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The Somatomedin Hypothesis: 2001

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ABSTRACT

Since the original somatomedin hypothesis was conceived, a number of important discoveries have allowed investigators to modify the concept. Originally somatic growth was thought to be controlled by pituitary GH and mediated by circulating insulin-like growth factor-I (IGF-I, somatomedin C) expressed exclusively by the liver. With the discovery that IGF-I is produced by most, if not all, tissues, the role of autocrine/paracrine IGF-I *vs.* the circulating form has been hotly debated. Recent experiments using transgenic and gene-deletion technologies have attempted to answer these questions. In the liver-specific *igf-1* gene-deleted mouse model, postnatal growth and development are normal despite the marked reduction in circulating IGF-I and IGF-binding protein levels; free IGF-I levels are normal. Thus, the normal postnatal growth and development in these animals may be due to normal free IGF-I levels (from as yet unidentified sources), although the role of autocrine/paracrine IGF-I has yet to be determined. (*Endocrine Reviews* **22:** 53–74, 2001)

I. Introduction

GROWTH is regulated by the integration of environmental signals (*e.g.*, nutritional and seasonal cues) with endogenous neuroendocrine responses to the genetic programs that ultimately determine the body plan. The insulin-like growth factors (IGFs) are integral components of multiple systems controlling both growth and metabolism. The modern wide-ranging field of IGF research evolved from experiments examining the role of pituitary-regulated growth-stimulating substances performed by Salmon and Daughaday nearly half a century ago (1). A cursory examination of the current literature reveals that the IGF system is of great interest in many seemingly disparate fields. For example, the IGFs play a critical role in both cell cycle control and apoptosis, two functions involved in regulation of tumorigenesis (2–5). IGFs have also been implicated in the context of the treatment of catabolic states associated with illness. Recombinant IGF-I administration to severely catabolic patients reduces the negative nitrogen balance associated with moderate caloric restriction (6), promotes recovery from hypoxic ischemic episodes in cardiac and nervous tissues in animal models (7), corrects the hyperglycemia of diabetic patients with insulin resistance (8), and has other important roles in physiological and pathophysiological conditions in pediatric and adult endocrinology (9). The IGF system is perhaps more complex than other endocrine systems, as genes for six high-affinity IGF binding proteins (IGFBPs) have been identified. The finding that IGFBPs are themselves regulated by protease activity, and that some IGFBPs may have IGF-I-independent actions, has added a further layer of complexity to the IGF system. The seemingly endless list of functions regulated by the IGFs is, of course, a great comfort to researchers hoping to stay employed in this field. However, such a broad scope of functions is highly problematic when considering the potential therapeutic uses for IGFs.

II. Original Somatomedin Hypothesis

The somatomedin hypothesis originated in early efforts to understand how somatic growth was regulated by factors secreted by the pituitary. In essence, the results of these

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experiments suggested that pituitary-derived GH did not act directly on its target tissues to promote growth. These experiments, performed almost 50 yr ago, used an assay that measured the incorporation of $^{35}SO_4$ into chondroitin sulfate from cartilage. The experiments were performed both *in vivo* (in rats), as well as *in vitro*, using costal cartilage. Hypophysectomy of rats markedly reduced the incorporation of $^{35}SO_4$ into chondroitin sulfate of epiphyseal cartilage (10). *In vivo* injections of pituitary extracts and purified bovine GH (bGH) effectively restored $^{35}SO_4$ incorporation into cartilage (1, 11). However, when bGH was placed on costal cartilage slices maintained *in vitro*, only a minimal effect was observed. These observations led the investigators to postulate that the effect of bGH on costal cartilage was probably indirect, utilizing an intermediary substance such as an endocrine hormone or growth factor (12). Further experiments demonstrated that normal rat serum was capable of stimulating the biological effect. Serum from hypophysectomized rats was ineffective. However, if hypophysectomized rats were injected with bGH, the serum from these animals was able to stimulate $^{35}SO_4$ incorporation. Thus the term "sulfation factor" was coined to indicate a circulating substance that is increased by GH and can stimulate sulfate uptake into cartilage.

Subsequent experiments provided further evidence that the effect of GH on cartilage growth was indirect. Serum from hypophysectomized rats treated with bGH, but not bGH itself, could stimulate DNA synthesis, as measured by ^{3}H-thymidine incorporation into cartilage (13). Furthermore, the mitogenic effects of bGH could be mimicked by a partially purified fraction of "sulfation factor," derived from the serum of patients with acromegaly, which is associated with elevated circulating GH levels. The term "somatomedin" was coined to reflect the ability of the substance to mediate the effects of GH (also referred to as "somatotropin") (14). The somatomedins were subdivided into subtypes, with somatomedin C later identified as the GH-responsive form.

Two decades (1978) after the existence of somatomedin(s) had first been postulated, IGF-I and IGF-II were purified and characterized. IGF-I was shown to be the somatomedin substance that was regulated by circulating GH in rats (15, 16). Both substances were termed "insulin-like," because of their ability to stimulate glucose uptake into fat cells and muscle (17). Thus, it was perhaps not surprising that their sequence and tertiary structure were similar to those of proinsulin. Both IGF-I and IGF-II share approximately 50% amino acid identity with insulin. The major structural difference between the IGFs and insulin is that the IGFs retain the C chain that is cleaved from proinsulin, and there is a small D extension to the A chain in the IGF molecules (16, 18, 19). While the structural similarity between insulin and IGF-I suggested a metabolic function, the belief predominated that the primary functional role of the IGFs was to act as growth factors. At this stage, the original somatomedin hypothesis remained the most widely accepted model of IGF action. This hypothesis put forward that growth is determined by GH acting primarily on the liver, where it stimulates IGF-I synthesis and release. IGF-I then circulates to the main target organs, such as cartilage and bones, and thus acts in an endocrine mode (Fig. 1A). Circulating IGF-I also provides a feedback effect within the somatotropic axis, with circulating IGF-I suppressing the further release of GH from the pituitary (20). A model whereby the hypothalamic-pituitary-liver axis controlled growth was attractive and certainly seemed to fit the models of other hypothalamic-pituitary axes of the time. However, subsequent experiments and findings have required an expansion of this hypothesis, in view of new evidence showing that IGF-I synthesis occurs in many tissues and is often regulated by a variety of local and endocrine factors.

III. The Alternative or "Revised" Somatomedin Hypothesis

The first significant challenge to the original somatomedin hypothesis came with the discovery that IGFs were expressed in most, if not all, tissues. In 1980 D'Ercole and co-workers (21) discovered that explants of fetal mouse tissues maintained in serum-free media showed higher levels of somatomedin-C in the culture medium as compared with extracts of the tissues themselves. Elevated somatomedin-C levels were observed in cultured explants of fetal mouse liver and limb bud (11-day gestation), intestine, heart, brain, kidney, and lung (17-day gestation). The authors concluded that this evidence for local somatomedin-C/IGF-I production strongly suggested that IGF-I had an autocrine/paracrine effect. Further support for the local production of IGF-I was garnered from studies by multiple investigators who were examining the tissue distribution of IGF-I mRNA using the IGF-I cDNAs recently obtained from multiple species (22–26). The investigators determined that the IGF-I gene was expressed in multiple tissues throughout embryonic and postnatal development and during adult life (26, 27). The widespread expression of IGF-I supported the earlier findings of D'Ercole and colleagues, indicating that this factor plays a role in the regulation of growth in multiple tissues and cells in a local, paracrine, or autocrine manner. Since GH was known to regulate hepatic IGF-I output, the effect of hypophysectomy on the abundance of the mRNAs for IGF-I was studied in various rat tissues. Injection of GH in hypophysectomized rats increased IGF-I mRNA not only in liver, but also in numerous nonhepatic tissues including lung, kidney, skeletal muscle, heart, and white adipose tissue (28, 29). These studies provided additional support for the hypothesis that IGF-I is produced locally in diverse tissues, where its expression might be regulated by GH or by other hormones.

Isaksson and co-workers (30) raised further questions about whether circulating IGF-I plays a role in mediating the actions of GH. Their studies demonstrated that direct injection of hGH into the cartilage growth plate of the hind limbs of hypophysectomized rats at days 14, 16, and 19 of age resulted in a significant increase in longitudinal bone growth. The contralateral limb, which received no hGH, did not show a significant increase in growth rate, indicating that the effect was local (30). They concluded that the circulating form of IGF-I is not required for stimulation of longitudinal bone growth, but rather that GH itself

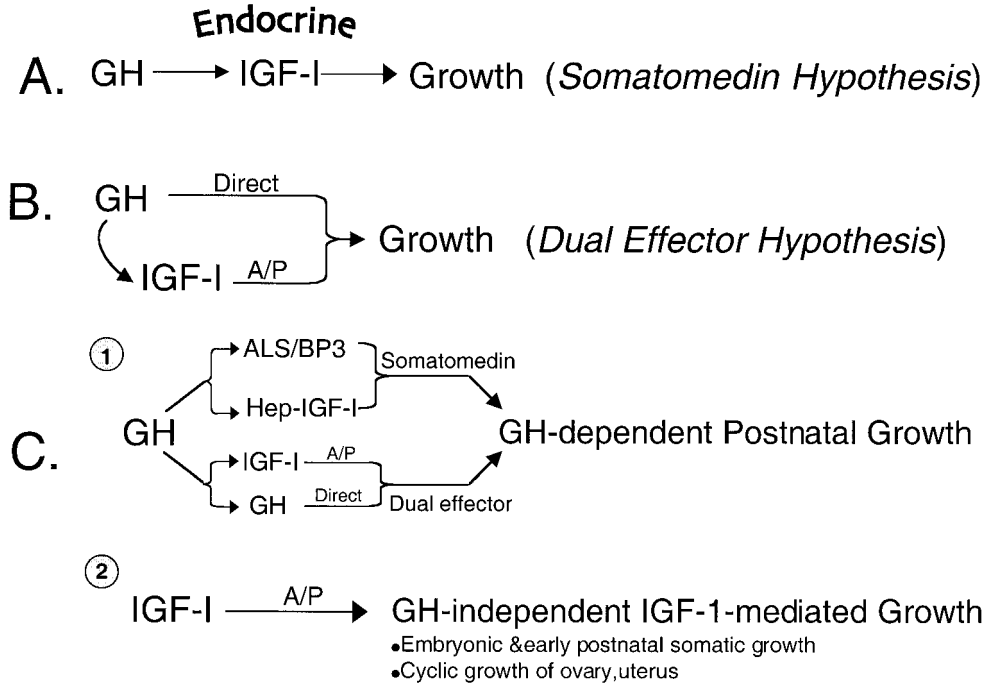

FIG. 1. The stages of evolution of the somatomedin hypothesis. The original somatomedin hypothesis postulated that somatic growth was regulated by GH's stimulation of hepatic IGF-I production, with IGF-I acting in an endocrine fashion on peripheral tissues to promote growth (A). The dual effector theory proposed an alternative view, involving direct effects by GH on peripheral tissues not mediated by IGF-I and GH-stimulated local IGF-I production for autocrine/paracrine (A/P) action (B). Current evidence suggests the situation is more complex than either of these hypotheses envisioned. It is now clear that GH, in addition to stimulating hepatic IGF-I synthesis, stimulates the formation of ternary IGF binding complex, including IGFBP-3 and the acid-labile subunit, which stabilizes IGF-I in the serum. While it is not precisely known how somatic growth is parsed between endocrine *vs.* local autocrine/paracrine GH-IGF-I systems, there is sufficient evidence to support a role for both systems in normal postnatal growth (C1). Finally, observations in *igf1* null mice showing significant growth retardation at birth and infertility in both sexes suggest that IGF-I has important, GH-independent effects on embryonic growth and reproductive function, since these effects are not seen in GH- or GHR-deficient animals (C2).

directly stimulates the cartilage. These authors raised the possibility that the effect of GH was mediated by local production of IGF-I. This concept was supported by other investigators who infused rat hindlimb arteries with rat GH or human IGF-I and observed increased growth of epiphyseal plates (31, 32). These results strongly indicated that GH has local effects that may be independent of the increase in the circulating "endocrine" form of IGF-I, thereby introducing an alternative to the original somatomedin hypothesis. Isaksson and co-workers (33, 34) postulated that GH was capable of stimulating the differentiation of epiphyseal growth plate precursor cells both directly and indirectly, by increasing their responsiveness to IGF-I. Furthermore, GH enhances the local production of IGF-I that, in turn, stimulates the clonal expansion of differentiating chondrocytes.

How can the earlier findings of Salmon and Daughaday, which suggested an endocrine role for IGF-I, and the more recent findings, which suggest an autocrine/paracrine role, be reconciled? The alternative somatomedin hypothesis could be viewed as a compromise, where both circulating "endocrine" IGF-I and locally produced IGF-I are responsive to GH and responsible for the effects of GH. In addition, the possibility that GH may have IGF-I-independent effects on tissues could not be excluded.

A. Dual effector theory

In 1985, Green and co-workers (35) proposed a new concept concerning the roles that GH and IGF-I play in growth and differentiation known as the "dual effector hypothesis." The dual effector hypothesis suggests that GH stimulates the specific differentiation of adipocytes, while IGF-I stimulates their clonal expansion (Fig. 1B). Their proposal was based on studies from the 1970s where several fibroblast-like cell lines were used as models for studying adipocyte differentiation, including 3T3-L1, BALB 3T3-T, and ST 13 cells that were obtained from Swiss 3T3 and BALB 3T3 whole mouse embryos (36–38). The adipocyte phenotype appears in these cells upon growth arrest once they reach confluence. As they convert to adipocytes, there is a marked increase in expression in insulin receptors, from approximately 7,000 receptors per cell to 250,000 insulin receptors per cell. The marked increase in insulin receptor number is associated with an increased sensitivity and responsiveness to insulin as shown by increased glucose uptake, lipid synthesis, and glucose oxidation (39–42). In parallel, IGF-I receptors, which are expressed at high levels in preadipocytes, are markedly reduced in the differentiated adipocyte. At this time, the differentiation, but not the growth, of 3T3 preadipocytes was found to be dependent on a serum "adipogenic factor." A hormone(s) secreted from the pituitary was considered a

possible candidate by Morikawa *et al.* (43), since pituitary extracts and recombinant human GH showed this activity, while serum from hypophysectomized rats showed reduced activity. Thus, as opposed to the *original* concept that IGF-I mediated all the functions of GH, they showed that preadipocytes could be induced to differentiate into adipocytes by GH (44). Furthermore, since somatomedins could not replace GH in their studies, they suggested that IGF-I induced clonal expansion of these differentiated cells (45). By analogy with the adipocyte work of Greene *et al.*, the Isaakson group extended the "dual effector" theory to the growth plate, proposing that GH acts directly at the growth plate germinal zone to stimulate the differentiation of chondrocytes. GH also acts to induce local IGF-I synthesis, which was thought to stimulate the clonal expansion of chondrocyte columns in an autocrine/paracrine manner (the body of work bearing on this hypothesis was reviewed in Refs. 33 and 46).

B. Recent evidence questioning the dual effector theory

The evidence upon which the dual effector hypothesis of how GH and IGF-I interact to regulate growth may well be flawed. It was subsequently clearly shown that insulin at high doses and IGF-I at physiological concentrations also cause preadipocytes to differentiate into mature adipocytes (41, 42, 47, 48) probably by activating the IGF-I receptors. However, the 3T3-F442A cell line (a subclone of mouse 3T3 cells that has the unusual property of undergoing adipogenesis) has been studied extensively as a model of IGF-I-independent differentiation by GH and has been valuable in elucidating the molecular mechanisms of GH action such as identifying specific activation of transcription factors such as Elk-1 and Sap-1a (49).

More recent studies have yielded data that are not consistent with the dual effector hypothesis. The suggestion that GH has direct, non-IGF-I-dependent effects on growth plate germinal zone cells has been confirmed in studies showing that GH stimulates increased proliferation of germinal zone cells (50, 51). While IGF-I was also shown to have this effect (51), the growth plate germinal zones are significantly expanded in *igf-I* null mice (52). Given the complete absence of IGF-I in these mice, it is inferred that elevated endogenous GH levels are responsible for this germinal zone effect. It remains to be determined whether GH's effect on the germinal zone may be mediated by local IGF-II production.

While the hypothesis that GH has direct, non-IGF-I-dependent effects on growth plate germinal cells has been adequately confirmed, the further suggestion that GH induces IGF-I synthesis in proliferative chondrocytes *in vivo* (33) is disputed. For example, both Shinar *et al.* (53) and Wang *et al.* (54) were unable to detect IGF-I mRNA in growth plate chondrocytes of rats or mice of any age, while both groups found abundant IGF-II mRNA in proliferative chondrocytes of both murine species. The finding of IGF-I mRNA and immunoreactivity in growth plate chondrocytes could be explained by cross-reactivity of IGF-I probes with IGF-II, or perhaps by strain-specific differences in local IGF expression. We have noted significant variability in local patterns of IGF-I expression between rats and mice and even between different strains of rats and mice (Ref. 55 and J. Zhou and C. A. Bondy, unpublished data).

Despite the continuing uncertainty about its mode of action, IGF-I clearly has an important role in longitudinal bone growth, since *igf-I* gene deletion results in dwarfism in mice (56, 57) and extreme short stature in humans (58). Analysis of long bone growth and growth plate characteristics in *igf-I* null mice has shown that growth plate chondrocyte numbers and proliferation are normal, despite a 35% reduction in the rate of long bone growth (52). Chondrocytes from *igf-I* null mice are, however, smaller than wild type at all levels of the growth plate. The terminal hypertrophic chondrocytes, which form the scaffold upon which linear growth extends, are reduced in linear dimension by 30%, accounting for most of the decreased longitudinal growth in *igf-I* null mice. Expression of the insulin-sensitive glucose transporter, GLUT4, is decreased, glycogen synthase kinase 3b is hypophosphorylated, glycogen stores are depleted, and ribosomal RNA levels are drastically reduced in *igf-I* null chondrocytes (52).

The data derived from examination of *igf-I* null mice suggest that IGF-I's role in longitudinal bone growth involves "insulin-like" anabolic actions that augment chondrocyte hypertrophy, rather than mitogenic effects on chondrocytes, as previously thought. The fact that IGF-II, rather than IGF-I, is normally expressed by proliferative chondrocytes (53, 54) and that IGF-II expression is not impaired by IGF-I deletion may explain the normal proliferation in the *igf-I* null growth plate. A new view of GH and IGF interactions in long bone growth at the level of the growth plate is illustrated in Fig. 2. The source of IGF-I that promotes chondrocyte hypertrophy remains uncertain. IGF-I mRNA is concentrated in the murine periosteum and perichondrium (53, 54) and is also expressed by muscle and fat cells; therefore, local tissue sources may provide enough IGF-I effect to enhance longitudinal bone growth. Certainly, circulating IGF-I derived from the liver may also serve this role.

In summary, with regard to the dual effector hypothesis, it seems clear now that GH does indeed have direct effects, not mediated by or dependent on IGF-I, on cells *in vitro* and *in vivo*. However the physiological significance of such findings remains unclear. GH is clearly not *essential* for the differentiation of cells such as adipocytes or chondrocytes, since these differentiated cell types are abundant in GH-deficient and GHR-deficient mice and humans. Indeed, adiposity is excessive in states of GH deficiency, and GH treatment significantly reduces the abundance of adipose tissue (59), suggesting that the lipolytic effects of GH are far more important than any effect it might have on differentiation of adipocytes. Moreover, as noted above, growth plate chondrocytes proliferate normally in the complete absence of IGF-I; therefore, IGF-I is apparently not required for their clonal expansion. Thus, while GH and IGF-I, and possibly IGF-II, have complementary roles in promoting long bone growth (Fig. 2), they are not exactly as proposed in the original dual effector scheme (33). Examples from other systems also show divergence from the dual effector scheme, *e.g.*, differentiating myoblasts are unable to proliferate (60), and IGF-I is itself capable of inducing myoblast differentiation into mature myotubes (61, 62).

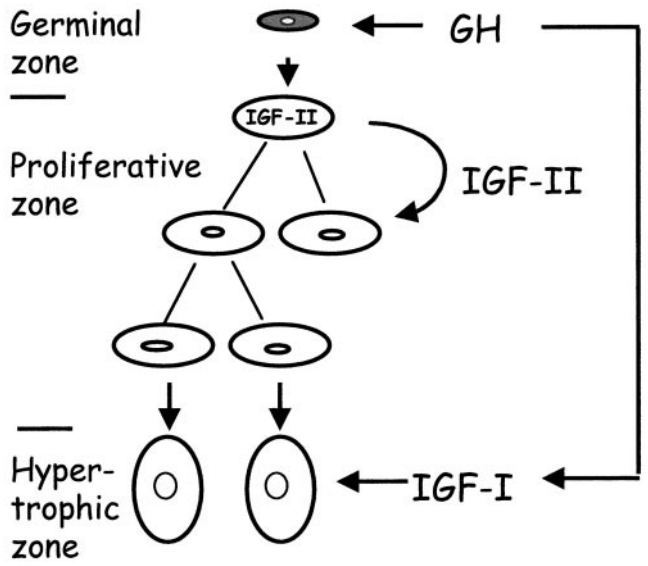

FIG. 2. This cartoon represents the epiphysial growth plate wherein sequential chondrocyte proliferation and hypertrophy drive long bone growth. GH acts on the germinal zone to stimulate increased proliferation and differentiation of chondrocyte precursors, which enter the growth plate as proliferative chondrocytes. IGF-II is expressed by proliferative chondrocytes and may be important for enhancing their mitotic activity, although this has not been shown. It is not known whether GH stimulates growth plate chondrocyte IGF-II production, although this seems likely. IGF-I is not required for chondrocyte proliferation, but is required for the full development of chondrocyte hypertrophy, since the reduced long bone growth in *Igf1* null mice is entirely accounted for by the attenuation of chondrocyte hypertrophy. According to this view, GH, IGF-I, and IGF-II each have unique and complementary roles in augmenting long bone growth.

IV. GH and the GH Receptor (GHR)

A. GH

GH and the GHR belong to a large family of cytokine peptides (63, 64) and receptors (65–67). GH is synthesized and stored by somatotroph cells within the anterior pituitary gland. In the male rat, GH is secreted in discrete pulses with low interpeak levels. Circulating IGF-I inhibits GH secretion, providing a feedback loop on the actions of GH in peripheral tissues. In contrast, GH secretion in the female rat exhibits a less pulsatile pattern and has relatively high interpeak levels (68–70). A similar sexual dimorphism in the secretory pattern of GH has been reported in humans (71). In humans, and other species studied, serum IGF-I levels and growth are positively correlated with peak amplitude of GH, rather than the concentration of GH between pulses. Studies in rodents in which an intermittent treatment regimen, corresponding to the naturally observed pulsatile release, also suggest that episodic exposure to GH is more potent than continuous infusion, with respect to stimulation of growth and increasing serum IGF-I levels (72, 73).

The "somatotropic axis" was originally described as being comprised of the hypothalamus, pituitary, and liver. The hypothalamus was thought to be the "control center," regulating the secretion of GH from the pituitary (74). While concepts of IGF function have evolved to accommodate new data, the basic concepts concerning central regulation of GH secretion have remained largely unchanged. Two hypothalamic factors, GH releasing hormone (GHRH) (75, 76) and the inhibitory hormone, somatostatin (SS) (77), act in concert to regulate GH secretion from pituitary somatotrophs.

Several new factors controlling GH release and novel pathways that regulate GH secretion have recently been reported (78, 79). These factors are primarily related to or derived from the metabolic status of the organism, which is consistent with the role of GH in regulating metabolism, as well as growth. FFA act directly on the pituitary to inhibit GH release, which is postulated to complete a feedback loop, since GH stimulates lipid mobilization (80, 81). The adipostat hormone leptin stimulates GH secretion at the level of the hypothalamus by regulating GHRH and SS activity (82–84). The effect of leptin on GH secretion may also involve neuropeptide Y (NPY), since leptin suppresses NPY expression, and infusion of NPY is known to suppress GH secretion (85, 86).

Another GH-secretory factor that is derived from a peripheral organ has recently been isolated. A synthetic hexapeptide, hexarelin, has long shown promise as an orally active GH secretagogue (87). Hexarelin belongs to a family of GH-releasing peptides (*e.g.*, GHRP-1, GHRP-2, GHRP-6) with demonstrable GH secretagogue activity (88). A G protein-coupled receptor expressed in the pituitary and activated by the small synthetic GH secretagogues (GHS-R) was cloned in 1996 (79). The cloned receptor was recently used to isolate Ghrelin, a 28-residue peptide, from stomach extracts (89). Ghrelin mRNA and immunoreactivity were found to be expressed at high levels in endocrine cells of the stomach, in addition to lower levels of expression of the hypothalamic arcuate nucleus. Ghrelin circulates at a considerable plasma concentration, in the order of 120 fmol/ml, suggesting that GH secretion is controlled by both hypothalamic (*i.e.*, neuroendocrine) and peripheral signals.

B. The GHR and GHR signal transduction

The actions of GH are mediated by the binding of GH to the transmembrane GHR, which is present on the surface of most cells. The GHR was the first member of the type I cytokine receptor family to be cloned, and all members share the same single-transmembrane domain structure. This family includes the closely related PRL receptor, as well as several of the interleukin and colony-stimulating factor receptors (67).

The GHR is subject to a number of posttranscriptional and posttranslational modifications during synthesis. The most significant of these is the generation of a soluble GH binding protein (GHBP), comprised of the GHR extracellular ligand-binding domain (90, 91). The mechanism used to generate the soluble GHBP varies across species. The nascent GHR mRNA in rodents was the first discovered to undergo alternate splicing of the nascent RNA transcript producing a truncated form (92), while GHBP from other species was originally believed to result from proteolytic cleavage of the full-length receptor (93, 94). It is generally believed that the primary function of the GHBP is to act as a physiological buffer, stabilizing GH in plasma; however, there is still no consensus as to what the physiological functions of truncated GHR isoform are (95–97).

As with most cytokine receptors, the GHR utilizes the

JAK-STAT signal transduction pathway (98) (Fig. 3). The activated GHR associates with JAK2 (janus kinase 2). JAK2 is a tyrosine kinase, which upon activation by GH phosphorylates STATs-1, -3, -5a, and -5b (signal transducers and activators of transcription) on tyrosines (98). Upon phosphorylation by GHR and JAK2, the STAT proteins translocate to the nucleus where they bind to specific DNA sequences and activate gene transcription (99, 100). The current evidence suggests that the STAT proteins are involved in programming the different effects observed in response to pulsatile or continuous activation of the GHR by GH. STAT 5b is believed to be responsible for regulating gene expression in the adult male liver in response to the male-specific pulsatile pattern of GH secretion (101).

GHR activation induces tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins IRS-1 and IRS-2. As discussed further below, IRS proteins are docking proteins that direct integrate signals from the IGF and insulin receptors to various downstream signaling pathways. GHR activation induces the association of IRS-1 and IRS-2 with phosphatidylinositol-3′-kinase (PI-3′kinase) in various GH-responsive cell types (102–104). GH-induced lipid synthesis and inhibition of noradrenaline-induced lipolysis in rat adipocytes is blocked by a specific inhibitor of PI-3′kinase. Thus, many of the metabolic effects of GH may be mediated via the IRS molecules, which are likely to be phosphorylated by JAK 2 (102–104). Activation of the insulin-like growth factor-I receptor (IGF-IR) also stimulates the phosphorylation of the IRS family of signaling proteins (9), thus providing for cross-talk between GH and IGF-I at the level of signal transduction. While the effect of GH on insulin receptor-related signal transduction events has been the subject of numerous studies, to the best of our knowledge there have been no studies examining the consequences of GH-induced IRS tyrosyl-phosphorylation on the mitogenic and metabolic effects of IGF-I.

GH has also been shown to induce increased influx of

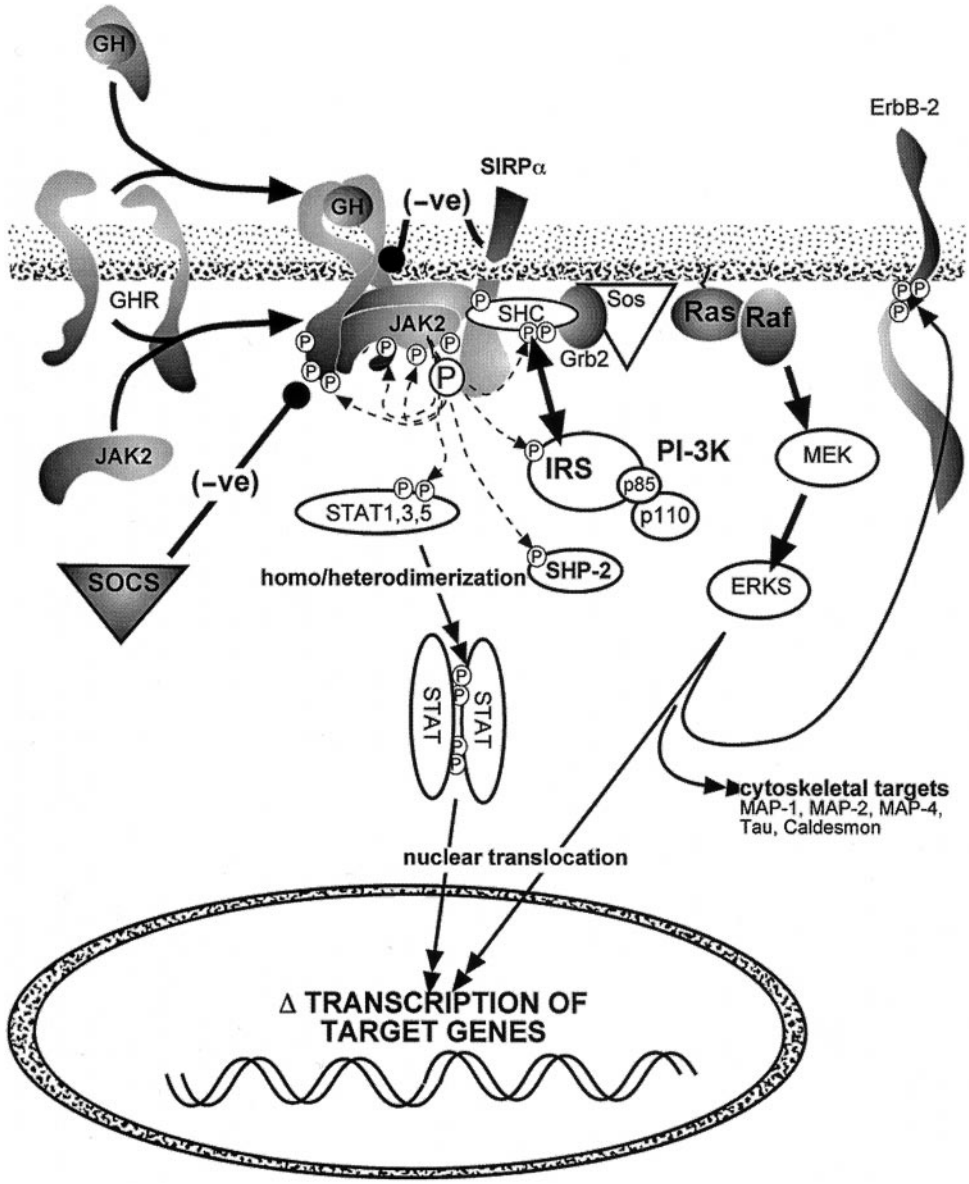

FIG. 3. Signaling pathways used by GH. JAK, Janus kinase; STAT, signal transducers and activators of transcription; SOCS, suppressors of cytokine signaling; SHP2, protein tyrosine phosphatase.

extracellular calcium via the plasma membrane voltage-dependent L-type calcium channels (105). This elevation of intracellular calcium is necessary for GH-induced transactivation of the Spi 2.1 promoter. GH-induced calcium influx may also be the proximal cause underlying the refractoriness to the effects of further GH exposure on lipogenesis and glucose uptake, the elevated intracellular Ca concentration causing the refractoriness (106, 107). GH may also increase intracellular diacylglycerol, which potently activates protein kinase C (PKC). This is associated with the generation of inositol triphosphate (IP3), suggesting that GH may stimulate a pathway involving either phosphoinositide hydrolysis [leading to IP3 accumulation followed by diacylglycerol (DAG) generation] or phosphatidylcholine breakdown, which can lead to DAG generation directly). Activation of PKC by GH results in activation of MAP kinases, p90rsk, and induction of c-*fos* (108–110).

GH stimulation has been shown to regulate gene expression of IGF-I and many of the IGF-binding proteins (IGFBPs). However, only the promoter for the acid labile subunit (ALS) of the IGFBP-3 complex has an identifiable GH-responsive element (111). Interestingly, *in situ* hybridization studies show that the mRNAs encoding IGF-I, IGFBP-3, and ALS are not colocalized within the same cells in the liver (112). Hepatocytes express mRNAs encoding IGF-I and ALS, whereas IGFBP-3 mRNA is exclusively expressed in adjacent endothelial cells of the hepatic sinusoids. Unlike hepatocytes, sinusoidal endothelial cells of the liver do not express detectable levels of GHR mRNA. Thus, the regulation of IGFBP-3 levels by GH is presumably indirect and most likely to be mediated by IGF-I, at least in rodents, as demonstrated by the marked reduction in circulating IGFBP-3 levels in liver-specific IGF-I gene-deleted mice despite elevated GH levels. Administration of rhIGF-I reversed this effect (Yakar *et al.*, manuscript submitted). Conversely, hepatocytes do not express detectable levels of IGF-I receptor mRNA. Thus, IGF-I presumably does not act on hepatocytes directly, but rather relies on the inhibition of GH to complete a feedback circuit. Alternatively, IGF-I may affect IGFBP-3 levels by a posttranslational event that may include stabilization of the protein and protection against proteases.

TABLE 1. Biological actions of GH

Stimulates	Inhibits
Longitudinal bone growth & bone remodelling	Insulin action
Chondrocyte proliferation	IGFBP-1 synthesis
Osteoblast proliferation and bone deposition	IGFBP-2 synthesis
Osteoclast proliferation and bone resorption	
Type I collagen synthesis	
Skeletal muscle growth (fiber, strength)	
Liver growth	
Deiodination of T_4 to T_3	
Lipolysis	
Ketogenesis	
Gluconeogenesis	
Protein synthesis/turnover	
Total body nitrogen balance	
Lactation	
IGF-I synthesis	
ALS synthesis	
IGFBP-3 synthesis	
Serine protease inhibitors (SPI) 2.1 and 2.2	
Immunomodulation (endocrine, autocrine-paracrine)	
B and T cell proliferation	
Natural Killer cell activity	
Macrophage activity	
Neutrophil activity	
Immunoglobulin production	
Cytokine production	

The effects of GH on growth and metabolism involves the simultaneous stimulation of anabolic processes and growth factors and inhibition of inhibitory factors (such as IGFBP-1 and IGFBP-2).

C. The physiological effects of GH

The physiological actions of GH are pleiotropic and involve multiple organs and physiological systems (Table 1). GH exerts many metabolic effects that persist throughout life. GH is essentially an anabolic hormone, inducing positive nitrogen balance and protein synthesis in muscle (113). Muscle size is increased in GH-deficient individuals undergoing replacement therapy with recombinant human GH (rhGH) at all ages (114, 115). Because GH enhances amino acid uptake into skeletal muscle, it has been suggested that this tissue is the primary target of the physiological effects of GH (116, 117). However, conflicting reports have suggested that other tissues may be equally important in the effect of GH on nitrogen balance. Furthermore, it remains controversial whether the effects of GH on nitrogen balance are direct or mediated by IGF-I (118).

GH therapy increases lean body weight primarily by enhancing protein synthesis, with little or no effect on protein degradation. Studies using [^{13}C]-leucine kinetics demonstrated that GH therapy increases whole body protein synthesis (119). GH also reduces fat mass, especially in individuals who have accumulated excess fat mass during prolonged periods of GH deficiency (59). A similar effect has been seen in geriatric patients treated with rhGH. Concomitant with the increase in lean body mass, nitrogen balance is shifted toward retention (120). It was of interest to determine whether GH effects on protein synthesis in muscle require IGF-I. To do this, the metabolic effects of GH, IGF-I, and insulin were compared by infusing these factors into the forearms of human volunteers while holding systemic amino acid levels constant. All three agents enhanced phenylalanine balance, although IGF-I was the most potent of the three. GH and IGF-I increased the uptake of phenylalanine, whereas IGF-I and insulin inhibited phenylalanine release, but GH did not (121). These studies strongly support the notion that many of the anabolic effects of GH in muscle may be IGF-I dependent. On the other hand, IGF-I may have other additional effects, such as inhibiting proteolysis.

Systemic administration of GH stimulates longitudinal bone growth and skeletal muscle growth, whereas treatment with IGF-I increases the size of lymphoid tissues (spleen and thymus) and kidney (122). GH has a more robust effect than IGF-I on longitudinal bone growth in animals, and the effects of these factors may be additive (123–125). However, many studies have shown a greater effect of rhGH administration

compared with rhIGF-I administration (126), and these results need to be interpreted carefully. GH administration systemically increases circulating levels of IGF-I, IGFBP-3, and ALS (Fig. 1C1). IGF-I administration, on the other hand, while transiently increasing circulating levels of IGF-I, inhibits GH secretion and may actually decrease IGFBP-3 and ALS levels, thereby leading to faster clearance of IGF-I from the circulation. Indeed, this has led investigators to begin clinical trials using a complex of IGF-I/IGFBP-3, rather than IGF-I alone. In hypophysectomized rats, the coadministration of IGFBP-3 with IGF-I markedly reduced the hypoglycemia associated with IGF-I treatment (127). The effects of coadministration of IGFBP-3 on the anabolic actions of IGF-I were variable, however, either showing no change or enhancing the effects of IGF-I on growth (127).

Evidence for synthesis of GH in a number of extrapituitary sites, including the lateral hypothalamus (128), lymphocytes, thymocytes (129), neutrophils (130), the placenta (131), and both normal and neoplastic mammary tissue (132), has been reported. These findings suggest that GH may have local paracrine/autocrine effects that might be distinct from, or in addition to, its classic effects that are known to be mediated by circulating IGF-I. These local paracrine/autocrine effects may be mediated either by local production of IGF-I or by other additional factors (49) (Table 2).

Intrauterine growth is apparently not dependent on GH, since infants with congenital absence of the pituitary and GH or GHR deletions are born near-normal in size. However, postnatal growth and development are dependent on normal pulsatile secretion of GH (133). This effect is especially prominent during puberty. GH stimulates both circulating and local levels of IGF-I, which culminates in a process of chondrocyte proliferation and differentiation in the epiphyseal growth plate of long bones, followed by calcification and incorporation into metaphyseal bone (134). In osteoblasts, GH induction of c-*fos* and c-*jun* expression requires a PKC-mediated signaling cascade. GH induces type-I collagen synthesis and proliferation of osteoblasts through an IGF-I-dependent process (135, 136). Furthermore, as described above, Isaksson and co-workers (30) have demonstrated that direct injection of GH into the cartilage growth plate of the rat induces longitudinal bone growth.

GH has a lipolytic action on fat and muscle, whereby circulating FFA and glycerol levels rise after acute administration of GH. This effect is apparently mediated by the inhibition of lipoprotein lipase, an enzyme involved in lipid accumulation in adipocytes (137, 138), and represents a major effect of GH on metabolic intermediates. Long-term effects of GH are decreased deposition of fat and increased fat mobilization. GH administration also causes mild reductions in low-density lipoprotein (LDL) cholesterol levels and small elevations in high-density lipoprotein (HDL) cholesterol (139). Acute administration of GH to fat and other tissue explants causes a temporary insulin-like effect on glucose uptake. In contrast, chronic exposure to GH leads to insulin resistance associated with hyperinsulinemia that is primarily due to a post receptor defect in insulin signaling (140). The acute insulin-like activity of GH on carbohydrate metabolism seen both *in vivo* and *in vitro* appears to be independent of both IGF-I and insulin, since these effects have also been observed in isolated tissue preparations and in cultured cells (141). While the exact mechanism(s) are not yet well defined, GH-induced tyrosine phosphorylation of IRS-1 and/or IRS-2 may be involved (103). Prolonged GH stimulation ultimately results in hyperglycemia that is associated with enhanced hepatic gluconeogenesis and glycogenolysis. These effects may be indirectly caused by the GH-induced lipolysis and elevated plasma FFA that inhibit insulin activity at its target tissues. This so-called "lipotoxic" effect was first noted by Randle and others (142), and became known as the glucose/fatty acid or Randle cycle.

D. GH actions not mediated by IGF-I

The strongest evidence for a growth-promoting effect of GH that is independent of IGF-I has come from the observation that growth plate germinal zones are significantly expanded in *igf-I* null mice (Fig. 2) (52). GH treatment also induces hepatomegaly in *igf-I* null mice (143). Furthermore, IGF-I is not the only growth factor regulated by GH (Table 2). After partial hepatectomy in rats, GH induces expression of the hepatocyte growth factor gene in liver (144). GH also modulates basic fibroblast growth factor gene expression in costal cartilage. In addition, GH regulates epidermal growth factor (EGF) and EGF receptor gene expression in kidney and liver (145), respectively. GH also increases levels of estrogen receptors in the uterus of guinea pigs (146). Bone morphogenetic proteins (BMPs) play important roles in the differentiation of multiple tissues. Both GH and IGF-I increase the expression of BMP-2 and -4. However, GH induces the ex-

TABLE 2. Local intermediates regulated by GH

Tissue	Intermediate
Liver	Hepatocyte growth factor (144), tyrosine phosphorylation of ErbB-2 by JAK2 (214)
Chondrocyte	bFGF (215)
Kidney	EGF (216)
Pancreatic β-cell	NGF receptors (217)
	Preadipocyte factor-1/delta-like protein (218)
Thymic stromal cells, Thymocytes	IL-1α, IL-1β
Osteoblasts	IL-6 (219)
Fibroblast	Bone morphogenetic proteins 2 and 4 (147)
	Serine/threonine phosphorylation of ErbB-2 via MAPK pathway (220)
Uterus, Mammary gland, Liver	Estrogen receptor (146, 221)
Primary adipocytes	Preadipocyte factor-1/delta-like protein (222)

GH regulates many growth factors other than IGF-I at a local level. bFGF, Basic fibroblast growth factor; IL, interleukin; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase.

pression of these genes even in the presence of an anti-IGF-I neutralizing antibody, suggesting that this effect of GH may also be independent of IGF-I (147). Similar results were obtained for when the effects of GH on colony formation of rabbit epiphyseal chondrocytes (148) and β-cell proliferation (149) were evaluated.

V. The IGF System

A. IGF-I and the IGF-I receptor

IGF-I is a member of the IGF family of growth factors and related molecules. The IGF family is comprised of ligands (IGF-I, IGF-II, and insulin), six well characterized binding proteins (IGFBP-1 though -6), and cell surface receptors that mediate the actions of the ligands (IGF-I receptor, insulin receptor, and the IGF-II mannose-6-phosphate [(M-6-P) receptor (2, 150, 151)]. Gene knockout studies revealed that the IGF-I receptor mediates the cellular responses of IGF-I and IGF-II. While the insulin receptor mediates the biological functions of insulin, genetic evidence suggested that the insulin receptor was responsible for some of the mitogenic actions of IGF-II *in utero* (152). Recently, a subset of alternately spliced insulin receptors have been described with an increased affinity for IGF-II (153). In contrast, the IGF-II/M-6-P receptor is not considered to have any major role in IGF signal transduction, but is primarily responsible for clearing, and thereby reducing, the levels of IGF-II during fetal development (154). On the other hand, the IGF-II/M-6-P receptor plays a major role in transporting lysosomal enzymes between intracellular compartments.

The IGF-I receptor and insulin receptor are very similar in structure and show approximately 60% identity overall at the amino acid level (155). However, certain regions of these receptors share very high degrees of homology, including the tyrosine kinase domain, which shows about 85% homology between the two receptors. Both receptors are comprised of α- and β-subunits with the α-subunit localized entirely extracellularly and the β-subunit spanning the membrane and localized primarily intracellularly (156). The receptors assemble a $\alpha_2\beta_2$-configuration with ligand binding being primarily mediated by the α-subunits, which form a binding pocket. The intracellular domains of the β-subunits contain the tyrosine kinase activity and tyrosine residues that become phosphorylated upon activation of the receptor (157).

Upon tyrosine phosphorylation of the IGF-I receptor, multiple endogenous substrates are recruited to "docking sites" formed by the phosphotyrosine residues (Fig. 4)(2). These include the IRS family of proteins (IRS-1 through -4), which associate with the IGF-I receptor through PTB (phosphotyrosine binding) domains and, like the SHC family of adapter proteins, with SH2 domains (158, 159). Both these docking proteins bind to the IGF-I receptor at the juxtamembrane region through the NPXpY motif (160, 161). These docking proteins are then able to recruit other substrates that lead to activation of a number of essential signaling cascades. These include SH2 domain-containing proteins, which bind to specific motifs that contain phosphotyrosine. For example, the p85 subunit of PI3′-K, an enzyme that phosphorylates intracellular lipids, binds to pYXXM motifs on the IRS molecules (162). Grb2 (growth factor receptor binding protein-2) binds to pYVNM motifs (163), whereas SHP-2 (a protein-tyrosine phosphatase) and phospholipase Cγ bind to pYIEV motifs within IRS molecules (159). SHC, on the other hand, apparently only binds Grb2. Recruitment of SHC to the activated receptor leads to a cascade of events involving association of SHC with Grb2, association of Grb2 with mSOS (a nucleotide exchange protein), activation of Ras (a small monomeric G protein) by mSOS, and ultimately, activation of the MAP kinase signaling pathway (164, 165). While the IRS proteins are also capable of recruiting Grb2, they apparently play a more prominent role in activation of the PI3′-K pathway. This pathway leads to activation of Akt kinases and p70S6 kinase (166).

B. IGFBPs

The characterization of six IGFBPs has prompted the realization that the IGF system is considerably more complex than previously thought. What follows is a necessarily brief outline of the IGFBPs and their possible functional roles. For a more detailed description, the reader is directed to several excellent and comprehensive reviews (150, 167, 168). Unlike insulin, in the circulation, the IGFs are bound by a number of well characterized high-affinity binding proteins (IGFBPs) (150). These circulating IGFBPs act as carrier proteins, transporting the IGFs out of the circulation to the target tissues and prolonging the half-life of the IGFs by protecting them from proteolytic degradation. While IGFBP-3 was the first IGFBP identified as being present in the ternary complex, recent data suggest that IGFBP-5 is also capable of forming a complex with IGF and ALS (169, 170). When the IGFs are released from this large molecular complex, smaller molecular complexes form with other IGFBPs, and these are responsible for transporting the IGFs out of the circulation. In addition to their major roles in the circulation, most target tissues also express IGFBPs where they further regulate the local action of IGFs (168). Under certain circumstances the IGFBPs inhibit IGF stimulation of the IGF-I receptors, whereas under other conditions they may enhance the IGF-stimulated IGF-I receptor activation. In addition, IGFBPs may have IGF-independent effects on cell function.

C. Physiological effects of IGFs

IGF-I plays an important role in both embryonic and postnatal growth. Mice carrying null mutations in the IGF-I gene are born small and grow very poorly postnatally (57, 154, 171). Since GH and GHR gene-deleted mice have relatively normal birth weights, this strongly supports a GH-independent effect of IGF-I in embryonic growth. Moreover, plasma IGF-I levels in humans correlate with body size. Constitutional tall children have elevated plasma IGF-I levels (172), whereas lines of mice selected for high IGF-I levels show increased body weight (173). Infusions of rhIGF-I also enhance body weight and size in a number of models, further suggesting a role for circulating IGF-I in growth. Often, injections of GH have been shown to be more effective in promoting growth, at least in bone, whereas IGF-I was more

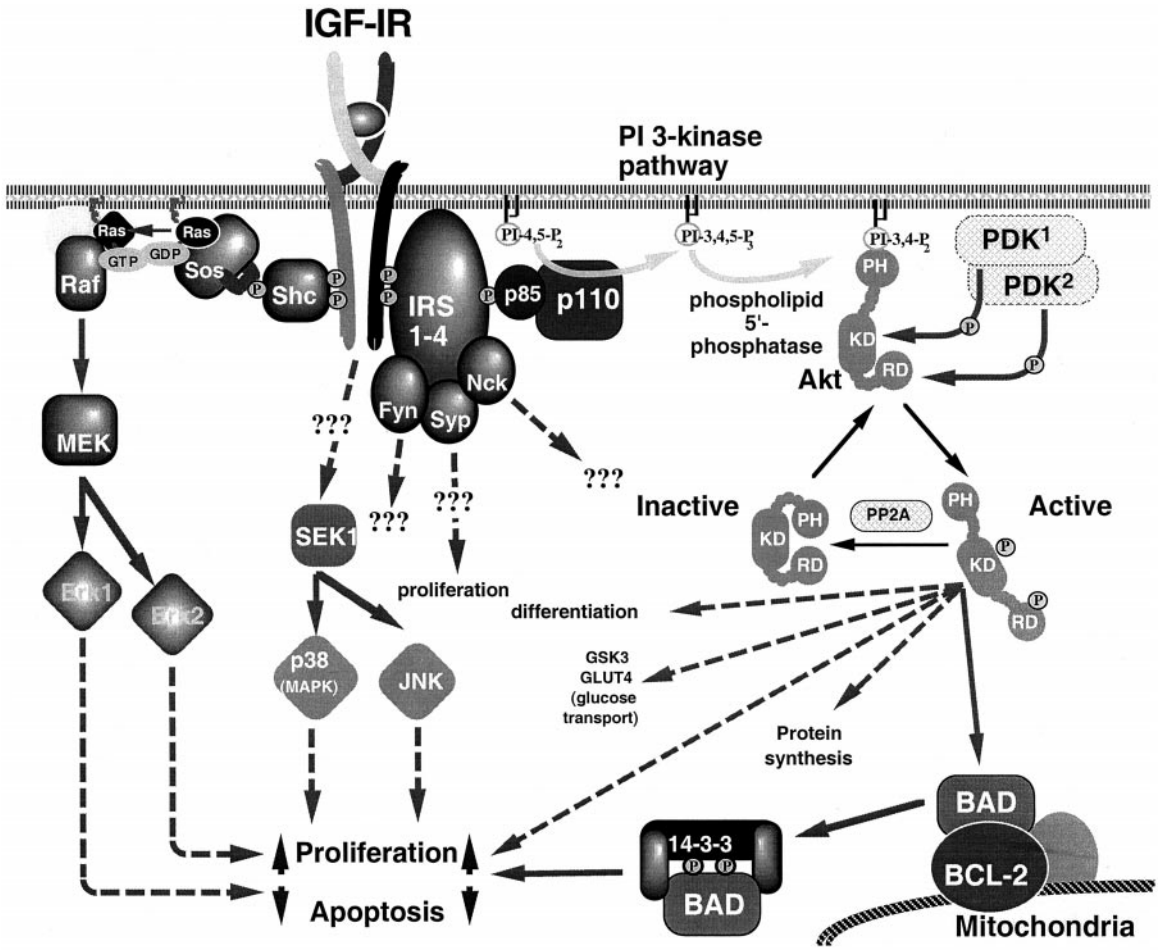

FIG. 4. Signal transduction cascades emanating from the activated IGF-I receptor. After autophosphorylation of the IGF-I receptor, Shc and IRS molecules are bound to the intracellular region of the IGF-I receptor β-subunit. Tyrosine phosphorylation of Shc and IRS molecules by the IGF-I receptor tyrosine kinase creates binding sites for other proteins in the signaling cascades such as Grb2, p85, Nck, Syp, and Fyn. These lead to activation of pathways such as MAP kinase and PI3′kinase pathways. A recently proposed model for the inhibition of apoptosis by Akt phosphorylation of BAD is also shown.

effective on kidney and spleen (122). These data may be interpreted to suggest that IGF-I may function alone in certain tissues, whereas it may mediate the effects of GH in other tissues (Table 3). IGF-I's role in ovarian follicular development and uterine growth is independent of GH, since GHR gene-deleted mice are fertile, whereas IGF-I gene-deleted mice are not (174). Furthermore, GH-resistant dwarfs are fertile (175). Since GH also increases the levels of certain IGFBPs, the higher potency of GH, relative to IGF-I, might be due to the fact that GH simultaneously stimulates the synthesis of IGF-I and produces a microenvironment that facilitates IGF-I action through modification of the IGFBP profile.

IGF-I and insulin have both shared and unique actions. Administration of IGF-I increases whole body protein metabolism by increasing protein synthesis as well as inhibiting proteolysis (121, 176). These actions are distinct from the metabolic effects of insulin, which acts primarily by inhibition of proteolysis. This evidence strongly supports the idea that IGF-I is acting via the IGF-I receptor and not the insulin receptor in muscle. However, IGF-I also enhances glucose uptake into peripheral tissues, which is an insulin-like effect (177). This latter effect could conceivably be mediated by either IGF-I or insulin receptors. It has been confirmed that hybrid IGF-I/insulin receptors do exist, although the function of such hybrid receptors is yet to be determined (178). A study by Mortensen and colleagues (179), showed that administration of recombinant IGFBP-1 leads to increased insulin secretion in the rat, suggesting that circulating IGFs may inhibit insulin release and thereby impart a tonic glycemic effect (179). In this context, IGFBP-1 might increase insulin secretion as a side effect of inhibiting the potential of free IGF-I in the circulation to elicit its hypoglycemic action. In contrast, GH stimulates glucose uptake with only a very short-term effect. Long-term administration of GH inhibits glucose uptake by inducing a state of insulin resistance (180). This effect is found to occur both at the liver and peripheral tissue level and is the result of a postreceptor effect (140). These findings further suggest that certain effects of GH occur independently from IGF-I.

In light of its insulin-like effects, rhIGF-I has been used successfully as an adjuvant to insulin therapy in patients with type 1 and type 2 diabetes. Plasma glucose concentrations fall after acute or chronic administration of rhIGF-I. This can be observed in those patients who are also insulin

TABLE 3. Cellular responses to IGF-I receptor activation

Function	Examples
Regulation of gene expression	Immediate early: c-*fos*, c-*jun*, junB, egr1 Osteoblast: Osteopontin, bone sialoprotein, procollagen(I), alkaline phosphatase, osteocalcin
Stimulation of myogenesis	Proliferation precedes differentiation Early activation of cell cycle genes (cyclin D1 and D2) Transient suppression of myogenic factors (myogenin, MRF4, myf5)
Apoptosis	Inhibition of apoptosis in response to environmental (*e.g.*, hypoxia) and chemical stimuli (*e.g.*, Fas, chemotherapeutic agents, growth factor withdrawal) Multiple pathways involved (AKT/protein kinase B, MAPK) leading to BAD dephosphorylation
Cell cycle progression	Activation of cell cycle genes "G^1-progression factor"
Immune response modulation	Regulation of cytokine production Regulation of cell proliferation (clonal expansion)
Adrenal steroidogenesis	Induction of steroidogenic enzyme genes Interacts with ACTH to stimulate cortisol release from adrenocortical cells
Sex steroid production	IGF-I enhances steroidogenic responsiveness to LH/hCG in Leydig cells Stimulates progesterone production in granulosa cells Synergistic interaction with FSH and estradiol

The major responses to IGF-IR activation by IGF-I or IGF-II are accompanied by relevant examples.

resistant (181–183). This effect of rhIGF-I is in direct contrast to GH therapy, which leads to increased insulin resistance. In type 1 patients with poorly controlled diabetes, levels of circulating IGF-I are reduced and GH levels are elevated. Administration of rhIGF-I often restores the high levels of GH to normal, leading to improved insulin sensitivity, and enhanced glucose uptake in peripheral tissues (184). In type 2 diabetic patients, who are commonly insulin-resistant, IGF-I therapy leads to some improvement, which is primarily due to enhanced muscle glucose uptake (185–188). Whether this effect is mediated by the IGF-I receptor, the insulin receptor, or hybrid receptors expressed in muscle remains to be determined (178, 189). Nevertheless, these data once again illustrate the distinct physiological effects of GH and IGF-I.

D. IGF-I's role in the cell cycle

It is worth noting that IGF-I's original growth-promoting activity was characterized in terms of protein synthesis ("sulfation factor," see above). The view of IGF-I as a mitogen was derived largely from *in vitro* studies of growth-arrested fibroblast cell lines [reviewed by Pardee (190)]. These *in vitro* studies established the view that growth factors act primarily in the G_1 phase of the cell cycle. Peptides such as PDGF, FGF, and EGF, termed "competence factors," stimulated quiescent cells to enter G_1, while IGF-I spurred progression through G_1 to S-phase and therefore was deemed a "G_1-progression factor" (191). More recent studies using IGF-I and IGF-I receptor "knockout" models suggest the situation is more complicated. Rubin and Baserga (192) investigated cell cycle kinetics in fibroblasts derived from *Igf1r* null embryos and found that these cells demonstrate a G_2/M phase duration 4-fold longer than comparable wild-type cells, while G_1 is not blocked but is doubled in duration. Moreover, progression through G_1 and S-phases is normal in the intact *Igf1* null animal, indicating that IGF-I has a minor, if any, role as a G_1 progression factor *in vivo* (193). However, *Igf1* null cells are profoundly retarded in their transit through G_2/M, at least in the estradiol-stimulated uterus (193), suggesting that IGF-I may be required for timely progression through later phases of the cell cycle. On the other hand, IGF-II is capable of diminishing the G_1 checkpoint after DNA damage (194). Further studies will undoubtedly resolve these important issues.

VI. Transgenic Tools for Analyzing the Somatotropic Axis

A. Transgenic mice overexpressing GH or IGF-I

The relative functions of GH and IGF-I have been studied in transgenic mice overexpressing either GH or IGF-I. In transgenic mice expressing human GH under the control of a metallothionein promoter, circulating GH levels were elevated 1,000-fold, relative to wild-type mice, whereas IGF-I levels were only increased by 2- to 3-fold, with the increase appearing at about 3 weeks after birth (195, 196). GHR binding and GHR mRNA have been detected in several tissues of the fetus and neonate (197, 198), while high levels of circulating GH have also been observed in fetal sheep (133). Thus, it is possible that postreceptor GH signaling pathways are activated during the postnatal stages of development, thereby leading to GH-dependent stimulation of growth and IGF-I synthesis.

Starting about 3 weeks postnatally, the growth rate of transgenic mice expressing the hGH gene accelerate such that the final weight of hGH transgenics is about twice that observed in normal littermates (133). Interestingly, this doubling in size corresponds more closely with the 2-fold increase in circulating IGF-I levels than with the greater elevations in circulating GH levels (199, 200). While these data were initially interpreted as being consistent with the somatomedin hypothesis, they are equally consistent with the alternative somatomedin hypothesis, *i.e.*, whereas postnatal growth by GH is IGF-I dependent, the GH effect can be mediated by either endocrine (circulating) or paracrine (locally produced) derived IGF-I.

More recent studies, comparing transgenic mice overexpressing GH to those overexpressing IGF-I, have yielded

interesting results. Mice overexpressing the IGF-I gene demonstrated a small but significant (~1.4 fold) increase in body weight, starting at about 2 months postnatally (199, 200). The weight increase was due to hyperplasia in a number of organs. There was, however, no increase in bone length or bone accumulation. Circulating GH levels were markedly reduced in these mice. Thus, on the basis of these results, one could speculate that bone growth requires concomitant elevation in both GH and IGF-I levels. Paradoxically, in another model where GH-deficient mice were crossed with IGF-I overexpressing transgenic mice, the resultant offspring exhibited increased organ and bone growth (201). Thus, it is possible that in the absence of GH, excess IGF-I may fully compensate for the lack of GH at the level of the target tissue. Furthermore, this study provides additional evidence against the dual effector hypothesis (33, 46).

B. Targeted deletion of the IGF system

Naturally occurring mutations in the IGF system have proved to be extremely rare. A single patient with both intrauterine and postnatal growth retardation has been found who has a deletion of the IGF-I gene (58). Since the low levels of circulating IGF-I are associated with elevated GH levels, this strongly supports the somatomedin hypothesis (that IGF-I is a major mediator of GH-induced postnatal growth). Furthermore, since both the human and murine *igf1* deletions demonstrate growth retardation *in utero*, before the ontogeny of normal GH expression, and more severe growth retardation *in toto* than GH or GHR mutants, it appears that IGF-I has extensive GH-independent growth-promoting effects. Indirect evidence that has been used to refute the original somatomedin hypothesis and support the revised hypothesis is the finding that GH-resistant (Laron-type) dwarfs do not respond to rhIGF-I therapy with "catch-up growth" nearly as well as GH-deficient children in response to rhGH therapy (202). These findings might support the notion that there are important paracrine effects of IGF-I that are somewhat GH dependent, but they may also reflect the inadequate maintenance of circulating IGF-I levels in the absence of GH-induced mobilization of the ternary complex (Fig. 1C).

Efstratiadis and colleagues (56, 154) used standard gene targeting technologies to create lines of mice lacking the *igf1, igf2, igf1r* and *igf2r* genes. These studies provided critical information regarding the roles of the IGF system during development, while also providing surprising data regarding the possibility of another receptor mediating the effects of IGF-II on fetal growth. Subsequent observations of GH- and IGF-I-deficient humans and mice have shown that the specific suggestions of the original dual effector schemes do not hold up (*e.g.*, that GH is required for adipocyte or chondrocyte differentiation). However, the concept that GH may have both direct and indirect growth-promoting effects is still viable. If, however, GH had major, IGF-I-independent growth-promoting effects, one would expect that the GHR knockout mouse would be smaller than the IGF-I knockout. However, the reverse is true (174), which is more consistent with IGF-I having effects on fetal and early neonatal growth that are independent of GH.

To further study the relationship between IGF-I and GH actions *in vivo*, we analyzed GH action in IGF-I knockout mice (171). The IGF-I null mice were generated by crossing mice in which exon 4 of the IGF-I gene is flanked by tandem repeats of the lox/P sequences with a line of mice expressing Cre (recombinase) driven by the EIIa promoter. The EIIa promoter is expressed at the early blastocyst stages of development and theoretically should cause recombination of the loxP-flanked allele. (An overview of the Cre/lox approach for gene targeting studies is discussed in more detail in *Section C*, below). Southern blot analysis revealed that the offspring of these crosses indeed demonstrated recombination of the IGF-I alleles. Homozygous animals failed to express detectable IGF-I mRNA in all tissues studied. Total deletion of the IGF-I gene was confirmed by separating IGF-I from its binding proteins by HPLC followed by measuring circulating IGF-I levels by a sensitive RIA technique. Using this approach, IGF-I was not detectable in the serum of IGF-I null mice at 6 weeks of age. Pups with homozygous deletions of the IGF-I gene were born at approximately 60% of the body weight of their normal littermates, suggesting a role for IGF-I in prenatal growth and development. However, their postnatal growth and development were even more markedly affected by the absence of IGF-I. At 7–8 weeks of age, these animals were only about 30% the weight of their normal littermates, and adults were infertile. These results were very similar to those described previously by Efstratiadis (56, 154) and Powell-Braxton *et al*. (57) using the traditional knockout approach.

Homozygous IGF-I-deficient mice were injected with rhGH (3 mg/kg subcutaneously, twice daily) from postnatal day 14 to day 56. Control animals received the diluent alone. Direct comparisons were made to wild-type littermates that received either rhGH or diluent over the same period. While GH injections significantly enhanced the growth of wild-type mice by 20%, as measured by body weight, in wild-type mice (from ~20 g to ~24 g), GH had no effect on growth in the IGF-I-deficient animals. Body length was similarly increased in the wild-type mice receiving rhGH but unchanged in the IGF-I-deficient mice. rhGH appeared to be active in the IGF-I-deficient mice, as these animals exhibited an increase in liver weight from approximately 7 g to about 8 g, as well as increases in *jun*B mRNA levels in the liver (143). Circulating GH levels were markedly elevated in the IGF-I-deficient mice (75 ng/ml *vs.* 6 ng/ml in the wild-type animals). Despite this dramatic elevation in circulating GH levels, and the administration of exogenous rhGH, there was no significant effect of GH on overall body growth or development in these animals. Thus, at least in mice, postnatal growth is dependent on IGF-I. While this may be true of overall body growth and development, this does not exclude the possibility that other effects of GH, such as liver growth (see below), are IGF-I independent, and probably occur at a local tissue level. Furthermore, these studies do not distinguish between the role of circulating IGF-I and local autocrine/paracrine forms of IGF-I, since the synthesis of both liver IGF-I and that expressed by some extrahepatic tissues such as bone are GH-dependent (26).

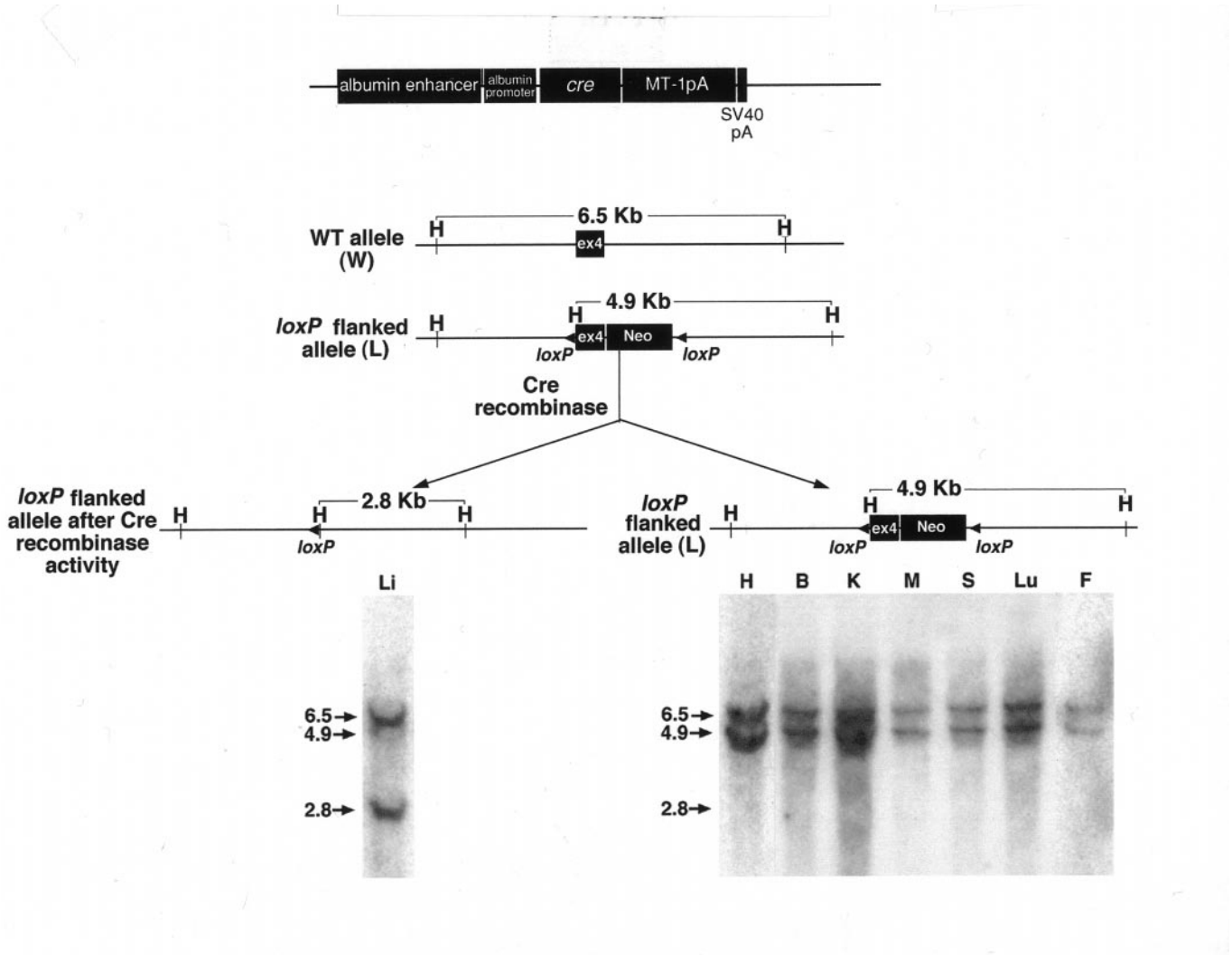

FIG. 5. Schematic diagram of the Cre-Lox/P system and Southern blot of the *igf1* gene in different tissues. Exon 4 of the *igf1* gene is flanked by two lox/P sequences in tandem. Cre expression is liver specific due to the albumin promoter and recombines the region between the two lox/P sequences. The flanked allele is cleaved from a 4.6-kb band on the Southern to a smaller 2.8-kb band. Wild-type alleles are 6.0 kb in size. As demonstrated, only the liver *igf1* gene was affected by the recombination event.

C. New approaches: conditional knockouts using the Cre-loxP system provide new insights into the function of circulating IGF-I

In recent years, *in vivo* gene targeting studies have enabled us to study the function of specific genes in the whole animal. However, a common criticism of this experimental approach is that disruption of a gene that is critical for normal development may prohibit using the resulting animals as an appropriate model to study normal function of the gene in the adult. Furthermore, it is possible that compensatory mechanisms, *e.g.*, the up-regulation of another gene with a similar function, may obscure the loss of function of the gene of interest. Thus, the ability to inactivate genes in a tissue and temporally regulated manner represents an incredibly powerful new tool to the molecular biologist. Genes may be excised from the genome utilizing a site-specific recombination technology adapted from bacteriophage. Bacteriophage P1 Cre recombinase, a 38-kDa protein, recognizes 34-bp DNA sequences called loxP sites (locus of cross-over P1) (203, 204). When the loxP sites are in tandem and flanking an essential exon of the gene of interest, Cre induces an intramolecular recombination and excision of the intervening DNA, resulting in deletion of the exon (Fig. 5). By placing the Cre recombinase under the control of tissue-specific or inducible promoters, the function of a gene in a certain tissue(s) can be examined in the absence of potentially critical developmental abnormalities. The approach is technically quite challenging, however, requiring the generation by homologous recombination in ES cells of the gene of interest flanked with loxP sequences and different lines of mice expressing Cre under the control of the desired promoter for tissue-specific Cre expression. This procedure is further complicated when the level of Cre recombinase expression is insufficient to achieve complete abrogation of gene function. Mice with a gene flanked by loxP sites therefore represent an extremely valuable resource.

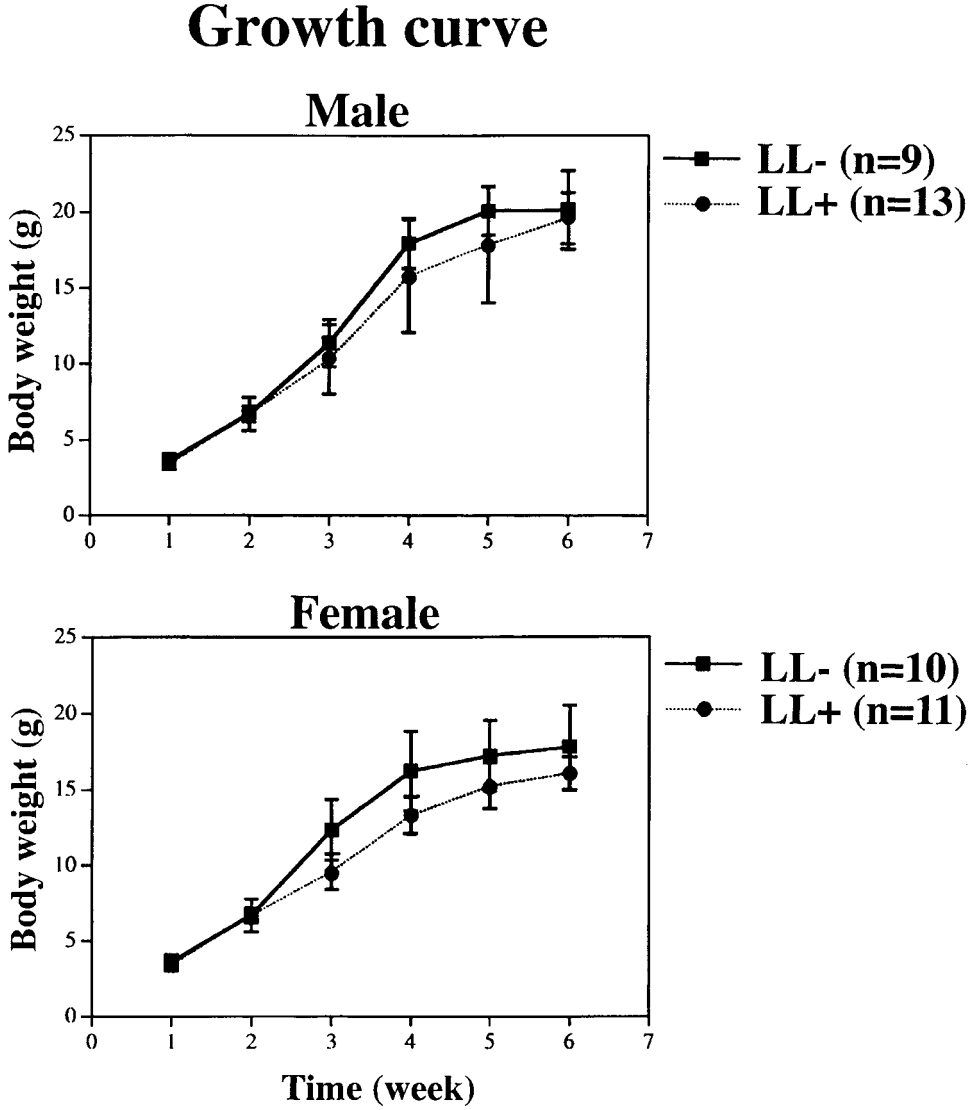

FIG. 6. Postnatal growth curves for liver-specific *igf1* gene-deleted animals. Body weights for male and female mice are shown separately. LL–, Mice that have exon 4 flanked by loxP sequences, but were cre–, *i.e.*, no loss of the *igf-1* gene from the liver. LL+ are mice with cre recombinase-deleted exon 4 of the *igf-1* gene in a liver-specific manner. These studies demonstrate that postnatal growth in liver-specific *Igf-I* gene-deleted mice is normal.

Using homologous recombination, we have established a mouse line in which two loxP sites in tandem (Fig. 5) flank the fourth exon of the IGF-I gene.

Exon 4 encodes amino acid residues 26–70 of the IGF-I peptide, including part of the B domain and the entire C, A, and D domains. This region of the peptide is solely responsible for IGF-I binding to its cognate receptor (IGF-I receptor) and has been previously targeted by others to create a total IGF-I knockout for developmental studies (154).

To create a liver-specific deletion of the IGF-I gene, we generated transgenic mice expressing Cre recombinase exclusively in the liver, by expressing Cre under the control of the albumin promoter. The albumin promoter is highly active in the liver and weakly active in certain other tissues. The albumin gene is activated late during fetal development, becoming easily detectable at about 10 days postnatally, with maximal expression levels occurring during the adult stages of development (205). Cross-breeding of the loxP-flanked IGF-I mice and the albumin-Cre expressing mice resulted in deletion of the IGF-I gene in the liver. Southern blot analysis revealed that the IGF-I gene-deletion effect was approximately 95% in the liver and undetectable in other tissues. Furthermore, IGF-I mRNA levels in liver, as determined by solution hybridization RNAse protection assays, were less than 1% of the levels in wild-type animals. In contrast, IGF-I mRNA levels measured in nonhepatic tissues such as heart, muscle, fat, spleen, and kidney were similar to control animals despite the increase in circulating GH (206). This lack of increase in nonhepatic tissue IGF-I mRNA is possibly due to these tissues being already maximally stimulated by factors other than GH, or that the lack of cyclical GH secretion affects the responsiveness of these tissues.

The effect of liver-specific IGF-I gene deletion on growth and development was then tested. Circulating IGF-I levels in these animals were markedly reduced at 6 weeks of age (only 25% of that in wild-type animals). This was associated with an approximately 4-fold increase in circulating GH levels, presumably due to the decrease in negative feedback control by circulating IGF-I on GH secretion by the pituitary. Measuring body weight twice weekly from ages 3 to 6 weeks assessed postnatal growth and development (Fig. 6). At age 6 weeks, mice were killed and body length (nose to anus) was

measured, femoral length was measured by x-ray, and the weights of individual organs were recorded. None of these measurements were significantly different between liver-specific IGF-I knockout animals and their wild-type littermates. The only exception was that the spleen showed a reduced size in the knockout animals. The splenic size may be a result of chronically reduced levels of circulating IGF-I. Sexual maturation was normal, as demonstrated by normal fertility, normal size litters, and normal lactation and weaning. Essentially, there were no phenotypic distinctions between the liver-specific IGF-I gene-deleted animals and their wild-type littermates. In a second model, we produced a liver-specific deletion of the IGF-I gene by using an inducible interferon promoter to drive the expression of cre. While the expression of cre is not liver specific, in this model the deletion of the IGF-I gene was largely specific to the liver and spleen. Once again, the circulating levels of IGF-I were reduced by about 75%; however, postnatal growth and development was considered normal (207).

Since the circulating (endocrine form) of IGF-I was markedly reduced and GH levels were correspondingly increased in these animals, we also considered the possibility that the normal growth and development of these mice was the result of compensation from nonhepatic tissues. Using the solution

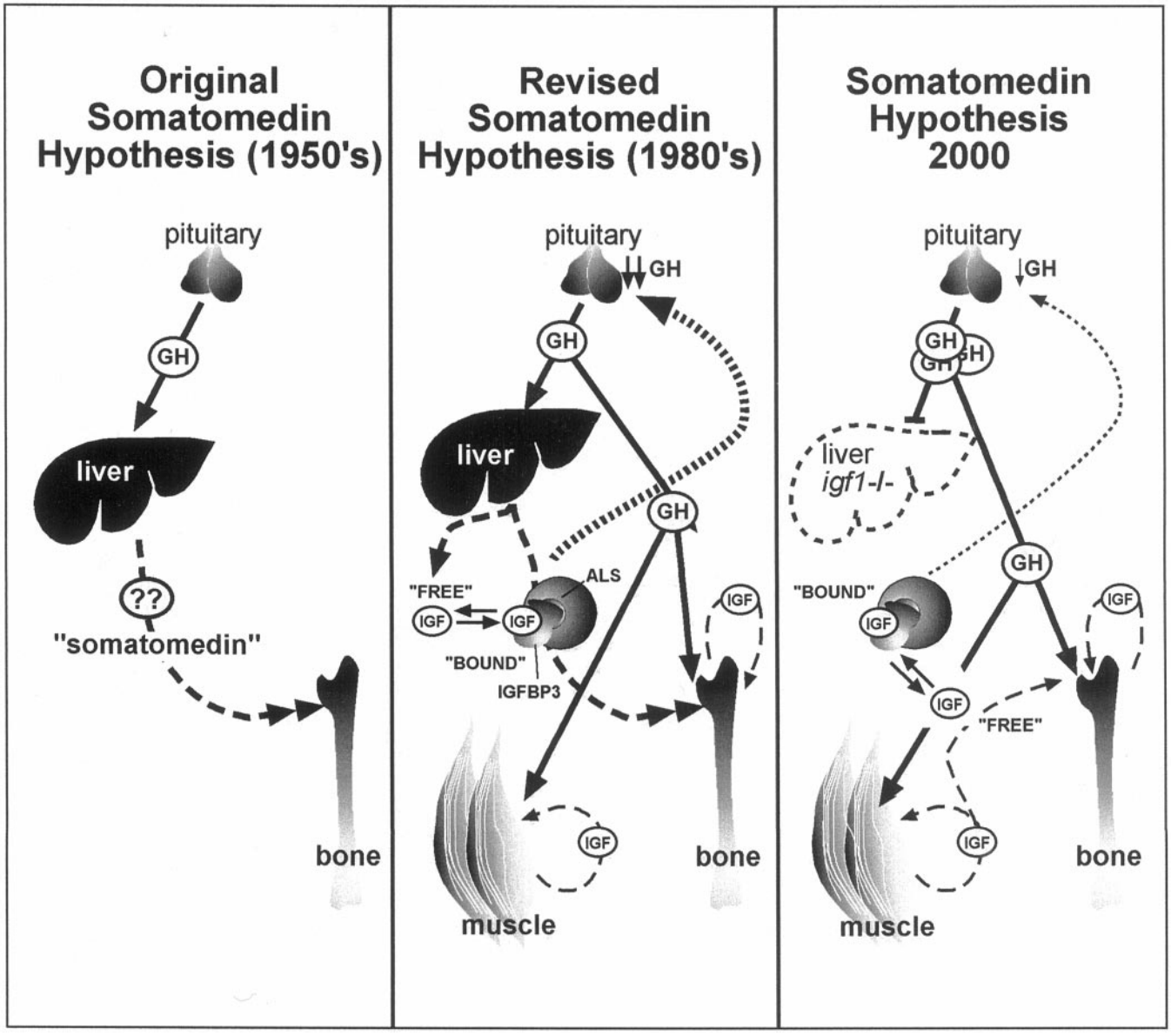

FIG. 7. Evolving concepts in the somatomedin hypothesis. *Left*, The original hypothesis proposed that GH controls somatic growth by stimulating the liver production of a circulating substance (somatomedin or IGF-I). *Middle,* The hypothesis was later modified after the discovery that IGF-I is expressed by almost all tissues of the body, and this led to the additional possibility of an autocrine/paracrine role for IGF-I. *Right,* The results of recent gene deletion experiments have questioned the role of liver IGF-I and the bound form of circulating IGF-I in controlling postnatal growth and development. Thus the marked reduction in circulating total IGF-I (and IGFBP-3) associated with elevated GH levels, postnatal growth, and development is normal. Free IGF-I is apparently normal and may be involved in this process in addition to any autocrine/paracrine IGF-I effect. The source of this free IGF-I is as yet undetermined.

hybridization/RNAse protection assay, which provides high specificity and sensitivity, IGF-I mRNA levels were measured in various tissues. In all tissues examined, including heart, muscle, fat, spleen, and kidney, IGF-I mRNA levels were not different from wild-type levels. From these results, we have concluded that these tissues do not show compensation, although we cannot exclude the possibility that compensation could occur at the level of IGF-I translation (208).

At this stage it is still unclear whether the normal growth and development in these mice are due entirely to local IGF-I production or whether the free circulating IGF-I levels are sufficient to maintain this function. Preliminary studies suggest that circulating free IGF-I levels are normal and that the reduction in total IGF-I is due to the marked reduction in circulating IGFBP-3 levels (S. Yakar, J.-L. Liu, Y. Wu, J. Frystyk, S. Chernausek, and D. Le Roith, manuscript submitted). This could explain why when IGF-I receptor mRNA levels were measured in various tissues they were not different from those in wild-type mice. Since lowered circulating IGF-I levels can result in up-regulation of IGF-I receptor gene expression (209, 210), the absence of such up-regulation is consistent with the notion of local IGF-I production and action. Alternatively, free IGF-I levels are sufficient to maintain endocrine IGF-I-induced growth. However, it is clear that no compensation is seen with IGF-II since IGF-II mRNA levels in tissue and IGF-II protein levels in serum were both undetectable in the IGF-I gene-deleted mice.

VII. Conclusion and Future Directions

A. Current understanding of the somatotropic axis

The evolution of our understanding of the complex, intertwined roles of GH and IGF-I in stimulation of somatic growth is summarized in Fig. 7. The original endocrine paradigm had IGF-I mediating the effects of GH on somatic growth and did not envision direct effects by GH on peripheral tissues or local production of IGF-I. The picture became more complicated as it became clear that some local tissues as well as the liver produced IGF-I, and that there were direct effects by GH, mediated by the GHR and not involving IGF-I. These dual observations suggested that GH had both direct and indirect (via IGF-I) effects on growth and emphasized local, autocrine/paracrine action by IGF-I.

Recent studies have also revealed considerably more complexity to the endocrine GH-IGF-I system, involving multiple level interactions between circulating and tissue IGFBPs. Observations of near-normal growth in mice with liver-specific IGF-I deletion and ALS deletion (211) led some workers to question the role of circulating IGF-I in somatic growth. Could it be that somatic growth is due primarily to GH-stimulated, locally produced IGF-I, while IGF-I in the circulation serves mainly to provide systemic negative feedback on GH secretion? It is important to remember, however, that most of the evidence for GH-stimulated local IGF-I production comes from rodents. Furthermore, in humans, there is generally a good correlation between circulating IGF-I levels and somatic growth, and treating patients systemically with recombinant IGF-I produces nearly normal growth. The failure of IGF-I treatment to achieve fully normal growth in GH-resistant children may be due to inadequate exposure to IGF-I, since in the absence of GH effect, ternary complex formation is impaired and IGF-I is rapidly cleared from the system. Alternatively, specific GH effects on tissues such as the growth plate in conjunction with IGF-I, as proposed in the dual-effector theory, may be required for optimal growth. On the other hand, there are some examples of near-normal growth in humans despite low GH levels, but with normal circulating IGF-I levels (212, 213). For the present, our view of the regulation of postnatal somatic growth includes both endocrine behavior of IGF-I, modulated by GH-induced binding complexes, and a local mode of action involving direct effects of both GH and IGF-I and possibly IGF-II at the growth plate (Fig. 1, C1). Finally, IGF-I has GH-independent actions in embryonic growth and reproductive system function (Fig. 1, C2).

B. What is the function of circulating IGF?

Much new knowledge has been gained in the previous decade concerning the function of the IGF system. We know now that it is an exceedingly complex system, in large part due to the essentially ubiquitous role played in controlling growth processes at the cellular level. We also know that the regulation of the IGF system is not limited to systemic GH. A major question that still remains to be answered, however, is why the liver produces an output sufficient to maintain such high levels of IGF-I (and IGF-II) in the circulation. Mice lacking hepatic IGF-I have apparently normal growth. Furthermore, a recent report has also shown that targeted deletion of the ALS gene, which also results in a large (60%) reduction in circulating IGF-I levels, is associated with only minor effects on growth rate and no discernable effects on glucose metabolism (211). Thus the maintenance of normal levels of IGF-I in the circulation is apparently not required for normal growth. Does circulating IGF therefore represent a pool with little bioactivity and destined only for clearance? What about free IGF-I?

We would suggest that a possible reason for maintaining such high levels of circulating IGF-I is to keep GH secretion in check. In both models of conditional deletion of the hepatic *igf-I* gene, circulating GH levels are elevated (206, 207). Granted that the studies published to date have been on young (6- to 8-wk-old) animals or have only reported relatively short-term effects of the deficit. We might need to look at older (5- to 6-month-old) animals to see lesions associated with long-term excess GH secretion (*e.g.*, glomerular hypertrophy, hyperinsulinemia and NIDDM, hypertriglyceridemia, hypertension, cardiomyopathies, increased occurrence of neoplastic diseases). At the time of writing, it has been shown that hyperinsulinemia is observed in liver *igf-I* null mice. Thus, we might be on the right track in reasoning that, rather than acting to *promote* growth, circulating IGF-I might actually serve to *restrain* the somatotropic axis. Only further studies will prove/disprove this hypothesis.

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Human Genome Information on the World Wide Web

The following three web sites present different but complementary views of the human genome:

http://genome.ucsc.edu
http://www.ncbi.nlm.nih.gov/genome/guide/
http://www.ensembl.org