Mapping of a Cytoplasmic Domain of the Human Growth Hormone Receptor That Regulates Rates of Inactivation of Jak2 and Stat Proteins*

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It has been previously demonstrated that growth hormone (GH)-stimulated tyrosine phosphorylation of Jak2 and Stat5a and Stat5b occurs in FDP-C1 cells expressing either the entire GH receptor or truncations of the cytoplasmic domain expressing only the membrane-proximal 80 amino acids. However, other receptor domains that might modulate rates of GH activation and inactivation of this cascade have not been examined. Here we have defined a region in the human GH receptor between amino acids 520 and 540 in the cytoplasmic domain that is required for attenuation of GH-activated Jak/Stat signaling. Immunoprecipitations with antibodies to Jak2 indicate that the protein tyrosine phosphatase SHP-1 is associated with this kinase in cells exposed to GH. To address the possibility that SHP-1 could function as a negative regulator of GH signaling, liver extracts from motheaten mice deficient in SHP-1 or unaffected littermates were analyzed for activation of Stats and Jak2. Extracts from motheaten mice displayed prolonged activation of the Stat proteins as measured by their ability to interact with DNA and prolonged tyrosine phosphorylation of Jak2. These results delineate a novel domain in the GH receptor that regulates the inactivation of the Jak/Stat pathway and appears to be modulated by SHP-1.

Growth hormone (GH) exerts its pleiotropic actions on a variety of tissues including fat, bone, soft tissue, and liver. One of the earliest events that occurs after GH binds to its cell surface receptor is the tyrosine phosphorylation of several cellular proteins, including the SH2 domain-containing transcription factors termed signal transducers and activators of transcription or Stats (1–4). Tyrosine phosphorylated Stat proteins bind enhancers that are present in genes whose transcription is rapidly induced by the treatment of cells with GH and other cytokines. One of these enhancers is the gamma response region (GRR) present in the promoter of the FcγRI receptor gene. This enhancer, which is required for IFN-γ-activated transcription of the FcγRI receptor gene, has a sequence similar to those of enhancers that are required for the activation of cellular genes by a variety of other cytokines. GRR binding activity can be measured in many cells in response to growth hormone treatment, and it serves as an assay for the tyrosine phosphorylation of Stat proteins (1, 5). Most of the cytokine receptors interact with members of the Jak family of tyrosine kinases, and Jak activation closely parallels and in many cases is required for Stat protein phosphorylation on tyrosine. Tyrosine phosphorylated Jak2 has been shown to associate with the GHR after the addition of ligand, which allows Stat1, Stat3, Stat5a, and Stat5b to also be phosphorylated (6).

The receptors for growth hormone and other members of this cytokine receptor superfamily have several conserved features including cysteine residues within their extracellular domains and two intracellular subdomains (termed box 1 and box 2) adjacent to the transmembrane region. To elucidate the domains in the GH receptor required for activation of Stat(s) and Jak2, cell lines containing deletions in the cytoplasmic domain of the human receptor have been analyzed for GH-stimulated tyrosine phosphorylation of Jak2 and GRR binding activity. These studies demonstrated the importance of box 1 and box 2 in GH activation of Jak2 kinase and the Stat transcription factors (5, 7–11). However, little if any information has been reported concerning the role of other domains within the cytoplasmic region of the receptor in the modulation of GH activation of the Jak/Stat pathway. It has been shown that the SH2 domain-containing PTP SHP-1 (PTP1C, SHPTP1, and HCP) plays a role in the dephosphorylation of Jak2 after erythropoietin (EPO) stimulation and functions to down-regulate the proliferative effects of both EPO and IL-3, activators of Jak2 (12–14). In the case of EPO activation of Jak2, SHP-1 is recruited through its SH2 domain to the receptor as a consequence of the tyrosine phosphorylation of the later (13). Several reports have also implicated a role for tyrosine phosphatases in IFN regulation of the Jak/Stat pathway, including the role of SHP-1 as a negative regulator of IFN signaling and PTPID (SHP-2) as a positive activator of both interferon and prolactin stimulation of the Jak/Stat pathway (14–16). These results suggested that it would be worthwhile to examine whether other components modulate GH stimulation of the Jak/Stat pathway.

MATERIALS AND METHODS

Cells—The FDC-P1 cell line was transfected with cDNAs of the human growth hormone receptor and cytoplasmic truncations thereof (17). Cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum, 50 μg 2-mercaptoethanol, 50 μg/ml gentamicin, 700 μg/ml G418, and 5 μM human growth hormone (17). Cells were starved overnight in the absence of GH and then incubated for 1–2 h in fresh medium minus serum prior to being treated with 10 μM GH for the times indicated.

Whole Cell Extracts—Cells (5 × 10⁷) were collected by centrifugation, washed with phosphate-buffered saline, and resuspended in ice cold

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† The abbreviations used are: GH, growth hormone; GHR, GH receptor; IFN, interferon; GRR, IFNγ response element; FcγRI, IFNγ-induced gene; EMSA, electrophoretic mobility shift assay; PTP, protein tyrosine phosphatase; EPO, erythropoietin; mel/me, motheaten mice; GHSF, GH-stimulated factor.
Supernatant was transferred to a new tube. The GRR (gamma response region) (5'-GAGATGTATTTCCCAGAAAAG-3') of the GH receptor (see Fig. 1) were analyzed for GH-induced activation of Stat proteins by their ability to bind to the GRR of the GH receptor and box 1 and 2 are shown at the top. The lower diagram indicates the position of the carboxyl-terminal stop codon (the mutated GHR COOH-residue plus one) expressed in each truncation used in these experiments. The positions of the tyrosines (denoted by Y) are also shown within the cytoplasmic domain of the receptor.

Experiments were initiated to examine whether regions of the cytoplasmic domain of the GH receptor other than the previously described box1-box2 Jak2-binding domain might be involved in the modulation of the GH-stimulated Jak/Stat signaling pathway. As an initial screen, lysates of FDC-P1 cells were incubated with anti-Jak2 antiserum (Upstate Biotechnology Inc.) and developed using ECL (Amersham Corp.) (1). In experiments that contained only the amino-terminal 461 residues (S462stop or D351stop, respectively), no loss of the GH-induced binding activity (labeled GHSF) was observed after 4 h (lane 5). A 4-h time point was not examined. The GHR truncations of the cell lines, as diagrammed in Fig. 1, and the time points are indicated above each panel. The GHSF complex induced by GH treatment of the cell lines contains Stat5, as analyzed by supershifts. C shows a separate set of experiments in which the amount of GHSF (growth hormone-stimulated factor) in cells expressing the full-length receptor, D351stop, and the tyrosine to phenylalanine substitutions Y469F, Y516F, or the Y469F/Y516F double mutation was analyzed on the PhosphorImager. The level of GHSF seen after treatment of cells with GH for 15 min was given an arbitrary value of 100.

RESULTS

Experiments were initiated to examine whether regions of the cytoplasmic domain of the GH receptor other than the previously described box1-box2 Jak2-binding domain might be involved in the modulation of the GH-stimulated Jak/Stat signaling pathway. As an initial screen, lysates of FDC-P1 cells that express either full-length or carboxyl-terminal truncations of the GH receptor (see Fig. 1) were analyzed for GH-induced activation of Stat proteins by their ability to bind to the GRR of the high affinity FcγR1 receptor. Several of these lines have been previously characterized and were found to express approximately equal numbers of GH receptors (17). We determined that Stat5a and Stat5b are the only known Stats to become tyrosine phosphorylated in these cell lines as a result of activation of the receptor containing no tyrosines, SHP-1 has two SH-2 domains.

Experiments were initiated to examine whether regions of the cytoplasmic domain of the GH receptor other than the previously described box1-box2 Jak2-binding domain might be involved in the modulation of the GH-stimulated Jak/Stat signaling pathway. As an initial screen, lysates of FDC-P1 cells that express either full-length or carboxyl-terminal truncations of the GH receptor (see Fig. 1) were analyzed for GH-induced activation of Stat proteins by their ability to bind to the GRR. Cell lines that contained only the amino-terminal 461 or 350 residues (S462stop or D351stop, respectively), no loss of the GH-induced Stat complex was observed after 1 h in the absence of GH, and much of the activated Stat was still present after 4 h (Fig. 2A, lanes 5 and 6). To further map the region in the cytoplasmic domain of the GHR responsible for this down-regulation of the Jak/Stat signaling cascade, a series of carboxyl-terminal truncations of the receptor were generated between amino acids 462 and 540, where the change in the rate of decay of the activated Stats occurred (Fig. 2B). Cell lines that expressed 520 amino acids or less of the GHR all showed delayed rates of attenuation of the GHSF, suggesting that the region between amino acids 520 and 539 mediates this function. Although this particular domain of the receptor contains no tyrosines, SHP-1 has two SH-2 do-
phosphatase SHP-1 as a negative regulator of IFN

10-min incubation of cells with GH stimulated the tyrosine
tated and examined on blots by probing with antiphosphoty-
Cellular extracts were prepared, and Jak2 was immunoprecipi-
with GH for 10 min and washed in medium as described above.
leled different rates of inactivation of Jak2, cells were treated
the differential rates of inactivation of the Stat proteins paral-
rylation of the Stat proteins (1–4). To determine whether
activation of the Stat proteins containing the GHSF complex.

The Jak2 tyrosine kinase is activated by tyrosine phospho-
alylation as a result of treatment of cells with GH, and activa-
tion of this kinase is linked to GH-stimulated tyrosine phos-
phorylation of the Stat proteins (1–4). To determine whether
the differential rates of inactivation of the Stat proteins paral-
led different rates of inactivation of Jak2, cells were treated
with GH for 10 min and washed in medium as described above.
Cellular extracts were prepared, and Jak2 was immunoprecipi-
tated and examined on blots by probing with antiphospho-
binaries that have been implicated in binding to a phosphory-
ated tyrosine in the EPO receptor (13). We decided to mutate
the two tyrosines located at amino acids 469 and 516 to ensure
that these residues were not involved in altering the half-life of
activation of the GH signal. A PhosphorImager was used to
determine the amount of GHFS Stat5-containing complex in
cell lines where these tyrosines were replaced with phenylala-
nines. The results of these experiments are shown in Fig. 2C.
Compared with the cell line that expresses 350 amino acids of
the GHR, the cell lines that either expressed the full-length
receptor or mutations of one or both tyrosines (Y469F, Y516F,
or Y469F/Y516F) all displayed similar rates of Stat inactiva-
tion. It therefore appears that tyrosine phosphorylation of the
receptor in the 462–540 region is not involved in the mecha-
nism for down-regulating the Stat-containing GHFS complex.

The Jak2 tyrosine kinase is activated by tyrosine phospho-
phorylation as a result of treatment of cells with GH, and activa-
tion of this kinase is linked to GH-stimulated tyrosine phos-
phorylation of the Stat proteins (1–4). To determine whether
the differential rates of inactivation of the Stat proteins paral-
led different rates of inactivation of Jak2, cells were treated
with GH for 10 min and washed in medium as described above.
Cellular extracts were prepared, and Jak2 was immunoprecipi-
tated and examined on blots by probing with antiphospho-
rosine antibodies (Fig. 3). In all of the cell lines examined, a
10-min incubation of cells with GH stimulated the tyrosine phos-
phorylation of Jak2 (Fig. 3A, compare lanes 1 and 2). After
removing GH, cells that expressed the full-length or amino-
terminal 539 amino acids of the receptor displayed rapid de-
phosphorylation of Jak2, which was complete within 30 min.
However, in cells expressing either the proximal 520 or 350
amino acids of the receptor, a delayed dephosphorylation of the
enzyme was observed. Reprobing the blots with Jak2 anti-
serum confirmed the presence of approximately equal amounts
of Jak2 protein in each sample. These results correlated with
the presence of the GH-induced Stat complex seen in Fig. 2 and
indicated that a region in the receptor between 521 and 540 is
required to inactivate GH stimulation of the Jak/Stat signaling
cascade.

Recent evidence has implicated the protein tyrosine phos-
phatase SHP-1 as a negative regulator of IFNα/β, EPO, and
IL-3 signaling by Jak1 or Jak2 (12–14). In the case of IFNα/β
activation of the Jak/Stat pathway, SHP-1 is constitutively
associated with the α subunit of the IFNα receptor and is
displaced from the signaling complex after the addition of IFNβ
(14). To determine whether SHP-1 might be responsible for
inactivation of the GH-stimulated Jak/Stat pathway, experi-
ments were performed to determine whether SHP-1 was asso-
ciated with the GHR. In co-immunoprecipitation experiments,
SHP-1 was often constitutively associated with the full-length
or truncated GHR and was lost after treatment of cells with
GH; however, this result was not consistent (data not shown).

To examine this interaction by an alternative approach, immu-
noprecipitations were performed to determine whether
SHP-1 associated with Jak2 because Jak2 is activated and
becomes associated with the GHR as a result of treatment
of cells with GH (6). Extracts made from GH-stimulated cells
were immunoprecipitated with Jak2 antiserum, and the result-
ing immunoblots were probed with either antiphosphotyrosine
(Fig. 4A) or SHP-1 antibodies (Fig. 4B). SHP-1 associated
with Jak2 after incubation of cells with GH at a time when the
kinase became tyrosine phosphorylated (Fig. 4, A and B, com-
pare lanes 1 and 3), suggesting that SHP-1 might function to
shut off signaling by dephosphorylating Jak2. Fig. 4C is a
reprobe of Fig. 4A with Jak2 antiserum to demonstrate that
approximately equal amounts of protein were present in each
sample.

Although the association/dissociation of SHP-1 with Jak2 was
demonstrated to be ligand-dependent, it was possible that the
changes in down-regulation of the signaling cascade that
were observed with the truncated receptors were not directly
correlated with the actions of SHP-1. To examine this issue in
greater detail, experiments were performed using mothetaten
mice (me/me). The me/me phenotype is a result of a mutation
in the SHP-1 gene such that this PTP is absent in these mice
(21, 22). The lack of expression of SHP-1 causes multiple he-
matopoietic abnormalities, including hyperplilation and
inappropriate activation of macrophages resulting in wide-
spread inflammation. Previous studies have shown that injec-
tion of rats with GH activates the Jak/Stat pathway in the liver
(4). To examine the role of SHP-1 in GH signaling, livers were
isolated from me/me mice and their unaffected littermates
after the mice were injected with either GH or saline. Cellular
extracts were prepared from a portion of the liver at the time
the animals were sacrificed (Fig. 5A, lanes 1, 2, 5, and 6). The
remaining tissue from animals injected with GH was incubated for varying times at 37 °C, and portions of the liver were extracted for analysis of activated Stats by EMSA. GH-stimulated Stat activation was assayed by EMSA in equivalent protein loadings and was found to be approximately 1.5-fold greater in livers isolated from me/me mice compared with WT mice. We have shown here that SHP-1 is associated with the GHR in the absence of treatment with GH and other cytokines such as EGF. We have also shown that SHP-1 is transiently associated with the GHR and then returns at later time points (14). In contrast to the effects of SHP-1 in the IFN signaling cascade, the GHR appears to be dependent upon tyrosine phosphorylation of the receptor (13). We have shown here that SHP-1 is transiently associated with the GHR and then returns at later time points (14). In contrast to the effects of SHP-1 in the IFN signaling cascade, the GHR appears to be dependent upon tyrosine phosphorylation of the receptor (13). We have shown here that SHP-1 is transiently associated with the GHR and then returns at later time points (14). In contrast to the effects of SHP-1 in the IFN signaling cascade, the GHR appears to be dependent upon tyrosine phosphorylation of the receptor (13).
lines that demonstrate prolonged tyrosine phosphorylation of the enzyme (see Fig. 3). Whether another PTP functions to control basal activation of GH-stimulated Jak/Stat activity is not clear. However, vanadate does activate the cascade in the absence of ligand in macrophages isolated from mice, suggesting that SHP-1 probably is not the only negative regulatory PTP in GH signaling (14). It is also clear that in livers from GH-treated mice as well as in all of the cell lines expressing the GHR, Jak2 is eventually dephosphorylated. This observation suggests that another PTP contributes to shutting off the system or can substitute for SHP-1 when it is not functional.

Although our results suggest a general model for SHP-1 regulation of GH signaling, questions still remain. The component(s) of the GHR/Jak2 complex that directly mediate association with SHP-1 as well as the molecular determinants of association (SH2-or non-SH2-mediated) remain to be defined. The region between 521 and 540 in the GHR which potentiates down-regulation of Jak2 activation by GH contains no tyrosine residues, and the two adjacent tyrosine residues at amino acids 469 and 516 appear to have no effect on down-regulation of signaling. It is therefore unlikely that tyrosine phosphorylation of the GHR is mediating this effect. However, it is possible that SHP-1 can interact directly or indirectly with the GHR at more than one site because signaling does eventually diminish in the truncated forms of the receptor. In fact, SHP-1 has been shown to associate with the GHR in the absence of ligand in lines expressing truncated forms of the receptor (data not shown). Alternatively, the carboxyl terminus of SHP-1, which has been implicated in its association with the insulin receptor (26), could be responsible. Studies using purified recombinant proteins should resolve this issue. Understanding the mechanisms by which SHP-1 is able to regulate cytokine signaling complexes is clearly of importance as its pivotal role in the regulation of cellular growth and differentiation becomes more and more evident.

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