In Vivo Analysis of Growth Hormone Receptor Signaling Domains and Their Associated Transcripts

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The growth hormone receptor (GHR) is a critical regulator of postnatal growth and metabolism. However, the GHR signaling domains and pathways that regulate these processes in vivo are not defined. We report the first knock-in mouse models with deletions of specific domains of the receptor that are required for its in vivo actions. Mice expressing truncations at residue m569 (plus Y539/S45-F) and at residue m391 displayed a progressive impairment of postnatal growth with receptor truncation. Moreover, after 4 months of age, marked male obesity was observed in both mutant 569 and mutant 391 and was associated with hyperglycemia. Both mutants activated hepatic JAK2 and ERK2, whereas STAT5 phosphorylation was substantially decreased for mutant 569 and absent from mutant 391, correlating with loss of IGF-1 expression and reduction in growth. Microarray analysis of these and GHR+/− mice demonstrated that particular signaling domains are responsible for the regulation of different target genes and revealed novel actions of growth hormone. These mice represent the first step in delineating the domains of the GHR regulating body growth and composition and the transcripts associated with these domains.

Over the last decade, extensive in vitro studies have identified signaling pathways triggered by growth hormone receptor (GHR) activation and the sequence motifs within the conserved cytoplasmic domain of the receptor that are required to initiate these pathways. These studies have established the critical signaling role of JAK2 tyrosine kinase, which is recruited to the trimeric GHR:GH complex at the box 1/2 motif (9). Hormone binding initiates JAK2 transphosphorylation and activation, resulting in phosphorylation of critical tyrosines within the cytoplasmic domain of the GHR, as well as other direct JAK2 substrates such as IRS-1 and -2. The distal cytoplasmic phosphotyrosines of GHR have been shown to recruit signal transducer and activator of transcription 5 (STAT5) and other proteins through SH2-domain interactions, whereas the proximal JAK2 activation domain is responsible for ERK1/2 and phosphatidylinositol 3-kinase (PI 3-kinase) activation (30), although it has been claimed that residues distal to m390 are required for ERK1/2 activation (residue 390, mouse sequence numbers given throughout) (38). There is some dispute regarding the distal tyrosine residues used to recruit STAT5 for activation, notably in relation to tyrosine m498 (8, 33); moreover, it has been claimed that tyrosines m341 and m346, proximal to boxes 1 and 2, may also generate active STAT5 (31).

In vitro studies have identified other signaling elements within the distal region of the GHR cytoplasmic domain, for example, a JAK2-independent calcium signaling element between residues m465 and m517 (30). SHP2 phosphatase can have a dual role when bound to the cytoplasmic domain of the GHR. It binds primarily to m606 to attenuate JAK2-STAT5 signaling but can also serve as an adaptor protein (30). GH-driven activation of STAT5 can also be attenuated by suppressor of cytokine signaling proteins (SOCS proteins). Tyrosine m498 and other proximal tyrosines are reported to bind SOCS-3, whereas residues m569 to m650 bind to other SOCS proteins, SOCS-2, and CIS (25). These SOCS proteins are believed to inhibit GH-induced gene transcription by competing with STAT5.

The relevance of these extensive in vitro studies to the in vivo state has not been established. Until the very recent publication by Milward et al. (19), there have been no publications of inactivating clinical mutations within the conserved 352 residue cytoplasmic domain of the GHR, other than an intron 9 donor splice mutation that effectively removes the cytoplasmic domain (1). STAT5b-deficient (STAT5b−/−) mice show a reduction in circulating IGF-1 (the central mediator of the growth actions of GH), and STAT5 response elements have recently been identified within the IGF-1 promoter (5, 35, 37, 41). One would predict that removal of tyrosines critical for docking of STAT5 would drastically reduce Igf1 transcript and consequently IGF-1 in serum, leading to reduction in postnatal growth. However, although STAT5−/− mice models do display growth retardation, this retardation is not as extensive as that seen in GHR gene-disrupted (GHR−/−) mice (4), indicating that other signaling pathways must play a significant physiological role in potentiating the growth signaling response of GHR. Moreover, the basis for regulation of the many other physiological roles of GH, such as the regulation of fat and carbohydrate metabolism, reproduction, bone turnover, and extended life span, need to be delineated in vivo by receptor mutation analysis. This is particularly relevant given that the sexual dimorphism in secretory pattern of GH is known to be...
responsible for the sexual dimorphism of many processes in rodents, particularly hepatic metabolism (34).

Here we report the creation and characterization of the first knockin mouse models designed to determine how and which specific regions of the GHR cytoplasmic domain are required for GH actions observed in vivo. Our phenotypic and microarray analyses with these mutant mice demonstrate that residues distal to m391 are critical for postnatal growth, STAT5 phosphorylation, and IGF-1 activation. However, in the liver the majority of regulated transcripts, including those for several novel GH actions, are associated with the proximal JAK2 activation domain.

MATERIALS AND METHODS

Gene replacement strategy. The GHR cytoplasmic domain was modified by homologous recombination with the GHR locus encompassing exons 9 and 10. Two targeting constructs were designed and generated incorporating a short homology arm of 1.4 kb upstream of exon 9 and a long homology arm of 4 kb, including exon 10 and the downstream intronic sequence.

Construction of targeting vector. Probes for the exon 9 and 10 portions of the GHR were generated by synthetic oligonucleotide creation (Genset Oligos, Lismore, Australia) or by PCR, respectively (10F, 5'-H11032-CCTGGGTCGAGTTCATTGAGC-3', 10R10, 5'-H11032-GCCCACTTACACCACCCAGC-3', 1-kb exon 10 product).

A 16-kb Sau3AI fragment containing exons 9 and 10 of the mGHR gene was isolated from a 129/SVJ mouse genomic phage library (Stratagene, La Jolla, Calif.). A 6.4-kb portion of this that contained exons 9 and 10 with a flanking intronic sequence was cloned into pBluescript by XbaI to use for targeting to embryonic stem (ES) cells (GenBank accession number AY271378). This fragment was subjected to QuikChange mutagenesis (Stratagene) to introduce relevant mutations to exon 10. Clone 1 was created by two rounds of mutagenesis to truncate the mature GHR at residue 569 (forward, 5'-H11032-GCA TGGAAGCCACGTCTTGTATAAAATAGAGCTTTAACCAAGAGG-3'; reverse, 5'-CTGCCAAGAAATTTCAGCATGAAACGGCTTTCATTTTACAGCAAGG-3'; reverse, 5'-GACTCAAAAAGAAGGCGCTGTTCATGCTGAAATTTTCTTGGCAG-3'). Clone 2 was treated to one round of mutagenesis to result in truncation of the mature GHR at residue 391 (forward, 5'-GCTGGTATCCTTGGAGCCTAGGATGGATTCTGGGCG-3'; reverse, 5'-CGCCCAGAATCATCCTAGGATGGATTCTGGGCG-3'). Both mutants were confirmed by automated sequence analysis (AGRF, University of Queensland, St. Lucia, Queensland, Australia). A floxed selection cassette (PGKneoNTRtkpA) (42) was then inserted between exons 9 and 10 by BamHI and XbaI engineered restriction sites (Fig. 1A).

Production of gene targeted mice. Targeting vector plasmids were linearized by NotI digest (cutting immediately upstream of the 5' flanking short arm) and purified by GeneClean II (Qbiogene, Carlsbad, Calif.) A 16-kb Sau3AI fragment containing exons 9 and 10 of the mGHR gene was isolated from a 129/SVJ mouse genomic lambda phage library (Stratagene, La Jolla, Calif.). A 6.4-kb portion of this that contained exons 9 and 10 with a flanking intronic sequence was cloned into pBluescript by XbaI to use for targeting to embryonic stem (ES) cells (GenBank accession number AY271378). This fragment was subjected to QuikChange mutagenesis (Stratagene) to introduce relevant mutations to exon 10. Clone 1 was created by two rounds of mutagenesis to truncate the mature GHR at residue 569 (forward, 5'-GCA TGGAAGCCACGTCTTGTATAAAATAGAGCTTTAACCAAGAGG-3'; reverse, 5'-CTGCCAAGAAATTTCAGCATGAAACGGCTTTCATTTTACAGCAAGG-3'; reverse, 5'-GACTCAAAAAGAAGGCGCTGTTCATGCTGAAATTTTCTTGGCAG-3'). Clone 2 was treated to one round of mutagenesis to result in truncation of the mature GHR at residue 391 (forward, 5'-GCTGGTATCCTTGGAGCCTAGGATGGATTCTGGGCG-3'; reverse, 5'-CGCCCAGAATCATCCTAGGATGGATTCTGGGCG-3'). Both mutants were confirmed by automated sequence analysis (AGRF, University of Queensland, St. Lucia, Queensland, Australia). A floxed selection cassette (PGKneoNTRtkpA) (42) was then inserted between exons 9 and 10 by BamHI and XbaI engineered restriction sites (Fig. 1A).

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The vector was electroporated into low-passage 129SvJ agouti ES cells, and homologous recombinants were selected for by G418 resistance, followed by genotyping. Positive heterozygote recombinants were Cre deleted by transient transfection with pMC-Cre (7) to remove the PGK neoNeoNeoNeo selection cassette from the genome. ES cells minus the selection cassette but retaining the desired GHR mutations (569 and 391) were confirmed by Southern blotting (described below). Cre-deleted ES cells were then injected into C57 black blastocysts and implanted in pseudopregnant foster mothers housed in a specific-pathogen-free facility. Chimeric offspring were mated with C57 black mice to produce germ line heterozygote offspring. Some chimeras were generated by injection of heterozygote ES cells that had not been Cre deleted. In this case, Cre deleter transgenic mice were obtained (29), which ubiquitously express Cre recombinase enzyme. These were mated with the F1 germ line mice and the chimeric males, which still carried the selection cassette.

Genotyping strategies. Southern blotting was used for genotyping as described previously (28). HindIII-digested genomic DNA was electrophoresed, transferred to a nylon membrane (Hybond N+; Amersham Pharmacia, Sydney, Australia), and hybridized to a probe corresponding to a 1.4-kb HindIII/XbaI fragment of upstream external intron 8/9 of the murine GHR. The probe was labeled with [α-32P]dCTP by random priming with a commercially available kit (Mega-prime DNA Labeling Systems; Amersham Pharmacia). The 7-kb fragment corresponded to the wild-type (WT) locus, whereas a 6-kb fragment was observed for the targeted locus (Fig. 1B, left panel). Analysis of Cre recombination was performed identically with the exception of EcoRI restriction digest, followed by hybridization to a probe corresponding to exon 10 as described earlier. In this case the WT locus yielded a 4-kb band, the targeted locus yielded a 5.3-kb band, and the Cre-deleted targeted locus yielded a 1.4-kb band (Fig. 1B, right panel).

Animals. Animals were housed in an SPF facility and treated in accordance with university guidelines, and all procedures were approved by the University of Queensland Animal Ethics Committee and the Australian Office of the Gene Technology Regulator. Water and feed pellets were available ad libitum in a 12:12-h light-dark cycle at 20 to 22°C. Fasting of the animals was performed overnight (16 h) with animals having water ad libitum. All animals passed standard virus screens quarterly throughout.

IGF-I measurements. Liver and ethanol-extracted serum IGF-1 was measured by using an IGF-1 radioimmunoassay kit (Biocline, Sydney, Australia) according to the manufacturer’s instructions.

RNA extraction and Northern blot analysis. Liver RNA samples from 42-day-old mice were isolated by using RNA-Beet reagent (Tel-Test, Inc., Friendswood, Tex.). Samples were separated on a denaturing gel (28) in morpholinepropane-sulfonic acid buffer and transferred onto an MSE membrane (Micron Separations, Westborough, Mass.). Hybridizations were performed by using Northern Max (Ambion, Austin, Tex.) and [α-32P]dCTP-labeled Megaprime Labeling System (Amersham Pharmacia). The 7-kb fragment corresponded to the wild-type (WT) locus, whereas a 6-kb fragment was observed for the targeted locus (Fig. 1B, left panel). Analysis of Cre recombination was performed identically with the exception of EcoRI restriction digest, followed by hybridization to a probe corresponding to exon 10 as described earlier. In this case the WT locus yielded a 4-kb band, the targeted locus yielded a 5.3-kb band, and the Cre-deleted targeted locus yielded a 1.4-kb band (Fig. 1B, right panel).

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knockin mice bearing cytoplasmic domain mutations through targeted homologous recombination in ES cells were created (Fig. 1A). The resulting mice express murine GHR proteins either truncated at proline m569, together with conversion of tyrosines m539 and m545 to phenylalanine (mutant 569), or truncated at lysine m391 (mutant 391) (Fig. 2A). The Y539/545F mutations were introduced to allow us to determine whether, in vivo, all STAT5 signaling originates distal to tyrosine m539 (as proposed by Hansen et al. [8]), or whether tyrosine m498 and potentially more proximal residues are required, as first proposed by Smit et al. (31). Homozygous mutant 569 or mutant 391 mice were generated by mating heterozygous mice carrying one copy of the 569 or 391 mutation, respectively. Correct homologous recombination was confirmed by restriction digest and Southern blotting, and expression of the mutant GHRs was demonstrated by Northern and Western blotting of hepatic tissue, together with sequencing of the mRNA transcript (Fig. 1B to D). The unavailability of an antibody recognizing the extracellular domain of the murine receptor in Western blots precluded determination of the molecular size of protein produced by the 391 truncation mutant. However, radioreceptor assays confirmed normal levels of specific binding of $^{125}$I-labeled bGH, i.e., $3.3\% \pm 1.1\%$ WT and $2.8\% \pm 0.8\%$ mutant 391 (mean $\pm$ the standard error of the mean [SEM]), expressed per milligram of membrane protein ($n = 3$ samples), to the receptor in this mutant.

**Signaling by mutant GHR.** The main aim of the present study was to determine the effects of mutations changing the intracellular signaling of GHR on growth and metabolism. Therefore, the relative phosphorylation of main effectors of GH stimulation, such as JAK2, ERK1/2, and STAT5, was compared 15 min after mice were injected with bGH or saline. In order to observe a direct response to GH, 19-day-old mice, which have low endogenous GH secretion (2) were used. Both mutants displayed a JAK2 phosphorylation response (Fig. 2B and E) and ERK1/2 activation (Fig. 2C and F) by GH that was not significantly different from that of WT mice with similar fold induction. In contrast, mutant 569 mice exhibited a sub-

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**FIG. 2.** Signaling in mutant mice in response to GH injections. (A) Predicted binding of signaling and adaptor molecules to the cytoplasmic domain of the GHR of the WT, mutant 569, and mutant 391. (B to D) Livers from the bGH- and saline-injected 19-day-old mice were used in immunoprecipitation analyses 15 min after the injections. Loading for each of the proteins was confirmed by using antibodies specific for an appropriate protein. (B) Antiphosphotyrosine (PY) immunoblot of JAK2 immunoprecipitated from liver homogenate. (C) Immunoblot for active ERK1/2 (Phospho-p44/42 mitogen-activated protein kinase) from liver homogenate. (D) Antiphosphotyrosine (PY) immunoblot of STAT5 immunoprecipitated from liver homogenate. (E to G) Densitometric quantification of the blots from panels B to D. Signals for the activated JAK2 (E), ERK1/2 (F), and STAT5a/b (G) were normalized for loading. The data for all graphs are presented as means $\pm$ the SEM.
maximal STAT5 tyrosine phosphorylation response (29 ± 10% of WT [mean ± the SEM; n = 6]), whereas no response was detectable in mutant 391 (Fig. 2D and G).

Postnatal growth rate and organ weights. Homozygous mice carrying the mutations remained phenotypically normal until ca. 3 weeks of age, after which a clear deviation in growth rates became apparent (Fig. 3), being more obvious in the mutant 391 than in mutant 569 (Fig. 3A to C). The 391 mutants grew significantly more than the GHR−/− (green), (B and C) Postnatal growth curves (B, females; C, males). All mice were weighed over a period of 60 days. The results are presented as mean body weight ± the SEM (n = 8). Growth was significantly reduced in all mutants compared to WT (P < 0.001). (D) Organ weights were recorded at 60 days postnatally. The results are shown as relative to body weight and are expressed as means ± the SEM (n = 6 to 8). The sizes of some organs were significantly reduced (all changes are marked with a single asterisk due to space constraints; P < 0.01 for all changes, except P < 0.05 for spleen in mutant 391 and liver in GHR−/− mice). (E) Subcutaneous fat pad sizes at 4 (●) and 10 (□) months in male mice. The results are expressed relative to body weight and are means ± the SEM (n = 3 to 4). ***, P < 0.01; *, P < 0.05. (F) Glucose levels in 10-month-old males after a 16 h fasting (n = 3) and are shown as means ± the SEM (*, P < 0.01).

FIG. 3. Postnatal growth reduction of the GHR mutant mice. (A) Photograph of WT and mutant homozygous 60-day-old male mice. (B to D) Color coded as follows: WT (black), mutant 569 (blue), mutant 391 (red), and GHR−/− (green). (B and C) Postnatal growth curves. (D) Organ weights were recorded at 60 days postnatally. The results are shown as relative to body weight and are expressed as means ± the SEM (n = 6 to 8). The sizes of some organs were significantly reduced (all changes are marked with a single asterisk due to space constraints; P < 0.01 for all changes, except P < 0.05 for spleen in mutant 391 and liver in GHR−/− mice). (E) Subcutaneous fat pad sizes at 4 (●) and 10 (□) months in male mice. The results are expressed relative to body weight and are means ± the SEM (n = 3 to 4). ***, P < 0.01; *, P < 0.05. (F) Glucose levels in 10-month-old males after a 16 h fasting (n = 3) and are shown as means ± the SEM (*, P < 0.01).
Differential gene expression in mutant 569, mutant 391, and GHR−/− mice compared to the WT. A set of 403 transcripts, representing 398 individual genes, which were differentially expressed across all animal groups and which met the above criteria, were identified. Twenty transcripts were common between the three receptor mutants in comparison to WT mice, with 13 unique to mutant 569, 59 unique to mutant 391, and 268 unique to the GHR−/− line (Fig. 4A and Table 1). Further analysis was performed with a subset of the 398 genes that passed stringent MAS 5.0 criteria (change \( P < 0.0025 \) and \( \Delta \)Log2 > 1 or \( \Delta \)Log2 < 1) by using an analysis of variance t-test with a cutoff score of \( P < 0.0005 \). Such a combination of criteria guarantees the lowest possible number of false positives (18, 24). Using these criteria, we have identified five genes to be regulated exclusively by m569-650 (Y539/545-F). The fold changes for these genes did not vary significantly between the mutant 569, 391, and GHR−/− lines and \( P > 0.0005 \). There were four genes upregulated to a similar extent in all groups (Gstt1, Ang, Papp2, and Serpina6) and only one gene similarly downregulated (Fabp5) with at least one of the genes, Serpina6 (corticosteroid binding globulin) known to be a direct GH target. This implies that most of the active STAT5, as well as SHP2, which binds in the m569-650 sequence, plays only a minor role in GH signaling to the genome. This upregulation of four of five genes and the fact that the majority of transcripts

FIG. 4. IGF-1 axis in GHR mutant mice. (A) Densitometric quantification of a Northern blot analysis of the IGF-1 levels in GHR mutant mice and WT littermates. Transcripts of 0.9 to 1.2 kb were used for this analysis. The data are shown as percentages of WT and are displayed as means ± the SEM (n = 10). (B) Levels of IGF-1 serum as measured by radioimmunoassay (see Materials and Methods) were significantly decreased in mutant 569, mutant 391, and GHR−/− mice in comparison to WT (\( P < 0.001 \)). The results are shown are means ± the SEM (n = 7 to 11). (C) Western ligand blot profile of IGFBP levels in serum in mice at 42 days shows a significant decrease in the levels of IGFBP3 (\( P < 0.001 \)), with no change detected in the remaining IGFBPs.
changed in mutant 569 (Fig. 6A) were upregulated reinforces the fact that the most distal part of the receptor is important for negative modulation of GH-induced gene expression. Furthermore, the set of 20 common genes (Table 1), which were regulated concordantly in the mutant 569, mutant 391, and GHR−/− mice, was identified. These genes can be reasonably assigned as STAT5-regulated genes since they included known STAT5-regulated genes such as Igf1, Igfals, Socs2, P450 cytochrome, Cyp2b9, and some metabolic enzymes. Eleven of these genes upregulated in mutant 569, mutant 391, and GHR−/− mice were upregulated (e.g., Stih2, Hao3, and Ang), and nine were downregulated (e.g., Igfals, Igfl, Egfr, and Comt); among these genes at least seven are currently known to be direct targets of GH-induced signaling. Increased downregulation of the Igf1 transcript in the 391 mutant suggests that the residual 30% of active STAT5 plays an important role in regulation of Igf1 (and presumably many other) transcripts, and Igfl mRNA levels are critically dependent on STAT5 both in vitro and clinically, based on loss-of-function studies (13, 41).

The analysis of the 121 transcripts altered in the mutant 391 showed that the majority of transcripts were not affected in mutant 569 and that 41 of them were not different from the GHR−− mice (e.g., Apcs, Es31, Cdkn1c, Ndufb8, Igfl-pending, LifR, and Mug-ps1) (Table 2 and Fig. 6A). The expression of these genes, as well as genes regulated preferentially in mutant 391 mice (Hsd3b3 and Hsd3b6), may be directed by the signaling proteins docking at all or some of the three tyrosines that were removed by the 391 mutation (residues m402, m465, and m498), which would include residual STAT5. The promoters of these genes may be either particularly sensitive to STAT5 or dependent on other signaling pathways within the m391-569 sequence. Such pathways include the Ca2+ signaling element between m465 and m517 and potentially other uncharacterized pathways.

The largest number of altered transcripts was observed for GHR−− mice (n = 330), providing a striking contrast to the 121 regulated transcripts for mutant 391 mice despite a very similar extent of growth retardation. These genes, presumably directly regulated by JAK2 and associated ERK1/2 and PI 3-kinase pathways, as well as negatively by SOCS proteins, include many metabolic genes, as well as genes regulating signaling, proliferation, translation, and transporter proteins. Such genes represent the core functions of the hepatocyte and are most likely regulated by elements common to all cytokine and tyrosine kinase receptors. Some of these genes have been shown to be directly regulated by GH (e.g., Igfl, Socs2, or Comt) (36) (Tables 1 and 2); some are likely to be regulated by transcription factors induced by GH signaling. In fact, the important differences between the GHR−− mice and the mu-

![Image](https://example.com/image.jpg)

**Fig. 5.** Regulation of hepatic nuclear factors in GHR mutant mice. (A and B) Western blot analysis of the expression of HNF3β (A) and HNF1α (B) in the livers of 10-month-old mice fasted for 16 h. Both HNFs were immunoprecipitated from the liver homogenates, and immunoglobulin G light chain was used as a loading control. (C and D) Densitometric quantification of the results from panels A and B are shown as means ± the SEM (n = 3). ***P < 0.001.

### Table 1. Genes regulated concordantly in mutant 569, mutant 391, and GHR−/− mice

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<thead>
<tr>
<th>Gene</th>
<th>Fold change&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Known regulation by GHR&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>Mutant 569/WT</td>
<td>Mutant 391/WT</td>
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<td>Ang</td>
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- Genes upregulated in all three groups.
- Genes downregulated in all three groups.
- Values indicate changes with WT values as a baseline. * transcript absent in WT mice; †, transcript absent in mutant mice as determined by MAS 5.0 software.
- +, regulation detected.
tant mice were the upregulation of transcription and elongation factors (e.g., Nr4a1, Bcl6, Egr-1, Dbp, and Tcea3) and the upregulation of translational factors (e.g., Sui1-rs1, Eef1d, and Ict1), none of which was significantly changed in mutants 569 and 391. This suggests that in GHR−/− mice a number of transcripts regulated by these factors would be expressed at a higher level than in WT mice. This could account for a substantial proportion of the genes uniquely increased in the GHR−/− mice compared to the mutants and WT (e.g., Rgs16, Cops3, Ppap2a, Gphn, and Gkap42-pending).

Classification of the regulated genes by GO highlights the main differences and similarities between the four lines of mice (Table 3 and http://research.imb.uq.edu.au/~mwaters/ghr/). We have explored the relationships between cell lines by using hierarchical clustering based on all transcripts (Fig. 6B) or based on metabolic genes (Fig. 6C). Mutant 569 and WT were most similar in both analyses, and although mutant 391 was similar to mutant 569 and WT based on overall gene expression, mutant 391 appeared to be more similar to GHR−/− mice in relation to metabolic genes. Both of these observations support our phenotypic findings (Fig. 2 and 3), in that mutant 391 retains JAK2 and ERK1/2 signaling and thus...
should be more similar to mutant 569 and WT than to GHR−/− mice. On the other hand, the levels of STAT5-dependent transcripts such as Igf-1 and Socs-2 in mutant 391 were more similar to GHR−/− mice (Table 2 and Fig. 6D). Mutant 391 and GHR−/− also shared similar alterations in transcripts encoding a number of transporters, signaling molecules, and cytochromes, as well as electron transport proteins. This GO analysis supports the view that we have delineated functional domains within the cytoplasmic signaling unit of the GHR in vivo.

**DISCUSSION**

A main aim of this study was to define the region(s) of the GHR cytoplasmic domain responsible for enhancing postnatal
growth, since no cases of GH insensitivity had been reported for the signaling domain other than those involving the JAK2 binding box 1 sequence. The approach used here allows us to conclude that the distal 80 residues and the tyrosines previously thought to be responsible for all or most of the STAT5 generation account for 70% of STAT5 generation and 44% of the GH-responsive postnatal growth in the male (30) (Fig. 6E). The remaining 30% of STAT5 signaling appears adequate to retain relatively normal gene expression, even of genes known to be STAT5 responsive. This includes the main Igf1 transcript, which was decreased by <33%. The overall correlation between STAT5 activation and Igf1 transcript level is concordant with recent studies implicating STAT5 in the generation of IGF-1 (41). Surprisingly, the modest decrease in Igf1 transcripts in the 569 line was associated with a substantial drop in IGF-1 transcript, [Igf1 transcript], as shown by their increase in the STAT5a/b null line. The lack of STAT5 activation and IGF-1 in serum in mutant 391 may represent the IGF-1-independent, GH-dependent element in postnatal growth (14%) identified by Lupu et al. (16) based on IGF-1−/− and GHR−/− crosses. In that study, IGF-1 receptor-dependent postnatal growth amounted to 70% of total postnatal growth, with 17% of postnatal growth being independent of either GH or IGF-1 (16). This GH-dependent, but IGF-1-independent growth may correspond to that seen in myeloid cells stably expressing 351 truncated GHR, which proliferate normally in response to GH (39). It is also of interest that the 391 mutant, lacking ability to generate STAT5a/b, did not show sexual dimorphism in growth, as was also observed in the STAT5b−/− mice (35). This correlates with an inability to express the male-specific MUP transcripts and protein in the 391 line, as well as feminization of its cytochrome P450 profile in the liver.

Our in vivo finding that ERK activation is normal with the 391 truncation is in contrast to the findings of some in vitro studies (38) but not others (3, 33). A second in vitro conclusion that was not verified in vivo is the lack of involvement of tyrosine m498 and proximal tyrosine in the generation of activated STAT5 (8). In support of this, other in vitro studies (32) have proposed that tyrosines m498 and m545 are responsible for most of the STAT5 activation. As is evident here, tyrosines m401, m447, and m498 (most likely tyrosine m498 [30]) can generate ca. 30% of active STAT5 in the liver. The lack of ability of the 391 truncated mutant to generate STAT5 eliminates a role for tyrosines m333 and m338 in generating active hepatic STAT5.

The present study has used the novel approach of combining transcript analysis by microarray with the creation of mice bearing targeted mutations within the cytoplasmic sequence of the GH receptor in order to define the role of signaling domains within the receptor. We have been able to define a limited set of 35 hepatic transcripts (5 exclusively) which are regulated by the carboxy-terminal 80 residues, and the two adjacent tyrosines (Fig. 6A). This domain generates the majority of active STAT5, and this is likely to be the instrumental agent in regulating these mainly metabolic genes. Accordingly, some of the P450 cytochromes that are known STAT5a targets, as shown by their increase in the STAT5a−/− mice, had their expression increased by deletion of this region (22). Microar-

<table>
<thead>
<tr>
<th>Type or function</th>
<th>Mutant 569</th>
<th>Mutant 391</th>
<th>GHR−/− control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td>12</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>Cytochrome/electron transport</td>
<td>5</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>Glutathione/antioxidant</td>
<td>2</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Transporters</td>
<td>1</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Proliferation/differentiation/growth</td>
<td>2</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Cell-to-cell interactions</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Transcription/translination</td>
<td>0</td>
<td>0</td>
<td>23</td>
</tr>
<tr>
<td>Signaling</td>
<td>1</td>
<td>13</td>
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</tr>
<tr>
<td>Protein processing</td>
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</tr>
<tr>
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<td>7</td>
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<tr>
<td>Ribosomal proteins</td>
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<td>15</td>
<td>91</td>
</tr>
<tr>
<td>Total</td>
<td>35</td>
<td>121</td>
<td>330</td>
</tr>
</tbody>
</table>
ray analysis of STAT5a/b−/− double-mutant mice should allow further definition of these regulated genes.

Despite the major involvement of residues m391 to m650 in regulating growth, the loss of the remaining receptor signaling results in a substantially greater number of altered transcripts (n = 330, compared to 121 for the mutant 391). The majority of transcripts changed in mutant 391 are downregulated in contrast to GHR−/− mice (Fig. 6A). This observation can be explained by the presence of binding sites in this truncated mutant, for negative regulators such as SOCS proteins, whereas all repression normally provided by GH signaling is lost in GHR−/− mice. In addition, these changes are accompanied in GHR−/− mice by upregulation of a number of important transcription factors (e.g., Nr4a, Bcl6, Dhp, and Egr-1), as well as translational regulators.

The present study has provided substantial new data on the physiological roles of GH in hepatic function, since large-scale microarrays have not previously been applied to GHR gene-disrupted mice. Indeed, the only microarray study using Clontech Atlas gene arrays (with 1,176 genes) on the GHR−/− mice. At least some of these changes would account for the serum protease inhibitor family were decreased. This could potentially decrease protein turnover and activation and lead to abnormal accumulation and/or actions of proteases. The changes observed in transcripts encoding metabolic enzymes, for example, in lipid and cholesterol metabolism (Scd1, Decr1, Ech1, and Acatal) confirm a previous study showing GH regulation (36). Changes in genes involved in cholesterol metabolism involved not only genes necessary for its synthesis and cellular uptake but also for cholesterol conversion to bile acids (Cysa). The latter was highly decreased, which can be expected to elevate the hepatic cholesterol levels. The changes in cholesterol availability would also affect steroid metabolism, and there were changes in transcripts encoding genes regulating this pathway. Transcript for one of the enzymes (Hsld3b5) was reduced in all mutants; however, in mutant 391 five other genes of this family were decreased. A number of transcripts encoding sugar-metabolizing enzymes (e.g., Glpdc and Pfklb1) were increased in the GHR−/− mice, with mild changes in mutant 391 and no changes in mutant 569. One of the important changes observed was an increase in transcripts encoding proteins of the beta-oxidation (Acadl, Hao3, and 1300002P22 Rik), electron transport chain (family of NADH dehydrogenases and cytochrome oxidases), and trichloroacetic acid cycle (Idh3g and Suclg1) would indicate higher energy production in GHR−/− mice. At least some of these changes would account for many of the phenotypes observed in GH-deficient patients and animals. However, the finding of obesity in both lines in later life does not correlate with the observed changes in hepatic lipid metabolism. The answer in this case is likely to be in the adipose tissue itself. GH-deficient lit/lit mice exhibit obesity in maturity, as did the older mutant mice in the present study. A likely cause of this is the deficiency of STAT5a, which is required for GH-dependent lipolysis in adipose tissue (6). There may also be a contribution to lipogenesis from the continuing drive to the distal receptor domain from elevated GH levels in plasma resulting from the lowered levels of IGF-1 in plasma.

An interesting finding in the young adult mice was the identification of a number of transcripts known to be important in regulating insulin sensitivity. These include the fatty acid-binding proteins 2 and 5, lipin 2, insulin-degrading enzyme, cortisold binding globulin (Serpina6), and the induced in fatty liver dystrophy 2 transcript (lfld2). Increased insulin sensitivity and decreased IGF-1 and insulin levels found in long-lived GHR−/− mice are concordant with these findings (4), although the elevated blood glucose in older animals (associated with obesity) would argue against this in the long term. Interestingly, 14 transcripts associated with life span extension in Caenorhabditis elegans are similarly regulated in the GHR−/− mice, raising the possibility that the life extension is not related to insulin. It will be important to determine whether mutant 569 with lowered IGF1 and mild growth retardation displays longevity similar to the heterozygous IGF1R+/- mouse (11), or if this is only seen with extreme suppression of IGF-1, as seen in the mutant 391, or correlates with loss of GH stimulated PI 3-kinase and ERK1/2 activity, as seen in the GHR−/− mice.

The present study has described the role of the various parts of the cytoplasmic domain of GHR in generating growth signal, indicated significant changes in metabolism associated with mutations of GHR (Fig. 6E), and provided evidence for novel roles of GH. In particular, the changes observed in GHR−/− mice indicated a role of GH in regulating mRNAs for transcription factors critical in promoting inflammation (Ppary, Nr4a1, and Bcl6) and transcripts for complement genes, for Rgs16 and Zap 70, and for interleukin-1 receptor antagonist, which together could account for the mortality observed when GH treatment is given to critically ill patients. In many cases the known roles of GH are more extensive than previously thought as, for example, in the regulation of antioxidant and glutathione metabolism, serum proteins, Serpin genes, RNA/DNA-binding proteins, chaperones, and ribosomal proteins, the latter presumably increasing translation efficiency. The results presented here also show that the GH-regulated metabolic functions can be successfully studied in our GHR mutant animals. Such studies will, however, require a physiological stress (e.g., induction of diabetes or use of specific diet) to determine how various enzymes and other proteins are regulated by remaining GH signaling. Further defining of the in vivo signaling pathways responsible for the regulation of expression of GH-induced genes will be facilitated by other targeted mutations to the cytoplasmic domain of the GH receptor.

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References


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IN VIVO ANALYSIS OF GH RECEPTOR SIGNALING


ERRATUM

In Vivo Analysis of Growth Hormone Receptor Signaling Domains and Their Associated Transcripts


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Volume 25, no. 1, p. 66–77, 2005. Page 67, Fig. 1D: The middle and right lanes of the top panel should be labeled “569+/−” and “569,” respectively, and the middle lane of the bottom panel should be labeled “391+/−.”