Mechanisms of Growth Hormone (GH) Action

IDENTIFICATION OF CONSERVED Stat5 BINDING SITES THAT MEDIATE GH-INDUCED INSULIN-LIKE GROWTH FACTOR-I GENE ACTIVATION*

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The biological effects of growth hormone (GH)1 on somatic growth and tissue regeneration have been inextricably linked with the actions of insulin-like growth factor-I (IGF-I) ever since the somatomedin hypothesis was first formulated over 47 years ago (1). Much is now known regarding the molecular physiology and biochemistry of both proteins (2, 3), and although several of the actions of GH do not require IGF-I (4) and IGF-I is not exclusively regulated by GH (5, 6), their interdependent roles in controlling normal growth during childhood and maintaining tissue integrity during aging have been both confirmed and amplified in the intervening years (7–10).

GH initiates its physiological effects after binding to the transmembrane GH receptor and activating JAK2, a receptor-associated intracellular tyrosine protein kinase (2), thereby setting into motion a series of protein phosphorylation cascades that lead to the activation of several transcription factors including AP-1 and Stats1, 3, 5a, and 5b among others (11). It has been known for over a decade that GH rapidly and potently induces IGF-I gene transcription in vivo (12), leading to the sustained production of IGF-I mRNAs and protein (12, 13), yet the signaling pathways connecting the GH receptor and cytoplasmic Jak2 to the nuclear IGF-I gene have remained uncharacterized. Contributing to this challenge is the fact that the IGF-I gene is more complicated that its simple protein structure would have predicted. In mammals, the gene is transcribed from two promoters, each with unique leader exons, and its initial transcripts undergo both alternative splicing and differential polyadenylation to yield multiple mature mRNA species (14).

Recent studies from our laboratory have implicated the transcription factor Stat5b as a key component in acute GH-stimulated IGF-I gene activation in rats (15), thereby extending previous observations pointing to roles for Stat5b and to a lesser extent Stat5a in controlling normal skeletal growth in mice (16, 17) but not elucidating molecular mechanisms. Here we use a combination of in vivo observations in GH-deficient rats and biochemical reconstitution experiments in tissue culture cells to identify a region within the IGF-I gene that binds Stat5b in a GH-dependent manner and is capable of mediating GH-stimulated IGF-I gene activation via this transcription factor.

EXPERIMENTAL PROCEDURES

Materials—Antibodies to Stats5 (C17X) were from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies to anti-FLAG (M2) were from Sigma. Recombinant rat GH was from the National Hormone and Pituitary Program, NIDDK, National Institutes of Health. Oligonucleotides were synthesized at the Oregon Health & Sciences University Core Facility. Transit-LT1 was purchased from Mirus (Madision, WI), and 5b fragment of rat IGF-I intron 2 was added 5′ to the promoter by standard molecular cloning methods. The mouse GH receptor in pdDNA3 was a gift from Dr. F. Talamantes (University of...
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**Table I**

| Gene   | Location | DNA sequence | Product size (bp) |
|--------|----------|--------------|------------------|
| IGF-1  | Exon 1   | 5'-TGAGTTCTTTCTGTTTACACCG-3' | 397              |
| IGF-1  | Intron 1  | 5'-CCCATGATCGCCCTTCCTCC-3'   | 316              |
| IGF-1  | Intron 2  | 5'-GCAATAGCCGCTCTCTGGCGCCG-3' | 316              |
| Spi 2.1| Exon 3   | 5'-TGGAGTGGTGATGTTTACGAGAGA-3' | 225              |
| c-fos  | Intron 3  | 5'-TGAGTTCTTTCTGTTTACACCG-3' | 202              |
| β-Actin| Exon 3   | 5'-TTTTAGAGGATCCATGCAAATGGACGTACG-3' | 202              |
| β-Actin| Intron 3  | 5'-AGGAAAGGCGGCCGCTGAAGAGGT-3' | 202              |

**Table II**

| Gene   | Location | DNA sequence | Product size (bp) |
|--------|----------|--------------|------------------|
| IGF-1  | Promoter | 5'-GCCCTCCTGTCGCTCAACCTTTAAAA-3' | 364              |
| IGF-1  | Exon 1   | 5'-CCCCTGCTCTGTTAATGACACAA-3'   | 200              |
| IGF-1  | Intron 2  | 5'-GGCCCAAGCTAGCCGATGGTTA-3'    | 200              |
| IGF-1  | Intron 3  | 5'-CTTCCCATGGAAGAGATCTTTGGA-3'  | 318              |
| Spi 2.1| Promoter  | 5'-CTTCCCATGGAAGAGATCTTTGGA-3'  | 224              |
| c-fos  | Promoter  | 5'-TGAGTTCTTTCTGTTTACACCG-3'    | 224              |
| β-Actin| Exon 3   | 5'-GGGCACTACACGCTACGTTCCAGA-3'  | 202              |
| β-Actin| Intron 3  | 5'-AGGAAAGGCGGCCGCTGAAGAGGT-3'  | 298              |

California, Santa Cruz, CA), and rat Stat5b in pcDNA3 was from Dr. Christin Carter-Su (University of Michigan, Ann Arbor, MI). The latter was modified by site-directed mutagenesis to add an NH2-terminal FLAG epitope tag, and point mutations were introduced to create constitutively active Stat5b (Stat5bCA, N699H) as described previously (15). Thymidine kinase luciferase was from Dr. Susan Berry (University of Minnesota, Minneapolis, MN). It was modified by the addition of portions of rat IGF-I intron 2 as indicated in Fig. 5.

**Transient Transfections and Reporter Gene Assays—**COS-7 cells (from ATCC) were incubated in media under conditions described previously (15) and were co-transfected with expression plasmids for the mouse GH receptor and wild type Stat5b or Stat5bCA using TransIT-LT1 and a protocol from the supplier. After incubation for 24 h, serum-free medium was added containing 1% bovine serum albumin for 8 h followed by the addition of recombinant rat GH (40 nM final concentration) or vehicle for 30 min. Cells were then harvested, and nuclear proteins were isolated (19). For reporter gene assays, cells on 6-well tissue culture dishes were co-transfected with mouse GH receptor (750 ng) and wild type Stat5b (750 ng) and the promoter-reporter plasmids indicated in Figs. 4 and 5 (750 ng). Cells were incubated for 24 h followed by the addition of serum-free medium containing 1% bovine serum albumin and either rat GH or vehicle for 18 h. Cells were then harvested, and cell lysates were used for luciferase assays. All of the results were normalized for total cellular protein values.

**Animal Studies—**Male Sprague-Dawley rats, hypophysectomized by a transauricular route at age 7 weeks, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed at the OHUS Animal Care Facility on a 12-h light/dark schedule with free access to food and water and received care according to National Institutes of Health guidelines. All of the procedures were approved by the OHUS Animal Care and Use Committee. Glucocorticoids (cortisone phosphate, 400 μg/kg/day) and thyroxine (10 μg/kg/day) were replaced by daily subcutaneous injection, and GH deficiency was confirmed by failure to grow during a 2-week observation period. Following this interval, rats were injected intraperitoneally with either vehicle (saline) or 1.5 μg/g recombinant rat GH. At intervals, rats were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and sacrificed. Liver proteins were isolated as described previously (19) as well as RNA and DNA outlined below.

**DNA-Protein Binding Studies—**Electrophoretic mobility shift assays were performed as described previously (15, 19) with 10 μg of COS-7 or rat hepatic nuclear proteins and 5′-fluorescein-labeled double-stranded oligonucleotides from rat GHRE-1 (top strand, 5′-GGGCTTCTCCTGGAGAAAG-3′), GHRE-2 (top strand, 5′-TCTCTCTCTTCTGAGGGAACGAGGAGAAGA-3′), or with a binding site for Spi1 (top strand, 5′-ATTCCGATCGGGGCGGAGGAC-3′). After incubation of proteins and DNA for 30 min at 4°C, products were separated by electrophoresis through non-denaturing 4%–12% polyacrylamide gels in 0.5× TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 8.3) at 120 V for 2 h at 20°C. Results were detected using a Molecular Imager FX and Quantity One software (Bio-Rad). Antibody supershift and competition experiments were performed as described previously (19). The top strand of competitor oligonucleotides is as follows: Spi1 Stat5 binding sites, 5′-ACGCTCTATAATCTTTAGTTTGCCGATGAGAGG-3′.

**RNA Analysis—**Hepatic nuclear RNA was isolated as described previously (12). RNA concentration was determined spectrophotometrically at 260 nm, and its quality was assessed by agarose gel electrophoresis. Nuclear RNA (5 μg) was reverse-transcribed with random hexamers in a final volume of 20 μl using a RT-PCR kit (Invitrogen). PCR reactions were then performed with 0.5 μl of cDNA (15). Primer sequences are listed in Table I. The linear range of product amplification was established in pilot studies for each primer pair, and the cycle number that reflected the approximate midpoint was used in final experiments. This varied from 20 to 28 cycles. Results were quantified by densitometry after electrophoresis through 1.5% agarose gels.

**Chromatin Immunoprecipitation Assays (ChIP)—**Initial steps were modified from published protocols (20). For each time point, a 200-ng fragment of rat liver was minced and incubated at 20°C in 10 ml of Dulbecco’s modified Eagle’s medium plus 1% formaldehyde on a rotating platform for 15 min followed by the addition of 1.5 ml of 1 X glycine and incubation for an additional 5 min. After centrifugation at 200 × g for 5 min at 20°C, the pellet was suspended in 1 ml of phosphate-buffered saline, transferred to a 2-ml Dounce apparatus, and homogenized with 10 strokes of a tight-fitting pestle. After brief centrifugation in a microcentrifuge, the pellet was suspended in 400 μl of lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 1% SDS, pH 8.1, plus protease inhibitors) and incubated for 15 min at 4°C. Each sample was sonicated at 4°C using a total of 5 pulses for 15 s each of a Branson Micro-tip sonicator at setting 5 interspersed with 30 s incubations on ice. After centrifugation at 14,000 × g for 10 min at 4°C in a microcentrifuge, the supernatant was diluted 10-fold with immunoprecipitation buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, pH 8.1, plus protease inhibitors) and 1 ml aliquots were used for immunoprecipitation as described.
previously (20) with 3 μl of the Stat5 C17X antibody and 45 μl of a 50% slurry of protein A-agarose beads. DNA was extracted from the immunoprecipitates using the Qiaquick PCR DNA purification kit and a protocol from the supplier and was suspended in 50 μl of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. PCR reactions were performed with 1 μl of DNA using the primer pairs listed in Table II. The linear range of product amplification was established for each primer pair in pilot studies, and the cycle number that reflected the approximate midpoint was used in final experiments. This varied from 28 to 30 cycles. Results were visualized after electrophoresis through 1.5% agarose gels.

RESULTS

Identification of a GH-regulated Stat5 Binding Site within the Rat IGF-I Gene—In past studies, we mapped a GH-stimulated DNase I-hypersensitive site termed HS7 to the second intron of the rat IGF-I gene and demonstrated that alterations in this chromosomal region coincided with GH-stimulated in vivo induction of IGF-I gene transcription (12). More recently, we found that Stat5b was required for acute activation of IGF-I gene transcription by GH (15). The latter observations prompted the analysis of the IGF-I gene including HS7 for potential Stat5 binding sequences. A map of the first three exons of the rat IGF-I gene is illustrated in Fig. 1 and includes tandem promoters P1 and P2, their leader exons 1 and 2, respectively, and exon 3, which encodes the NH2-terminal half of the mature IGF-I peptide (14). HS7 is located approximately midway between exons 2 and 3 as indicated. The DNA sequence illustrated below the map represents part of the 3′ end of HS7. It contains two putative Stat5 binding sites labeled GHRE-1 and GHRE-2, respectively, which both conform to the consensus of 5′-TTCNNNGAA-3′ (21). Fig. 1B demonstrates that this 84-bp region of the rat IGF-I gene is fairly well conserved in two other mammalian species and that GHRE-1 is preserved as a potential Stat binding site among rat, mouse, and human IGF-I genes. Identical nucleotides between two species are shaded in gray and among all three species are shaded in black. GHRE-1 and GHRE-2 are boxed.

Chromatin immunoprecipitation experiments were performed to determine whether the IGF-I HS7 region was capable of binding Stat5 in vivo in a GH-dependent way. As shown in Fig. 2A, in liver chromatin from GH-deficient rats, little Stat5 could be detected in association with HS7. Within 15 min of systemic hormone injection, Stat5 was found in chromatin at the HS7 site and this protein-DNA interaction persisted for the...
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Fig. 3. Assessing binding of Stat 5b to GHRE-1 and GHRE-2. A, results of gel-mobility shift assays using double-stranded oligonucleotides for either GHRE-1 or GHRE-2 and nuclear protein extracts from COS-7 cells transfected with expression plasmids encoding the mouse GH receptor and either wild type (WT) or constitutively active NHE-terminal FLAG-tagged rat Stat5b and incubated with rat GH (40 nM) or vehicle for 30 min. In cells transfected with wild type Stat5b, a protein-DNA complex is seen only after GH treatment (lane 2 versus lane 1 and lane 6 versus lane 5, arrow), whereas in cells expressing constitutively active Stat5b, a complex is observed in the absence of hormone (lanes 3 and 7, arrow). Lanes 4 and 8 demonstrate that an antibody to the FLAG epitope causes a supershift of each DNA-protein complex (upper arrow). A thin arrow denotes the location of free probe (FP). B, inducible binding after in vivo GH treatment of rat hepatic nuclear protein extracts to a double-stranded oligonucleotide for GHRE-1 as assessed by gel-mobility shift experiment (lanes 1–4). Lanes 5–8 show that binding of the same nuclear protein extracts to a double-stranded oligonucleotide for Sp1-1 is relatively constant after GH. The locations of FP and each gel shift are indicated by arrows. C, specific binding of rat hepatic nuclear protein extracts to oligonucleotides for GHRE-1 and GHRE-2 as assessed by gel-mobility shift experiment (lanes 1–4). Lanes 5–8 show that binding of the same nuclear protein extracts to a double-stranded oligonucleotide for Sp1-1 is relatively constant after GH. The locations of FP and each gel shift are indicated by arrows.
**control of IGF-I gene transcription by GH through Stat5**

**a**

![Graph A](image1)

**b**

![Graph B](image2)

**Figure 5.** GHRE-1 and GHRE-2 mediate GH-stimulated and Stat5b-regulated gene transcription. Results of luciferase assays in COS-7 cells transiently transfected with expression plasmids encoding the mouse GH receptor and wild-type rat Stat5b and incubated with rat GH (40 ng) or vehicle for 18 h. A, luciferase reporter genes were included that contained the minimal thymidine kinase (TK) promoter alone or with either 932 or 84 bp of DNA from the HS7 region of the rat IGF-I gene (thin lines). GHRE-1 and GHRE-2 are indicated by shaded circles. B, luciferase reporter genes were used that contained the minimal TK promoter alone or with 84 bp of DNA from HS7 encoding either wild type or mutant versions of GHRE-1 and GHRE-2. Mutant sequences are indicated by an X (for GHRE-1, TTCCTGGAA was changed to TCTCTGGTA, and for GHRE-2, TTCCTAGAA was changed to GTCTTAGTA). For both A and B, the graphs summarize the results of 3–5 independent experiments, each performed in duplicate (*, p < 0.01; **, p < 0.02 versus no GH or no Stat5b). Luciferase values for the TK promoter ranged from 4,000 to 8,000 relative light units/10 s.

probes, whereas constitutively active Stat5b was able to bind in the absence of hormone. Antibody supershift experiments confirmed that Stat5b interacted with each oligonucleotide.

We next evaluated the binding of endogenous hepatic Stat5 to the putative GHREs in HS7. Shown in Fig. 3B are results using labeled GHRE-1 and nuclear protein extracts from GH-deficient rats acutely treated with hormone. Inducible binding was observed beginning within 15 min after systemic GH injection and reached a maximum by 30–60 min. Under the conditions used in these experiments, no binding to a GHRE-2 oligonucleotide could be detected (data not shown), implying that it is a lower affinity site compared with GHRE-1. As a control, gel-mobility shift assays were performed with a double-stranded oligonucleotide that recognizes members of the Sp1 family of transcription factors (24) that are not regulated by GH. As expected, constant protein-DNA interactions were observed over the same time course, indicating that both the quantity and quality of nuclear protein extracts were similar at each time point after hormone treatment.

The relative affinity of GHRE-1 for hepatic Stat5 was assessed using liver nuclear proteins isolated at 60 min after injection of GH and a variety of unlabeled competitor oligonucleotides. As shown in Fig. 3C, GHRE-1 competed avidly with itself for nuclear protein binding, whereas GHRE-2 competed poorly, even at a ×100 molar concentration. The contiguous Stat5 binding sites from the Spi 2.1 gene promoter competed with GHRE-1 but only at ×100 concentration, and the unrelated binding site for the Oct-1 transcription factor (24) was ineffective. Taken together, the results in Fig. 3 show that GHRE-1 and GHRE-2 are bona fide Stat5 binding elements.

**Discussion**

A long-standing challenge in the field of growth biology has been to understand the biochemical mechanisms by which GH activates IGF-I gene expression. Here we identify binding sites for the transcription factor Stat5 in a region within the second intron of the rat IGF-I gene previously shown to undergo a GH-stimulated chromatin transition coincident with hormone-induced IGF-I transcription (12). We show that this segment of the IGF-I gene binds Stat5 in vivo in a GH-dependent manner that is sufficient to mediate hormone-regulated and Stat5b-dependent reporter gene expression in the context of both the
major IGF-I gene promoter and a minimal thymidine kinase promoter. Taken together, these results show that Stat5b is a key protein responsible for the induction of IGF-I gene transcription by GH and define the molecular architecture of this transcriptional response.

Previous gene knock-out studies have established a role for Stat5b and to a lesser extent Stat5a in the physiological pathways responsible for normal somatic growth in rodents (16, 17). In the absence of these proteins, mice developed up to a 30% deficit in postnatal linear growth that was associated with an ~50% decline in serum levels of IGF-I (16, 17). More recently, we found that forced expression of a dominant-negative version of Stat5b in GH-deficient rats blocked the acute activation of IGF-I transcription by GH, whereas constitutively active Stat5b stimulated IGF-I gene expression in the absence of hormone (15). Both groups of observations implicated Stat5b in IGF-I gene regulation but did not identify specific biochemical mechanisms, a question that we now have addressed.

Members of the Stat family of transcription factors bind as dimers to DNA response elements consisting of variants of the palindromic nucleotide sequence 5'-TTCN 3' where N signifies any deoxyribonucleotide (21). Ehret et al. (25) recently showed that Stats 1 and 5 preferentially bind to sites where a functional Stat5 binding sequence. We rule out this possibility because forced expression of a dominant-negative version of Stat5b in GH-deficient rats blocked the acute activation of IGF-I transcription by GH, whereas constitutively active Stat5b stimulated IGF-I gene expression in the absence of hormone (15). Both groups of observations implicated Stat5b in IGF-I gene regulation but did not identify specific biochemical mechanisms, a question that we now have addressed.

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GH and IGF-I play central roles in human physiology. They are essential for normal growth during fetal and postnatal development and are critical for tissue maintenance and repair during aging (2–5, 7, 8). In contrast, normal levels of both proteins have been found to have potential pathogenic consequences, being linked by epidemiological studies to several cancers (32). Thus, a full understanding of the molecular mechanisms by which GH regulates IGF-I gene expression will be necessary for developing effective therapeutic strategies to use these potent agents safely (33).