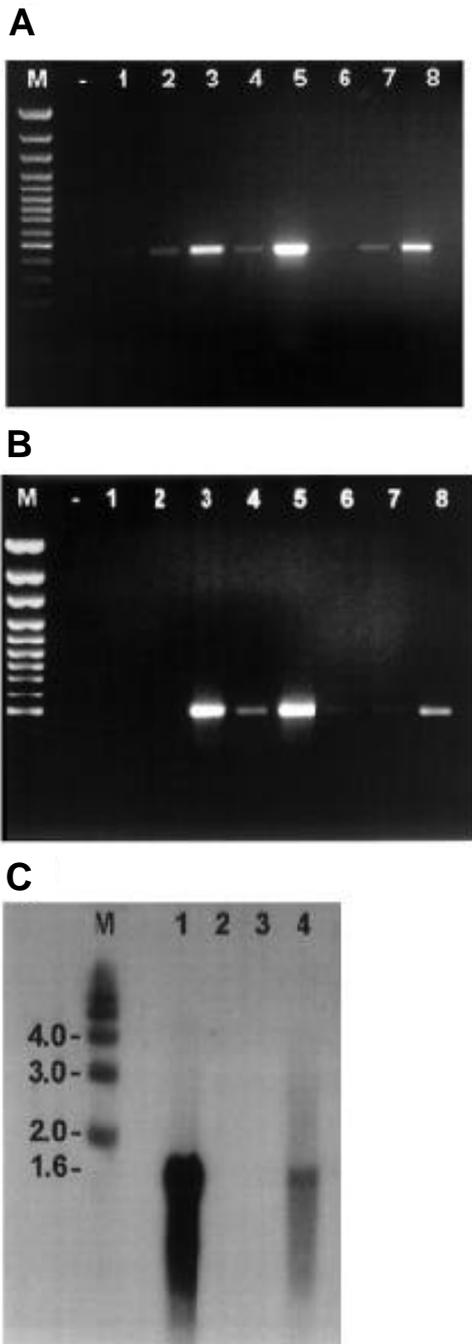
FIG. 2. Analysis of hIGFBP-1 expression in transgenic mice. A: Tissue distribution of hIGFBP-1 mRNA expression in transgenic mice. hIGFBP-1 mRNA was amplified by RT-PCR using the primer pair HBP1 F/HBP1 R, which specifically amplify a 445-bp product from hIGFBP-1 cDNA. The amplification profile was 30 cycles of 95°C 1 min/58°C 1 min/72°C 1 min. The samples are as follows: lane 1, brain; lane 2, heart; lane 3, kidney; lane 4, lung; lane 5, liver; lane 6, small intestine; lane 7, spleen; lane 8, uterus. M, 100-bp DNA ladder; –, RT-PCR of nontransgenic liver RNA. B: Endogenous IGFBP-1 expression in tissues of wild-type mice. RNA from wild-type tissues was amplified by RT-PCR using the primer pair MBP1F (5'-ACCTGCTGCACCCGCCACGAG-3') and MBP1R (5'-TTCAGTTATGTACAT TAAAATACTGG-3'), which amplify a 517-bp fragment of murine IGFBP-1 cDNA using the same amplification profile as above. The tissues and lane order are the same as in A. M, 100-bp DNA ladder; –, RT-PCR negative control (no template). C: Hepatic hIGFBP-1 transcript size. A Northern blot of liver RNA probed with the hIGFBP-1 cDNA. Lane 1 contains RNA from human liver; lanes 2 and 3 contain liver RNA from nontransgenic mice; and lane 4 contains liver RNA from a transgenic mouse of line c1:2-2.
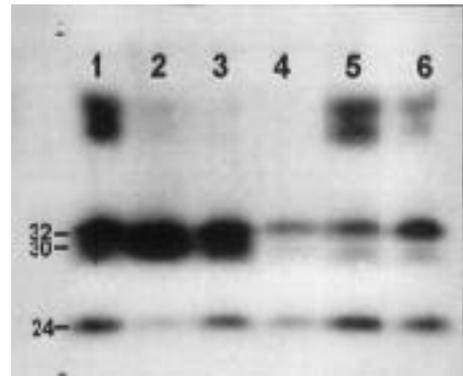

FIG. 3. Western ligand blot of fasting sera from transgenic mice (lanes 1–3) and wild-type controls (lanes 4–6). Murine and hIGFBP-1 co-migrate with an apparent molecular weight of ~30 kDa. IGFBP-2 (32 kDa) and IGFBP-4 (24 kDa) are indicated. IGFBP-1 abundance in transgenic mice was estimated to be fivefold greater than wild-type mice by densitometry (P < 0.05).

Analysis of hIGFBP-1 expression. The tissue distribution and transcript size of hIGFBP-1 mRNA in transgenic mice were determined by Northern blotting and RT-PCR analysis (Fig. 2). hIGFBP-1 mRNA was most abundant in the liver, followed by the kidney and uterus, with much lower levels in heart, small intestine, spleen, and brain (Fig. 2A). The sites of expression and relative abundance of hIGFBP-1 mRNA in tissues of transgenic mice are very similar to those of the endogenous murine gene in nontransgenic mice (Fig. 2B). The liver transcript size was estimated to be 1.6 kb by Northern blot analysis, which is identical in size to the transcript size in human liver (Fig. 2C; data shown only for line c1:2-2).

Serum IGFBP-1 levels in transgenic mice. Total IGFBP-1 levels (i.e., murine plus hIGFBP-1) in sera of transgenic mice were assessed by Western ligand blot analysis. Figure 3 shows a Western ligand blot of fasting sera from transgenic mice and wild-type controls. In sera of transgenic mice, there is a marked increase in the abundance of a 30-kDa IGFBP corresponding to IGFBP-1.

Estimation of serum hIGFBP-1. Total and nonphosphorylated hIGFBP-1 were quantitated in sera of transgenic mice using specific IRMAs. The mean fasting total hIGFBP-1 in sera of transgenic mice was 174.1 ± 29.7 µg/l (mean nonphosphorylated hIGFBP-1 was 99.1 ± 32 µg/l). hIGFBP-1 was undetectable in sera of wild-type controls, confirming the specificity of both assays.

Physiological regulation of hIGFBP-1. To determine whether hIGFBP-1 is responsive to normal physiological stimuli in transgenic mice, we compared hepatic gene expression and circulating levels of hIGFBP-1 in relation to nutritional status. Matched fasting and nonfasting sera from transgenic mice were assayed for total hIGFBP-1, nonphosphorylated hIGFBP-1, and insulin (Table 1 and Fig. 4). The nonfasting hIGFBP-1 level was reduced by 81% compared with fasting levels. The normal inverse relationship between insulin and IGFBP-1 is maintained in transgenic mice (Fig. 4B). Figure 5 shows the results of competitive RT-PCR analysis of liver RNA from fasted and fed mice. There is a 12-fold reduction in expression levels in the nonfasting state, accounting for the decrease in the serum level of the protein.

Response to IGF-I administration. The hypoglycemic response to exogenous IGF-I is attenuated in transgenic mice

TABLE 1
Glucose, insulin, and hIGFBP-1 in fasting and nonfasting sera of transgenic mice

	Fasting		Nonfasting	
	Male	Female	Male	Female
Glucose (mmol/l)	7.95 ± 0.9	8.0 ± 0.5	12.25 ± 0.6	11.8 ± 0.4
Insulin (µg/l)	0.21 ± 0.03	0.4 ± 0.1	1.85 ± 0.5	1.76 ± 0.4
Glucose/insulin	45.4 ± 2.6	39.8 ± 9.9	19.5 ± 5.9	11.3 ± 2.6
Total hIGFBP-1 (µg/l)	191.2 ± 56.1	160 ± 29.9	35 ± 6.5	31.7 ± 5.2
Nonphosphorylated hIGFBP-1 (µg/l)	130.6 ± 60.5	73.3 ± 31.5	ND	ND
Animals per group	8–10	8–12	8–10	8–12

Data are means ± SE for the indicated number of animals. ND, not determined.

(Fig. 6). Blood glucose levels in transgenic mice were higher at all time points after the glucose challenge compared with controls, although this was only significant at 60 and 120 min. There is a marked sexual dimorphism in the response to IGF-I. In wild-type males, there is an increase in blood glucose levels at 30 min and then a rapid decline. This initial increase in blood glucose is abolished in male transgenic mice. In contrast, both wild-type and transgenic females show a rapid fall in blood glucose levels, the fall being more pronounced in wild-type animals.

Glucoregulation in transgenic mice. Comparison of fasting and nonfasting blood glucose and insulin levels in transgenic and wild-type littermates at age 16–20 weeks indicated no significant differences between groups. However, there was a marked difference in the response to an intraperitoneal glucose challenge in transgenic mice (Table 2 and Fig. 7). The 30-min insulin levels were 2.5-fold greater in transgenic males (37% increase in females), although blood glucose levels were similar to controls. Fasting insulin and glucose levels in transgenic males did not differ significantly from sex-matched controls. The glucose/insulin ratio is significantly lower in transgenic males after a glucose challenge. Transgenic males had significantly higher 30-min glucose and insulin levels compared with females. Analysis of data from control mice indicates no similar differences.

Although the fasting and 30-min hIGFBP-1 levels were similar, there was a significant increase in the relative proportion of the nonphosphorylated isoform in sera of transgenic mice (Table 2). IGF-I levels did not differ between groups in either basal or stimulated sera (Fig. 7B). Transgenic mice have normal glucose tolerance based on blood glucose levels 30, 60, and 120 min after a glucose challenge (data not shown).

Age-related changes in glucoregulation. We measured fasting blood glucose, total hIGFBP-1, and insulin and performed intraperitoneal glucose tolerance tests at age 40–44 weeks to determine if sustained overexpression of hIGFBP-1 in transgenic mice is associated with more profound abnormalities of glucoregulation. There was a marked deterioration in glucocompetence with age, as demonstrated by increased fasting blood glucose and insulin levels in transgenic male mice (Fig. 8A). Fasting hIGFBP-1 levels were reduced, reflecting the relative hyperinsulinemia. Intraperitoneal glucose tolerance tests indicated a marked impairment in the ability to dispose of a glucose load compared with age- and sex-matched controls (Fig. 8B). Fasting blood glucose and insulin levels were moderately elevated in female transgenic mice compared with sex-matched controls, but the data did not reach statistical significance. This was also true of glucose
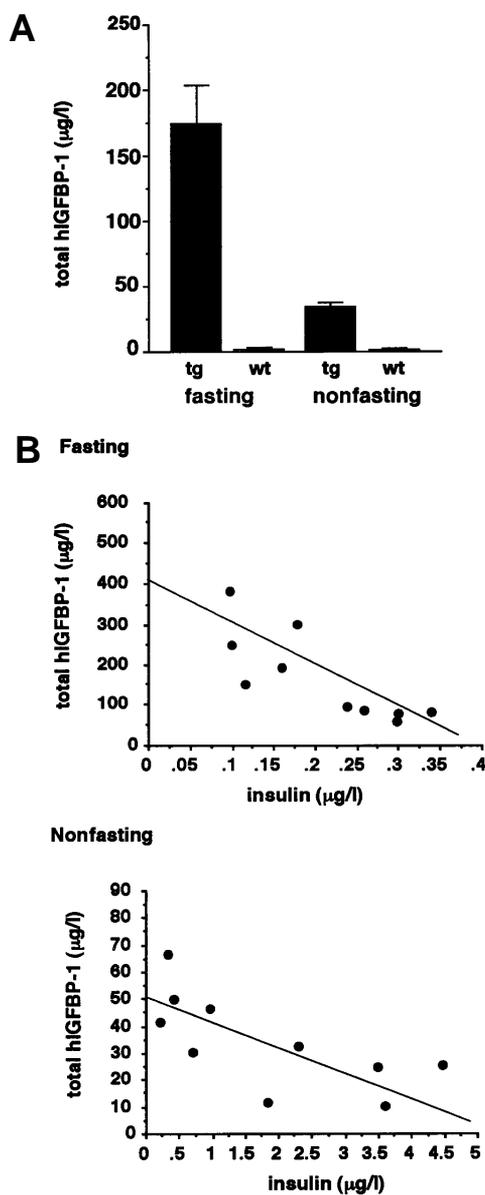
FIG. 4. Insulin and hIGFBP-1 levels in sera of transgenic mice. Matched fasting and nonfasting serum samples were assayed for total hIGFBP-1 and insulin. A: Total hIGFBP-1 levels in fasting and nonfasting sera of transgenic (n = 22) and control mice (n = 25). B: Comparison of insulin and hIGFBP-1 levels in fasting and nonfasting sera of transgenic mice by simple regression. Data from male mice (n = 10) aged 16–20 weeks are shown. The correlation coefficients for fasting and nonfasting sera are 0.69 (P < 0.05) and 0.66 (P < 0.05), respectively.
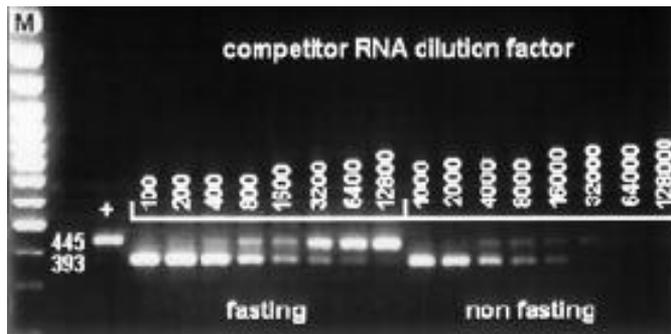
FIG. 5. Competitive RT-PCR analysis of hepatic hIGFBP-1 gene expression in transgenic mice. A representative ethidium bromide–stained gel shows amplification of liver RNA from fasting and non-fasting transgenic mice in the presence of twofold dilutions of the competitor RNA. Amplification of the target generates a band of 445 bp compared with 393 bp for the competitor using the same primer pair and amplification profile indicated in the legend to Fig. 2A. The log of the ratio of the absorbance of target to competitor (A_t/A_c) against the log of the competitor dilution was plotted. Extrapolation from $A_t/A_c = 0$ (i.e., equal amplification of target and competitor) indicates a 12-fold induction of target mRNA in the fasting group ($P < 0.01$). Data were derived from three separate amplifications of RNA pooled from six animals per group.

tolerance, which showed a mild but statistically insignificant impairment.

Growth of hIGFBP-1 transgenic mice. The body weights of transgenic and control mice were measured to examine possible effects of hIGFBP-1 overexpression on postnatal growth (Fig. 9). Transgenic mice were ~10% lighter than wild-type littermates at weaning, but by 8 weeks of age this deficit had narrowed such that body weights were essentially identical. The parity in body weight was maintained until the last recorded measurements at 12–16 weeks. A significant inverse correlation was observed between fasting serum hIGFBP-1 and body weight and IGF-I levels in adult transgenic mice (Fig. 9B). Measurements of serum IGF-I levels correlate well with body weights and sex in both groups, but no significant differences were observed between transgenic and control animals.

DISCUSSION

This study was undertaken to investigate the physiological significance of the inverse relationship between insulin and IGFBP-1 and its role in regulating glucose metabolism and glycemia. The role of IGFBP-1 in the acute regulation of serum free IGFs and the manner of its regulation by insulin and contra-insulin hormones, as well as the reciprocal relationship between insulin and IGF-I, are consistent with a model whereby the insulin/IGF-I/IGFBP-1 axis coordinates the metabolic and hypoglycemic activities of insulin and the IGFs in relation to fuel supply.

We have developed transgenic mice that overexpress hIGFBP-1 to investigate this hypothesis. The transgene is a human cosmid clone encompassing the entire structural gene and all the cis acting sequences necessary to direct appropriate tissue-specific and regulated gene expression. This approach avoids many of the problems associated with the use of heterologous promoters, such as atypical expression sites and low levels of expression. More importantly, the use of a constitutively active promoter would not provide
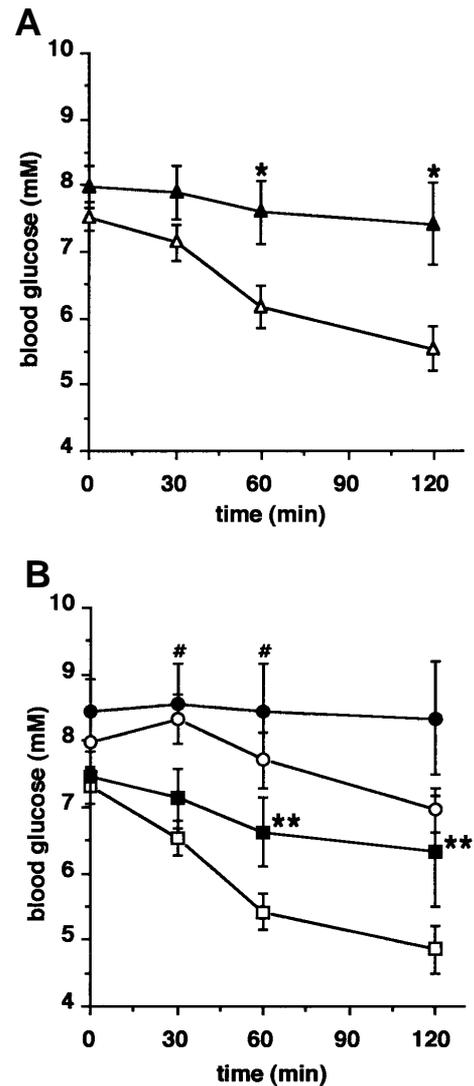

FIG. 6. Hypoglycemic effects of exogenous IGF-I. Recombinant human IGF-I (0.2 µg/g body weight) was administered subcutaneously to overnight fasted mice. Blood glucose was measured at the indicated intervals. Data are means ± SE for 25 wild-type (△) and 26 transgenic animals (▲) (A). In B, the data are presented to demonstrate sex differences in the response to IGF-I. The groups are denoted as follows: ○, wild-type male (n = 8); ●, transgenic male (n = 14); □, wild-type female (n = 17); ■, transgenic female (n = 12). *P < 0.02 for transgenic vs. wild-type; **P < 0.05 for sex-matched transgenic vs. wild-type; #P < 0.05 for transgenic males vs. females.

insights into the changing nature of the axis in response to nutritional status. An important advantage of using the human gene is that although the human protein is fully compatible with the rodent IGF/IGFBP system, it is sufficiently distinct to allow us to distinguish it from the murine homologue at the mRNA and protein level. The availability of reagents for the detection of specific isoforms of hIGFBP-1 allows analysis of the phosphorylation status of the protein.

The tissue distribution of hIGFBP-1 mRNA in transgenic mice is more similar to the human than the murine pattern. In transgenic mice, renal hIGFBP-1 expression is low compared with the liver; but in wild-type mice, endogenous IGFBP-1 mRNA is equally abundant in the liver and the kidney (22). Serum levels of hIGFBP-1 in transgenic mice are in the pathophysiological range. Fasting IGFBP-1 levels in nor-

TABLE 2
Glucose tolerance tests: basal and stimulated serum chemistry in male hIGFBP-1 transgenic mice and sex-matched controls

	Basal		Stimulated	
	Transgenic	Wild type	Transgenic	Wild type
Glucose (mmol/l)	7.55 ± 0.4	7.3 ± 0.5	15.4 ± 0.7	13.7 ± 1.4
Insulin (µg/l)	0.24 ± 0.04	0.25 ± 0.1	1.7 ± 0.32	0.69 ± 0.08*
Glucose/insulin	42.3 ± 7.8	43.6 ± 8.4	10.8 ± 1.1	22.6 ± 5.6†
Total hIGFBP-1 (µg/l)	75.9 ± 18	—	74 ± 27.3	—
Nonphosphorylated hIGFBP-1 (µg/l)	26.6 ± 10.6	—	41.5 ± 20.3	—
Nonphosphorylated/total hIGFBP-1	0.28 ± 0.06	—	0.49 ± 0.1*	—
IGF-I (µg/l)	517.5 ± 37.6	548.8 ± 64.2	507.4 ± 20.3	559.3 ± 62.4
Animals per group	8–11	6–10	8–11	6–10

Data are means ± SE for the indicated number of animals. Matched serum samples were collected before and 30 min after an intraperitoneal glucose challenge. Statistically significant differences between transgenic and age-matched control animals in stimulated sera and between the nonphosphorylated/total hIGFBP-1 ratio in basal and stimulated sera of transgenic mice are shown. *$P < 0.05$; †$P < 0.02$.

mal human serum are in the range of 50–80 ng/ml, falling to <10 ng/ml in the nonfasting state.

hIGFBP-1 levels in sera of transgenic mice vary in response to nutritional status, and the normal inverse relationship between hIGFBP-1 and insulin levels is evident. Hepatic hIGFBP-1 mRNA levels are reduced in the nonfasting state consistent with the inhibitory effect of insulin on hepatic IGFBP-1 gene transcription. However, the relationship between insulin and hIGFBP-1 levels is not absolute. It can

be seen that even in the nonfasting state when insulin levels are maximal, hIGFBP-1 levels in the circulation, although reduced compared with fasting levels, are considerably higher than would be expected. Therefore, regulated overexpression of hIGFBP-1 in transgenic mice is a model of reduced sensitivity of IGFBP-1 to insulin regulation.

This is the only transgenic model of IGFBP-1 overexpression to show appropriate post-translational modification of the protein. IGFBP-1 has both inhibitory and stimulatory effects on IGF
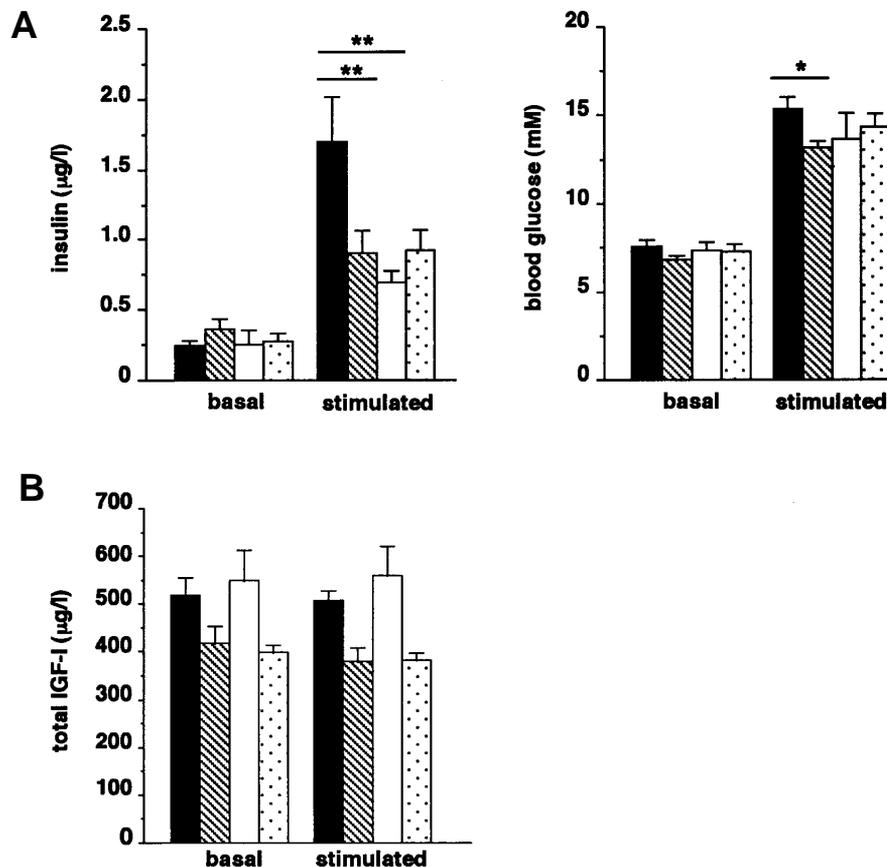

FIG. 7. Glucose tolerance tests in transgenic mice. Transgenic and age-matched controls (16–20 weeks) were fasted overnight and then given glucose intraperitoneally. Matched basal and stimulated samples were assayed for insulin and glucose (A) and total IGF-I (B). Data are presented as means ± SE for the number of animals shown in Table 2. Groups are as follows: ■, transgenic male; □, wild-type male; ▨, transgenic female; ▧, wild-type female. *$P < 0.01$; **$P < 0.05$.
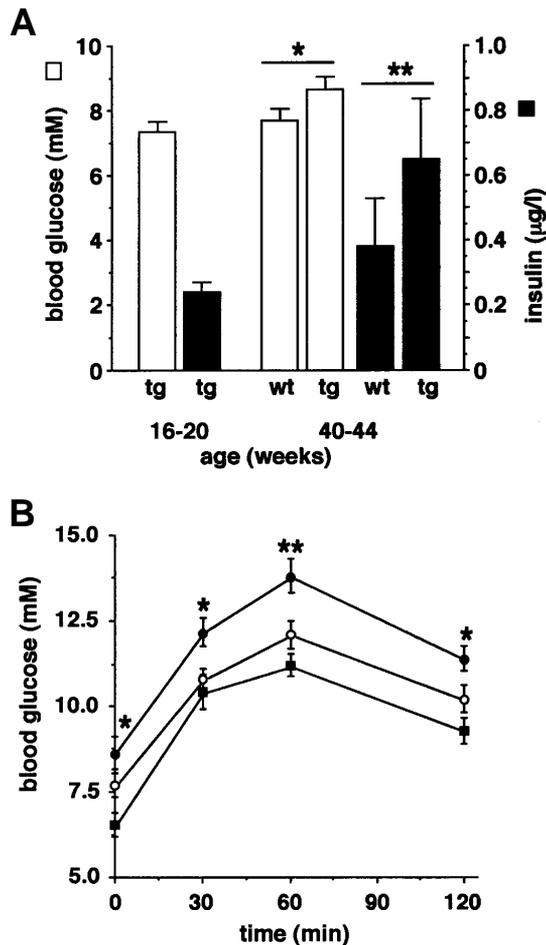
FIG. 8. Age-related decline in glucocompetence in hIGFBP-1 mice. A: Fasting blood glucose (□) and insulin (■) were measured at age 40–44 weeks in transgenic and age-matched controls. Data are presented as means ± SE for 10 transgenic and 12 wild-type males. Data from transgenic males aged 16–20 weeks are included for comparison. B: Glucose tolerance tests were performed on overnight fasted mice. Blood glucose was assayed at the indicated intervals. Data are means ± SE for 10 transgenic and 12 wild-type males. Groups are denoted as follows: ○, wild-type male; ●, transgenic male. Transgenic males aged 16–20 weeks (■) are included for comparison. *P < 0.05; **P < 0.02 for transgenic vs. wild-type males.
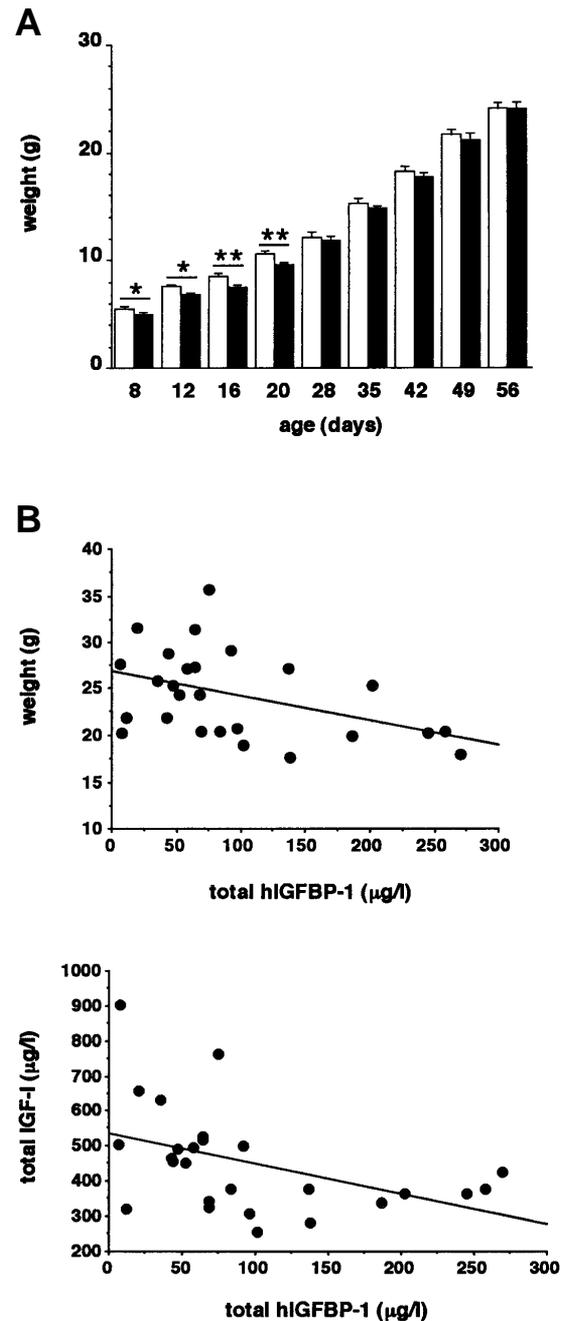

FIG. 9. Postnatal growth of hIGFBP-1 transgenic mice. A: Transgenic mice and wild-type littermates were weighed at the indicated time intervals. To control for the effects of litter size, only litters of 6–8 animals were considered. Data are presented as means ± SE for transgenic (■) (n = 10–36) and wild-type (□) (n = 10–24) animals. *P < 0.005; **P < 0.0005. B: Relationship between total hIGFBP-1 and IGF-I and body weight in transgenic mice. Total hIGFBP-1 and IGF-I levels were assayed in fasting sera of adult transgenic mice, and body weights were recorded. Data were analyzed by simple regression. The correlation coefficients and P values for the comparisons are as follows: r = 0.44, P < 0.05 for total hIGFBP-1 vs. IGF-I; r = 0.43, P < 0.05 for total hIGFBP-1 vs. body weight.

activity in vitro (23,24). This differential action appears to be due to the phosphorylation status of the protein, which can vary depending on its origin. Phosphorylated IGFBP-1 has a three- to fivefold greater IGF binding affinity than the nonphosphorylated protein. In vivo regulation of IGFBP-1 phosphorylation has not been convincingly demonstrated, and its physiological significance remains unclear. However, the striking differences in the phosphorylation status of IGFBP-1 in different feto-maternal compartments argues in favor of it having an in vivo role in modulating IGFBP-1 function, at least in fetal growth and development (25).

IGFBP-1 in normal human serum is present predominantly as a single, highly phosphorylated species (>95%). The proportion of phosphorylated hIGFBP-1 in sera of transgenic mice is significantly lower (60–70%). This may be due to the fact that murine species lack hepatic protein kinases with the appropriate substrate specificity to phosphorylate hIGFBP-1 on the serine residues (serine 98 and 169) that are not con-

served in murine IGFBP-1. Alternatively, the decrease in hIGFBP-1 phosphorylation may represent an adaptive response to increased IGFBP-1 synthesis, increasing the proportion of the nonphosphorylated (i.e., less inhibitory) form of the protein. The increase in the proportion of nonphos-

phorylated hIGFBP-1 after a glucose challenge is intriguing, particularly when total hIGFBP-1 levels remain unchanged. In physiological terms, there is a compelling rationale for such a phenomenon. Nonphosphorylated IGFBP-1 would be less inhibitory to the insulin-like actions of the IGFs at a time when this activity needs to be maximized, i.e., in response to a glucose challenge. Insulin itself, or perhaps glucose, may stimulate hepatocytes to alter the pattern of protein phosphorylation. Hormonal regulation of IGFBP-1 phosphorylation has been reported in humans, but the physiological significance and mechanisms involved require clarification (26).

We have demonstrated specific abnormalities of glucoregulation in hIGFBP-1 mice. At age 16–20 weeks, hIGFBP-1 transgenic mice have normal fasting glucose and insulin levels. Even after a glucose challenge, blood glucose levels in transgenic mice are similar to controls, albeit at the expense of a twofold increase in insulin secretion. The coordinated actions of insulin on glucose transport and IGFBP-1 expression enhance the ability to respond to a glucose challenge when insulin levels are high, and the insulin-like activity of the IGFs can be expressed because of the insulin-mediated suppression of IGFBP-1. The finding of normal fasting blood glucose in transgenic mice and the observation that insulin secretion is only perturbed following a glucose challenge are consistent with this analysis.

Our hypothesis is that the insulin/IGFBP-1 axis is important in coordinating the metabolic and hypoglycemic activities of insulin and the IGFs and that dysregulation of the axis may give rise to abnormalities of insulin action, leading to insulin resistance and ultimately glucose intolerance. The rationale is that if insulin-mediated reduction of IGFBP-1 levels in the nonfasting state is not absolute, then serum IGFBP-1 has the potential to attenuate the hypoglycemic actions of the IGFs. In our view, this would precipitate nonfasting hyperinsulinemia by a number of mechanisms acting in synergy and which are related to a reduction in IGF bioavailabilty. Attenuation of the hypoglycemic actions of the IGFs by IGFBP-1 may elicit a compensatory increase in insulin secretion to maintain normal blood glucose levels. The reduced inhibitory action of the IGFs on insulin secretion would have a similar outcome. Because IGF-I is reported to increase insulin sensitivity in humans and rodents, the reduction in IGF bioactivity could lead to desensitization of insulin-responsive tissues, namely skeletal muscle. The increase in insulin secretion after a glucose challenge in hIGFBP-1 mice is of the order of 2.5-fold greater than in controls. The cumulative effect of this level of insulin hypersecretion over a long period of time would inevitably lead to a decrease in tissue sensitivity to insulin, stimulating yet further insulin secretion. This prediction is borne out in hIGFBP-1 mice with the onset of fasting hyperglycemia and hyperinsulinemia and glucose intolerance with advancing age. The progression from normoglycemia and hyperinsulinemia to overt fasting hyperglycemia, hyperinsulinemia, and glucose intolerance in hIGFBP-1 is similar to some animal models of type 2 diabetes.

Abnormalities of hepatic insulin sensitivity offer the most plausible scenario by which the insulin/IGFBP-1 axis may be perturbed. Defects in insulin action at the receptor or postreceptor level could attenuate the inhibitory action of insulin on IGFBP-1 gene transcription. Liver-enriched transcription factors such as the hepatocyte nuclear factor (HNF) family (HNF-1 to -4) are important in regulating the expression of genes such as IGFBP-1 that are highly expressed in the liver. Recently, mutations in the HNF-1β and HNF-4α genes have been reported in patients with maturity-onset diabetes of the young (MODY), a rare form of type 2 diabetes (27,28). The role of the three known MODY genes in susceptibility to the more common late-onset form of type 2 diabetes remains uncertain. Finally, the phenomenon of "metabolic programming" is thought to underlie the epidemiological association between low birth weight and the incidence of type 2 diabetes in adult life (29). It has been suggested that abnormalities of the insulin/IGFBP-1 axis may be programmed in utero in response to fetal undernutrition (30). Circulating levels of IGFBP-1 and insulin are increased in fetuses with intrauterine growth restriction (31), and several studies have shown that children who were small at birth have impaired glucose tolerance and insulin resistance in adult life (32,33).

Our findings are distinct from those reported in IGFBP-1 transgenic mice in which gene expression is regulated by the mouse phophoglycerate kinase (PGK) promoter (PGK-rIGFBP-1). PGK-rIGFBP-1 mice exhibit basal and stimulated hyperglycemia and hyperinsulinemia and have impaired glucose tolerance from an early age (34). At a comparable age, hIGFBP-1 mice have normal fasting and nonfasting blood glucose and insulin but exhibit hyperinsulinemia after a glucose challenge. The increase in insulin secretion in response to a glucose challenge in PGK-rIGFBP-1 mice is less than that of wild-type controls (1.6- vs. 3.99-fold increase over basal levels). The glucose/insulin ratio falls in hIGFBP-1 mice but increases in PGK-rIGFBP-1 mice after a glucose load. Another divergence in the phenotypes is the age-related decline in serum insulin levels in PGK-rIGFBP-1 mice, in contrast to the increase in insulin secretion in hIGFBP-1 mice between the ages of 4 and 10 months.

hIGFBP-1 transgenic mice have a moderate degree of growth restriction in early postnatal life before weaning, but there appears to be "catch-up growth," as the weight difference is no longer evident by 8 weeks of age. In contrast, the growth restriction in PGK-IGFBP-1 transgenic mice follows a different pattern. The growth trajectories of transgenic and wild-type mice diverge early in postnatal life and continue into adult life with no evidence of catch-up growth.

Given the similarity in the weights of adult hIGFBP-1 transgenic and wild-type littermates, the negative correlation between hIGFBP-1 levels and IGF-I levels and body weight in adult transgenic mice is surprising. Gross body weight is a crude measurement of growth, because it does not distinguish between linear and nonlinear growth, i.e., increased adiposity. Therefore, it is possible that a reduction in linear growth may be counterbalanced by an increase in adiposity with no net change in body weight. We have demonstrated specific defects in glucoregulation in hIGFBP-1 mice that are consistent with insulin resistance. The conventional view that obesity predisposes to the development of insulin resistance is well established, but an alternative, though not mutually exclusive hypothesis, is that resistance to insulin-mediated glucose disposal in muscle can result in an increase in adiposity (35). Altered nutrient partitioning, leading to an increase in triglyceride synthesis and storage in adipose tissue, may be a consequence of diminished muscle glucose utilization. In addition, the hyperinsulinemia associated with insulin resistance may have direct anabolic actions on adipose tissue. Further investigation of lean body mass and adiposity

in hIGFBP-1 transgenic mice will be necessary to determine if this applies.

The observation of early postnatal growth restriction suggests that its origins may be in utero life. Increased hepatic expression and circulating IGFBP-1 levels are a consistent finding in animal models of intrauterine growth restriction and in human studies of small-for-gestational-age fetuses. We have recently demonstrated hepatic transgene expression in hIGFBP-1 transgenic conceptuses (P.A.C., C.C. Pillai, J.P.M., unpublished observations).

In conclusion, we have generated transgenic mice that overexpress hIGFBP-1 as a model system to study the regulation and function of the insulin/IGF-I/IGFBP-1 axis in an in vivo physiological context. The analyses performed here confirm the fundamental importance of the normal inverse relationship between insulin and IGFBP-1 in glucoregulation. We present evidence that sustained dysregulation of the axis leads to profound abnormalities of glucose homeostasis that have some similarities to the pathophysiology of type 2 diabetes.

REFERENCES

1. Sherwin RS, Borg WP, Boulware SD: Metabolic effects of insulin-like growth factor I in normal humans. Horm Res 41 (Suppl. 2):97–101, 1994
2. Jones JI, Clemmons DR: Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3–34, 1995
3. Baxter RC: Insulin like growth factor binding proteins as glucoregulators. Metabolism 44 (Suppl. 4):12–17, 1995
4. Pao C-I, Farmer PK, Begovic S, Goldstein S, Wu G-J, Phillips LS: Expression of hepatic insulin-like growth factor-I and insulin-like growth factor-binding protein-1 genes is transcriptionally regulated in streptozotocin-diabetic rats. Mol Endocrinol 6:969–977, 1992
5. Johnson TR, Blossey BK, Denko CW, Ilan J: Expression of insulin like growth factor I in cultured rat hepatocytes: effects of insulin and growth hormone. Mol Endocrinol 3:580–587, 1989
6. Porksen N, Hussain MA, Bianda TL, Nyholm B, Christiansen JS, Butler PC, Veldhuis JD, Froesch ER, Schmitz O: IGF-I inhibits burst mass of pulsatile insulin secretion at supraphysiological and low IGF-I infusion rates. Am J Physiol 272:E352–E358, 1997
7. Zenobi PD, Graf S, Ursprung H, Froesch ER: Effects of insulin-like growth factor-I on glucose tolerance, insulin levels, and insulin secretion. J Clin Invest 89:1908–1913, 1992
8. Moses AC, Young SCJ, Morrow LA, O'Brien M, Clemmons DR: Recombinant human insulin-like growth factor I increases insulin sensitivity and improves glycemic control in type II diabetes. Diabetes 45:91–100, 1996
9. Acerini CL, Harris D, Matyka KA, Watts AP, Umpleby AM, Russell-Jones DL, Dunger DB: Effects of low-dose recombinant human insulin-like growth factor-I on insulin sensitivity, growth hormone and glucagon levels in young adults with insulin-dependent diabetes mellitus. Metabolism 47:1481–1489, 1998
10. Ooi GT, Tseng LY, Tran MQ, Rechler MM: Insulin rapidly decreases insulin-like growth factor-binding protein-1 gene transcription in streptozotocin-diabetic rats. Mol Endocrinol 6:2219–2228, 1992
11. Powell DR, Suwanichkul A, Cubbage ML, DePaolis LA, Snuggs MB, Lee PDK: Insulin inhibits transcription of the human gene for insulin-like growth factor-binding protein-1. J Biol Chem 266:18868–18876, 1991
12. Lewitt MS, Baxter RC: Insulin-like growth factor-binding protein-1: a role in glucose counterregulation. Mol Cell Endocrinol 79:146–152, 1991
13. Munoz MT, Barrios V, Pozo J, Argente J: Insulin-like growth factor I, its binding proteins 1 and 3, and growth hormone-binding protein in children and adolescents with insulin-dependent diabetes mellitus: clinical implications. Ped Res 39:992–998, 1996
14. Clauson P, Brismar K, Grill V: Insulin like growth factor-I and IGF binding protein-1 in a representative NIDDM population (Abstract). Diabetologia 38 (Suppl. 1):A132, 1995
15. Kobayashi K, Amemiya S, Sawanobori E, Higashida K, Ishihara T, Kobayashi K, Kato K, Nakazawa S: Role of IGF binding protein-1 in the dawn phenomenon and glycemic control in children and adolescents with IDDM. Diabetes Care 20:1442–1447, 1997
16. Strasser-Vogel B, Blum WF, Past R, Kessler U, Hoeflich A, Meiler B, Kiess W: Insulin-like growth factor (IGF)-I and -II and IGF-binding proteins-1, -2, and -3 in children and adolescents with diabetes mellitus: correlation with metabolic control and height attainment. J Clin Endocrinol Metab 80:1207–1213, 1995
17. Rajkumar K, Barron D, Lewitt MS, Murphy LJ: Growth retardation and hyperglycemia in insulin-like growth factor binding protein-1 transgenic mice. Endocrinology 136:4029–4034, 1995
18. Ehrenborg E, Larsson C, Stern I, Janson M, Powell DR, Luthman H: Contiguous localization of the genes encoding human insulin-like growth factor binding proteins 1(IGBP1) and 3 (IGBP3) on chromosome 7. Genomics 12:497–502, 1992
19. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbour, NY, Cold Spring Harbour Laboratory Press, 1989
20. Julkunen M, Koistinen R, Aalto-Setala K, Seppala M, Janne OA, Kontula K: Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA. FEBS Lett 236:295–302, 1988
21. Khosravi MJ, Diamandi A, Mistry J: Immunoassay of insulin-like growth factor binding protein-I. Clin Chem 43:523–532, 1997
22. Murphy LJ, Barron D, Seneviratne C: Hormonal regulation of insulin like growth factor binding protein-1 expression and the development of transgenic mouse models to study IGFBP-1 function. In Current Directions in Insulin Like Growth Factor Research. LeRoith D, Raizada R, Eds. New York, Plenum Press, 1994, p. 279–291
23. Busby WH Jr, Klapper DG, Clemmons DR: Purification of a 31,000-dalton insulin-like growth factor binding protein from human amniotic fluid: isolation of two forms with different biologic actions. J Biol Chem 263:14203–14210, 1988
24. Elgin RG, Busby WH Jr, Clemmons DR: An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. Proc Natl Acad Sci U S A 84:3254–3258, 1987
25. Koistinen R, Angervo M, Leinonen P, Seppala M: Phosphorylation of insulin-like growth factor-binding protein-1 from different sources. Growth Reg 3:34–37, 1993
26. Westwood M, Gibson JM, Williams AC, Clayton PE, Hamberg O, Flyvbjerg A, White A: Hormonal regulation of circulating insulin-like growth factor-binding protein-1 phosphorylation status. J Clin Endocrinol Metab 80:3520–3527, 1995
27. Horikawa Y, Iwasaki N, Hara M, Furuta H, Hinokio Y, Cockburn BN, Lindner T, Yamagata K, Ogata M, Tomonaga O, Kuroki H, Kasahara T, Iwamoto Y, Bell GI: Mutation in hepatocyte nuclear factor-1 beta gene (TCF2) associated with MODY. Nat Genet 17:384–385,1997
28. Furuta H, Iwasaki N, Oda N, Hinokio Y, Horikawa Y, Yamagata K, Yano N, Sugahiro J, Ogata M, Ohgawara H, Omori Y, Iwamoto Y, Bell GI: Organization and partial sequence of the hepatocyte nuclear factor-4 alpha/MODY1 gene and identification of a missense mutation, R127W, in a Japanese family with MODY. Diabetes 46:1652–1657, 1997
29. Lithell HO, McKeigue PM, Berglund L, Mohsen R, Lithell UB, Leon DA: Relation of size at birth to non-insulin dependent diabetes and insulin concentrations in men aged 50–60 years. BMJ 17:406–410, 1996
30. Langford K, Blum W, Nicolaides K, Jones J, McGregor A, Miell J: The pathophysiology of the insulin-like growth factor axis in fetal growth failure: a basis for programming by undernutrition? Eur J Clin Invest 24:851–856, 1994
31. Giudice LC, de Zegher F, Gargosky SE, Dsupin BA, de las Fuentes L, Crystal RA, Hintz RL, Rosenfeld RG: Insulin-like growth factors and their binding proteins in the term and preterm human fetus and neonate with normal and extremes of intrauterine growth. J Clin Endocrinol Metab 80:1548–1555, 1995
32. Phipps K, Barker DJP, Hales CN, Fall CHD, Osmond C, Clark PMS: Fetal growth and impaired glucose tolerance in men and women. Diabetologia 36:225–228, 1993
33. Phillips DIW, Barker DJP, Hales CN, First S, Osmond C: Thinness at birth and insulin resistance in adult life. Diabetologia 37:150–154, 1994
34. Rajkumar K, Dheen ST, Murphy LJ: Hyperglycemia and impaired glucose tolerance in IGF binding protein-1 transgenic mice. Am J Physiol 270:E565–E571, 1996
35. Moller DE, Chang PY, Yaspelkis BP, Flier JS, Wallberg-Henriksson H, Ivy JL: Transgenic mice with muscle specific insulin resistance develop increased adiposity, impaired glucose tolerance and dyslipidemia. Endocrinology 137:2397–2405, 1996