c-Cbl Is a Negative Regulator of GH-Stimulated STAT5-Mediated Transcription

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We have previously demonstrated that cellular stimulation with GH results in the formation of a multiprotein signaling complex. One component of this multiprotein signaling complex is the adapter molecule c-Cbl. Here we have examined the role of c-Cbl in the mechanism of GH signal transduction. Forced expression of c-Cbl in NIH3T3 cells did not alter GH-stimulated Janus kinase 2 tyrosine phosphorylation nor GH-stimulated p44/42 MAPK activation and consequent Elk-1-mediated transcription. c-Cbl overexpression did, however, result in enhanced and prolonged GH-stimulated activation of phospho-Janus kinase 2 kinase. Forced expression of c-Cbl did not affect GH-stimulated STAT5 tyrosine phosphorylation, nuclear translocation, nor binding to DNA but markedly abrogated GH-stimulated STAT5-mediated transactivation. c-Cbl overexpression resulted in increased ubiquitination and proteosomal degradation of STAT5 and increased degradation of GH-stimulated tyrosine phosphorylated STAT5. Cellular pretreatment with the proteosomal inhibitor MG132 reversed the effect of c-Cbl overexpression with prolonged duration of GH-stimulated STAT5 tyrosine phosphorylation and restoration of STAT5-mediated transcription. Thus, c-Cbl is a negative regulator of GH-stimulated STAT5-mediated transcription by direction of STAT5 for proteosomal degradation.

THE c-Cbl PROTO-ONCOGENE was originally identified as a cellular homolog of v-Cbl oncogene that was cloned from Cas NS-1 murine leukemia virus (1). The c-Cbl gene product is a cytosolic protein that is expressed in a wide range of cell types, most abundantly in the testis, thymus, and in all hematopoietic cell lineages (2). It contains a highly conserved basic amino-terminal domain that contains a nuclear translocation signal, KKKTK, followed by a RING finger domain, a proline-rich domain that has been shown to function as a ligand for SH3 domain of many signaling molecules, a putative leucine zipper for dimerization and several potential tyrosine phosphorylation sites that could interact with many SH2 containing molecules. The role of c-Cbl in the signal transduction mechanism of various extracellular ligands has been actively investigated; mainly pertaining to its rapidity and prominently phosphorylated tyrosine residues, as well as the ability of c-Cbl to interact with many signal transduction molecules after phosphorylation by cellular stimuli (3–9). The first evidence to indicate that Cbl proteins regulate tyrosine kinase signaling came from genetic studies in Caenorhabditis elegans (10). Loss-of-function mutations in sli-1 (c-Cbl homolog in C. elegans) restored signaling for vulval induction and survival through a weakly active epidermal growth factor (EGF) receptor homolog (LET-23), whereas additional copies of sli-1 suppressed vulval induction. Following this report, it has recently been demonstrated that c-Cbl is a negative regulator of various signaling pathways initiated by cell surface receptor tyrosine kinases such as colony-stimulating factor 1, EGF, and platelet-derived growth factor (PDGF) (11–13). As an E3 ligase, one mechanism whereby c-Cbl regulates signaling of growth factors is to modulate the endocytotic internalization, ubiquitination and subsequent proteosomal destruction of the respective receptor molecules (11–13). Various studies have indicated that c-Cbl also negatively regulates nonreceptor tyrosine kinases such as Lyn, ZAP-70, Syk, Fyn, and Src (14–17) by enhancement of their ubiquitin-dependent degradation.

The GH receptor (GHR) is a member of the cytokine receptor superfamily that does not possess intrinsic tyrosine kinase activity (18). GH signal transduction has been postulated to be initiated by the binding of GH to the cell surface GHR, resulting in homodimerization of the receptor (18, 19). Dimerization of the GHR results in the association and activation of the associated Janus kinase 2 (JAK2). JAK2 is thought to subsequently tyrosine phosphorylate the GHR and molecules required to activate the various downstream signaling pathways (18, 20). The major groups of signaling molecules thus far identified to be activated by GH include: 1) other receptor (EGF receptor) (21) and nonreceptor (c-Src, c-Fyn (22), focal adhesion kinase (23)) kinases, although as in the case of the EGF receptor it may be used simply as an adapter protein; 2) members of the MAPK family including p44/42 MAPK (24–26), p38 MAPK (27) and JNK/stress-activated protein kinase (22) and the respective downstream pathways; 3) members of the insulin receptor substrate (IRS) group including IRS-1, 2, and 3, which may act as docking proteins for further activation of signaling molecules including phosphatidylinositol-3 kinase (PI3K) (28–32); 4) small Ras-like GTPases (33, 34); and 5) STAT family members including STATs 1, 3, 5a, and 5b (35–38), which constitute one major mechanism for transcriptional regulation by GH.
We have recently reported that GH stimulates the formation of a multiprotein signaling complex centered around CrkII and p130Cas (22). Other molecules contained in this complex include c-Src, c-Fyn, and focal adhesion kinase as well as c-Cbl, IRS-1, and Nck (22). We have subsequently analyzed the functional role of CrkII and determined that it specifically contributes to both the positive and negative modulation of the major pathways activated by GH (39). GH stimulates the tyrosine phosphorylation of c-Cbl and its association to the multiprotein signaling complex (22). However, the specific functional role of c-Cbl in the mechanism of GH signal transduction has not been defined. The expression of c-Cbl is ubiquitous and is expressed in multiple GH-responsive tissues such as adipose tissue (40), muscle (40), liver (41), and the mammary gland (42–44).

In this report, we have investigated the functional consequences of c-Cbl overexpression in NIH-3T3 cells on the ability of GH to activate its effector pathways. We demonstrate that c-Cbl overexpression is not involved in GH-stimulated JAK2 and p44/42 mitogen-activated protein activation and subsequent Elk-1-mediated transcription, despite enhancement of GH-stimulated PI3K activity. Interestingly, c-Cbl functions as a negative regulator of STAT5-mediated transcriptional activation. c-Cbl inhibition of GH-stimulated STAT5-mediated transcription is not mediated by interference with events leading up to DNA binding but rather is due to increased proteasomal degradation subsequent to ubiquitination of STAT5. Thus, we provide evidence for a novel mechanism to regulate STAT5-mediated transcription and therefore hormonal responsiveness of the cell.

**Materials and Methods**

**Materials**

Recombinant human GH (hGH) was a generous gift of Novo-Nordisk (Singapore). Sodium orthovanadate, phenylmethylsulfonyl fluoride (PMFS), and common chemicals and reagents were purchased from Sigma (St. Louis, MO). MG132 was purchased from Calbiochem (San Diego, CA). Polyclonal antiserum against JAK2 and monoclonal antiserum against phospho-STAT5a/b and phosphotyrosine (4G10) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antiserum against STAT5a/b and polyclonal antiserum against STAT5b and c-Cbl were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyvalent antiserum against ubiquitin was purchased from Novocastra Laboratories Ltd. (Newcastle Upon Tyne, UK). Phospho-Elk-1 (Ser383) polyclonal antibody, immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody and Elk-1 fusion protein were purchased from New England Biolabs, Inc. (Beverly, MA). Antibodies IgG conjugated to fluorescein isothiocyanate (FITC) and poly-deoxyinosinic-deoxycytidylic acid were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Secondary anti-IgG anti-mouse primary antibodies and the enhanced chemiluminescence (ECL) kit were kindly provided by Hisamaru Hirai (45). The empty vector, pUC-CAGGS and pUC-CAGGS containing c-Cbl cDNA (pUC-CAGGS-c-Cbl) used for transient transfection were kindly provided by Hisamaru Hirai (45). The fusion trans-activator plasmid pFA-Elk-1 and the reporter plasmid pFR-luciferase (Luc) were purchased from Stratagene (La Jolla, CA). The DNA 3’ labeling kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany), and Nck-associated protein-5 columns were purchased from Amersham Biosciences Inc. (Piscataway, NJ).

**Cell lines**

NIH3T3 cells stably transfected with pUC-CAGGS (designated NIH-3T3-vector) or pUC-CAGGS-c-Cbl (designated NIH-3T3-c-Cbl) were kindly provided by Hisamaru Hirai (45). The cells are maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin.

**Treatment of cells with hGH**

NIH3T3 cells were grown in medium containing 10% FCS for 48 h before complete serum deprivation for 15–18 h. Serum-deprived cells were treated with 50 nM hGH for the indicated time periods.

**[125I]-GH cell surface receptor binding assays**

NIH3T3 cells were grown in six-well plates with medium containing 10% FCS for 48 h before serum deprivation for 18 h. The medium was replaced with 2 ml of fresh cold serum-free medium and incubated at 4°C for 2 h. A 10-fold higher concentration of hGH was added to the first three wells and [125I]-labeled hGH (around 100,000 cpm) was added to all six wells. The plates were incubated at 4°C for another 4 h before washing twice with cold PBS. The cells were lysed in lysis buffer [0.1 M NaOH, 1% sodium dodecyl sulfate (SDS)] removed from the wells and counted using a γ-counter.

**Immunoprecipitation, SDS-PAGE, and Western blot analysis**

Cells were grown as described above and lysed in immunoprecipitation buffer [1% Triton X-100; 150 mM NaCl; 10 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 1% Nonidet P-40; 0.2 mM Na3VO4; 1 μg/ml protease inhibitor cocktail; and 0.1 mM PMSF]. Samples were then immunoprecipitated with polyclonal antibody against JAK2 (Upstate Biotechnology, Inc.) or STAT5b (Santa Cruz Biotechnology, Inc.). SDS-PAGE sample buffer (50 mM Tris, pH 6.8; 2% SDS; 2% β-mercaptoethanol and bromophenol blue) was added to each sample and the samples were boiled for 5 min. Samples were subjected to discontinuous SDS-PAGE with a 7.5% or 10% resolving gel and transferred to nitrocellulose membranes (Hybond C-extra) using standard electroblotting procedures. Membranes were blocked with 2% BSA overnight at 4°C and immunolabeled with monoclonal antibody against tyrosyl phosphorylated proteins (4G10) (1:10,000 dilution), phospho-specific STAT5a/b antibody (1:1,000 dilution) or polyclonal antibody against STAT5b (1:1,000 dilution), JAK2 (1:1,000 dilution) or ubiquitin (1:10,000 dilution) for 1 h at room temperature. Immunolabeling was detected by the ECL kit according to the manufacturer’s instructions. Blots were stripped and reprobed with the same antibodies used for their immunoprecipitation to ensure equal loading of the immunoprecipitated proteins. Blots were stripped by incubation for 30 min at 50°C in a solution containing 62.5 mM Tris-HCl, pH 6.7; 2% SDS; and 0.7% β-mercaptoethanol. Blots were then washed for 30 min with several changes of PBS, 0.1% Tween 20 at room temperature. Efficacy of stripping was determined by reexposure of the membranes to ECL. Thereafter, blots were reblotted and immunolabeled as described above.

**Confocal laser scanning microscopy**

At the end of the respective treatment period, cells were rinsed with ice-cold PBS, fixed in ice-cold 4% paraformaldehyde, permeabilized for 10 min with 0.1% Triton X-100, blocked in 2% BSA, and incubated with polyclonal antibodies against c-Cbl (1:100 dilution) or STAT5b (1:1,000 dilution) followed by antirabbit IgG conjugated with FITC. Labeled cells were visualized with a Carl Zeiss (Jena, Germany) Axiosplan microscope equipped with epifluorescence optics and a Bio-Rad Laboratories, Inc. (Hercules, CA) MRC1024 confocal laser system. Images were converted to the tagged-information-file format and processed with the Adobe Photoshop program.

**p44/42 MAPK assays**

p42/44 kinase assays were performed using the New England Biolabs, Inc. (Beverly, MA) assay kit according to the manufacturer’s in-
structions. Briefly, cells were serum deprived for 16 h, treated with 50 nm hGH, and lysed at 4 C in 1 ml of lysis buffer (20 mM Tris, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM glycerolphosphate, 1 mM Na₃VO₄, 0.1% PMSF, 1 mg/ml leupeptin) per sample. The lysates were centrifuged at 15,000 × g for 15 min at 4 C. The supernatant containing 200 μg protein per sample was incubated overnight at 4 C with 20 μl of immobilized phospho-specific p44/42 (Thr202/Tyr204) MAPK monoclonal antibody in a final volume of 20 μl in 1× lysis buffer. The mixture was incubated with gentle rocking overnight at 4 C. The pellets were washed twice with 500 μl of lysis buffer containing 0.1% PMSF and twice with 500 μl of kinase buffer (25 mM Tris, pH 7.5; 5 mM glycerolphosphate; 2 mM dithiothreitol; 0.1 mM Na₃VO₄; and 10 mM MgCl₂). The kinase reactions were performed in the presence of 2 μg of Elk-1 fusion protein and 200 μM ATP at 30 C for 30 min. Elk-1 phosphorylation was selectively detected by Western immunoblotting using a chemiluminescent detection system and a specific phospho-Elk1 (Ser383) antibody (1:1000 dilution).

PI3K assay

NIH3T3 cells were grown to 80% confluence, incubated for 15–18 h in serum-free medium, washed once in serum-free medium, and incubated with 50 nm hGH for the indicated time periods. Cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4; 0.25 mM sucrose; 1 mM EDTA; 0.1 mM sodium orthovanadate (Na₃VO₄); 0.1 mM diisopropyl fluorophosphate; 1% Triton X-100; 10 μg/ml protease inhibitor cocktail; and 1 mM PMFS) for 1 h at 4 C, scraped, and spun for 10 min at 4 C. The supernatants were collected and the proteins in the whole cell extracts were assayed using a Bio-Rad Laboratories, Inc. protein assay kit with BSA as standards. Eight hundred micrograms of each sample was immunoprecipitated overnight with 3 μg of polyclonal antibody against PI3K. Twenty-five milliliters of protein G plus/protein A agarose (Calbiochem) were added for 2 h and the agarose beads were washed twice with lysis buffer, twice with PBS and twice with assay buffer (40 mM Tris-HCl, pH 7.4; 5 mM MgCl₂; and 0.5 mM EGTA). A 50-μl assay mix (0.01 mg/ml phosphatidylserine, 0.2 mg/ml phosphatidylinositol, and 0.2 μM [γ³₂P]ATP) was added to each sample and incubated at 30 C for 10 min. The reaction was terminated by adding 200 μl of 1 M HCl and 400 μl MeOH:CHCl₃ (1:1). The organic phase was dried, redissolved in 20 μl MeOH:HCl:H₂O (5:95), spotted onto Silica Gel 60 plates (Merck, Darmstadt, Germany), and developed in MeOH:CHCl₃:H₂O:NH₄OH (35:45:5:2:8).

Densitometric analysis of band intensities

The intensities of the respective bands of proteins on the autoradiographs were quantified using a Bio-Rad Laboratories, Inc. GS-700 imaging densitometer and analyzed with the MultiAnalysis (version 1.0.1) program (Bio-Rad Laboratories, Inc.).

Oligonucleotides

Double-stranded serine protease inhibitor-GAS-like response element (SPI-GLE1), TGTTCTGAGAATA (46) was used as the probe in gel EMSA (GEMSA). The DNA fragment was labeled with [α-³²P] 2',3'-dideoxynucleosine 5'-phosphate using the DNA ³ labeling kit purchased from Roche Diagnostics GmbH. The labeled probe was purified through Nck-associated protein-5 column (Athersham Biosciences Inc.)

GEMSA

GEMSA was performed according to standard protocol. The binding reactions were performed by preincubating 10 μg of nuclear extracts of both GH treated and untreated NIH3T3-vector and NIH3T3-c-Cbl cells with 3 μg of poly-deoxyinosinic-deoxyctydilic acid (Roche) in 15 ml buffer containing 20% Ficoll; 60 mM HEPES, pH 7.9; 20 mM Tris, pH 7.9; 0.5 mM EDTA; and 5 mM dithiothreitol for 15 min on ice. For supershift analysis, the extracts were incubated with the antibodies recognizing STAT5B (1 μg), c-Cbl (1 μg), or control antibodies (1 μg) for another 10 min on ice. ³²P-3'-End-labeled double-stranded SPI-GLE1 probe (50 ng) was added, and the mixture was incubated for 5 min on ice followed by another 10 min at room temperature. The samples were electrophoresed on 4.5% nondenaturing polyacrylamide gels in 0.25X TBE buffer (22.5 mm Tris borate, pH 8.0; 0.5 mm EDTA) at 250 V at 4 C for 2 h. The gel was dried and visualized by autoradiography.

STAT5 reporter assay

NIH3T3 cells were cultured to 50% confluence in six-well plates. Transient transfection was performed in serum-free DMEM with Effectene according to the manufacturer’s instructions (QIAGEN GmbH), Cytomegalovirus-luciferase (CMV-LUC) (0.2 μg) of together with either 0.2 μg of reporter plasmid (SPI-GLE1-CAT) or 0.2 μg of reporter plasmid (SPI-GLE1-CAT) and 0.2 μg of pUC-CAGGS or pUC-CAGGS-c-Cbl were transfected per well in serum-free DMEM. Cells were incubated with Effectene/DNA for 12 h before. After a further 24 h, cells were washed in PBS and scrapped into lysis buffer (250 mM Tris-HCl, pH 8.0; 1 mM dithiothreitol). The protein contents of the samples were normalized and chloromphenicol acetyl transferase assays were performed. Results were normalized to the level of luciferase activity to control for transfection efficiency and calculated as the fold stimulation of unstimulated (non-hormone treated) cells.

Elk-1 reporter assay

NIH-3T3 cells were cultured to 60–80% confluence for transfection experiments in 6-well plates. Reporter plasmid pFR-Luc (1.0 μg) was transfected together with 0.02 μg of fusion trans-activator plasmid, pFA-Elk-1. Twenty-five microliters of Effectene for each microgram of DNA was used as per the manufacturer’s instructions. DNA-lipid complex was diluted to a final volume of 2 ml/well with serum-free medium, added to the cells and the cells were left in the 37 C incubator for 12–16 h. The media were then changed to fresh serum-free DMEM with or without 50 nm hGH for an additional 24 h. The cells were washed in PBS and lysed with 200 μl of 1× lysis buffer (25 mM Tris-phosphate, pH 7.8; 2 mM EDTA; 2 mM dithiothreitol; 10% glycerol; 1% Triton X-100) by a freeze-thaw cycle, and lysate was collected by centrifugation at 14,000 rpm for 15 min. The supernatant was used for the assay of luciferase activity. The luciferase activities were normalized on the basis of protein content well as on the β-galactosidase activity of pCMV-β-galactosidase vector.

Statistical analysis and presentation of data

All experiments were performed at least three times. Numerical data are expressed as mean ± S.D. Data were analyzed using the two-tailed t test or ANOVA.

Results

Characterization of c-Cbl overexpressing NIH3T3 cells

We have previously demonstrated that c-Cbl is tyrosyl-phosphorylated upon GH stimulation and is a component of a GH-stimulated multiprotein signaling complex (22). To determine the role of c-Cbl in the cellular effects of GH, we simply overexpressed c-Cbl in NIH3T3 cells by stable transfection with either pUC-CAGGS-c-Cbl (NIH3T3-c-Cbl) or with pUC-CAGGS vector alone (NIH3T3-vector). Confocal laser scanning microscopic analysis of c-Cbl expression in NIH3T3-c-Cbl and NIH3T3-vector cells clearly demonstrated overexpression of c-Cbl in NIH3T3-c-Cbl cells compared with vector-transfected control cells (Fig. 1A). c-Cbl was predominantly localized to the cytoplasm in both vector-transfected and c-Cbl-overexpressing NIH3T3 cells. Western blot analysis in Fig. 1B also confirmed the overexpression of c-Cbl in NIH3T3-c-Cbl cells. The level of c-Cbl expression in NIH3T3-c-Cbl cells was approximately 10 times that of vector-transfected control cells (NIH3T3-vector) as determined by densitometric analysis of the Western blot for c-Cbl (not shown).
Overexpression of c-Cbl does not down-regulate cell surface binding of hGH to the GHR

c-Cbl has been reported to be a negative regulator for many receptor tyrosine kinases such as the EGF receptor by facilitating their removal from the cell surface (14, 47–50). To determine if c-Cbl overexpression in NIH-3T3 cells affected cell surface GHR expression, we performed a cell surface [125I]-hGH binding assay to determine if both NIH3T3-c-Cbl and NIH3T3-vector cell lines possess comparable levels of GHR binding at the cell surface. The binding of [125I]-hGH to cell surface GHR in NIH3T3-c-Cbl cells was observed to be equivalent to that of NIH3T3-vector cells (Fig. 1C). Thus, overexpression of c-Cbl in NIH-3T3 cells does not alter the cell surface expression of the GHR, and this approach (overexpression of c-Cbl) can therefore be used to examine the role of c-Cbl in GH signal transduction.

c-Cbl overexpression does not affect the ability of GH to stimulate JAK2 phosphorylation

Phosphorylation and activation of JAK2 after GH binding to the receptor has been postulated to be required for subsequent transduction of the GH signal into the cell (18). We therefore first examined the effect of c-Cbl overexpression in NIH-3T3 cells on the ability of GH to stimulate tyrosine phosphorylation of JAK2. As is observed in Fig. 2, GH stimulated the tyrosine phosphorylation of JAK2 in vector transfected cells at both 5 and 15 min after exposure of the cells to GH. Overexpression of c-Cbl in NIH-3T3-c-Cbl cells did not significantly alter the ability of GH to stimulate the tyrosine phosphorylation of JAK2 (Fig. 2) with a slight but inconsistent increase in the tyrosine phosphorylation of JAK2 observed. Thus, c-Cbl overexpression does not significantly influence JAK2 stimulation by GH.

c-Cbl overexpression does not affect the activation of p44/42 MAPK

GH has been demonstrated in numerous studies to stimulate the activation of p44/42 MAPK in various cell systems (24, 33, 34, 51). We have also previously demonstrated GH-stimulated activation of p44/42 MAPK in NIH-3T3 cells used here in this study (39). We therefore examined the effect of c-Cbl overexpression on the ability of GH to stimulate p44/42 MAPK activity. As shown in Fig. 3A, GH rapidly activated p44/42 MAPK in NIH3T3-vector cells and also in NIH3T3-c-Cbl cells with similar maximal activation observed between the two cell lines. One of the mediators of the downstream cellular effects of p44/42 MAPK is transcriptional activation mediated by Elk-1 (52). We (27) and others (34) have previously demonstrated that activation of Elk-1-mediated transcription stimulated by GH is entirely mediated by p44/42 MAPK. By use of an Elk-responsive reporter construct we therefore demonstrated that both NIH3T3-vector and NIH3T3-c-Cbl cells exhibited similar activation of Elk1-mediated transcription in response to GH stimulation (Fig. 3B). Similarly, no effect of c-Cbl overexpression on GH-stimulated Elk-1-mediated transcription was observed upon transient transfection of the parental NIH3T3 cells with either vector or c-Cbl cDNA constructs (Fig. 3C). Thus, we conclude that c-Cbl overexpression does not alter the ability of GH to stimulate p44/42 MAPK activation and subsequent Elk-1-mediated transcription.
Materials and Methods

least three independently performed experiments. The data presented are representative of at least three independently performed experiments.

Cells were treated with 50 nM hGH and processed for immunoprecipitation with a polyclonal antibody recognizing JAK2 and subsequently membranes were then stripped and reblotted to demonstrate the equal loading of JAK2 protein. The data presented are representative of at least three independently performed experiments.

**c-Cbl overexpression enhances PI3K activation stimulated by GH**

C-Cbl has previously been reported to be associated with the p85 subunit of PI3K (53–57) and to be used by various cytokines for the activation of PI3K activity (56, 58). Furthermore, both c-Cbl and the p85 subunit of PI3K are present in the GH-dependent multiprotein signaling complex (22). We therefore examined the effect of c-Cbl overexpression on the ability of GH to stimulate PI3K activity. GH stimulation of NIH3T3-vector cells resulted in small but consistent increase in PI3K activity at 5 min and by 15 min after stimulation with GH the activity of PI3K returned to the basal level (Fig. 4). c-Cbl overexpressing cells exhibited a higher basal level of PI3K activity compared with NIH3T3-vector cells (Fig. 4, A and B) consistent with other published data on the effect of c-Cbl overexpression on PI3K activity (56). GH stimulation of NIH3T3-c-Cbl cells further stimulated activation of PI3K. Moreover, GH stimulation of NIH3T3-c-Cbl cells resulted in a dramatically prolonged and enhanced activation of PI3K activity at 15 min in comparison to the return to the basal level of PI3K activity observed in NIH3T3-c-Cbl cells. Thus c-Cbl is a positive regulator of GH-stimulated PI3K activity.

**c-Cbl overexpression in NIH3T3 cells does not affect the GH-stimulated tyrosine phosphorylation, nuclear translocation, nor DNA binding of STAT5**

The JAK-STAT pathway is one of the predominant pathways used by GH to mediate transcriptional activation (reviewed in Ref. 59). GH-stimulated JAK2 activation has previously been demonstrated to result in the tyrosine phosphorylation and transactivation of both STAT5 isoforms (38). Tyrosine-phosphorylation of STAT5 is required for activation of STAT5 and subsequent translocation into the nucleus for its participation in transcription. We therefore first examined the effect of c-Cbl overexpression on the ability of GH to stimulate the tyrosine phosphorylation of STAT5. To demonstrate the tyrosine phosphorylation status of STAT5, we used an antibody that recognized the phosphorylated form of both STAT5a and STAT5b since our STAT5 transactivation assay (see below) also utilizes both isoforms of STAT5. As observed in Fig. 5A, c-Cbl overexpression did not affect the ability of GH to stimulate the tyrosine-phosphorylation of STAT5. Because tyrosine phosphorylation of STAT5 does not necessarily result in nuclear translocation and subsequently binding to the DNA, we further examined the nuclear translocation of STAT5 by confocal laser scanning microscopy. We observed no difference in the ability of GH to stimulate nuclear translocation of STAT5 between NIH3T3-vector and NIH3T3-c-Cbl cells as shown in Fig. 5B. The majority of STAT5 was localized in the cytoplasm of both cell lines in the serum-deprived state and was subsequently translocated to the nucleus within 30 min after GH stimulation. Thus, the initial tyrosine phosphorylation and subsequent nuclear translocation of STAT5 as a result of cellular stimulation with GH is not affected by overexpression of c-Cbl.

We next examined whether c-Cbl overexpression affected the ability of GH activated STAT5 to bind to DNA via the γ-interferon-activated site-like element (GLE). We performed GEMSA using a GLE from the GH-regulated serine protease inhibitor gene 2.1 (SPI-GLE1) (46). No STAT5 binding to the GLE1 was observed in nuclear extracts from NIH3T3-vector cells in the basal state (Fig. 5C). After GH stimulation of NIH3T3-vector transfected cells, marked binding of STAT5 to the GLE1 could be observed, as previously reported for other cellular systems (60–62). Supershift analysis with a STAT5a/b specific antibody verified that the protein complex binding to SPI-GLE1 contained STAT5 (Fig. 5C). Overexpression of c-Cbl in NIH3T3-c-Cbl cells did not affect the ability of GH to stimulate binding of STAT5 to SPI-GLE1. Supershift analysis using antibody against c-Cbl also demonstrated that c-Cbl was not physically present in the STAT5-DNA binding complex. Thus, c-Cbl overexpression does not affect the ability of GH to stimulate the tyrosine phosphorylation, nuclear translocation or DNA binding of STAT5.

**c-Cbl overexpression inhibits GH-stimulated STAT5-mediated transcription**

STAT5 binding to the γ-interferon-activated site-like element in the promoter of the SPI 2.1 gene subsequently results in transcription (60–62). Concordantly, GH-stimulated STAT5-mediated transactivation via SPI-GLE1 in NIH3T3-vector cells (Fig. 6). Surprisingly, c-Cbl overexpression in NIH3T3-c-Cbl cells resulted in a dramatically reduced GH-stimulated STAT5-mediated transcription to between 5 and 30% of that seen in the NIH3T3-vector cells (Fig. 6A). To
exclude the possibility of clonal selection artifact giving rise to such a result, we therefore transiently transfected either the vector or c-Cbl cDNA into NIH-3T3 cells and examined the level of GH-stimulated STAT5-mediated transcription (Fig. 6B). We observed the same inhibition of GH-stimulated STAT5-mediated transcription in cells transiently transfected with c-Cbl cDNA as we observed for NIH3T3-c-Cbl cells stably transfected with c-Cbl cDNA (Fig. 6B). Thus overexpression of c-Cbl in NIH3T3 cells dramatically decreases STAT5-mediated transcription in response to GH. STAT5 is more heavily ubiquitinated in NIH3T3-c-Cbl compared with NIH3T3 cells.

From the above described experiments, we have observed that forced expression of c-Cbl abrogates GH-stimulated STAT5-mediated transcription even though the initial activation of STAT5 by GH was not hindered by c-Cbl overexpression. One possible explanation for this effect is that c-Cbl is functioning as an E3 ligase resulting in the ubiquitination and subsequent proteosomal degradation of STAT5. Thus,
enhanced degradation of STAT5 as a consequence of forced expression of c-Cbl would result in diminished STAT5-mediated transcription. We therefore first examined the ubiquitination status of STAT5a/b in both NIH3T3-vector and NIH3T3-c-Cbl cell lines. Immunoprecipitation using antibody against STAT5a/b and subsequent Western blot analysis with antire ubiquitin antisera demonstrated that STAT5 was somewhat more heavily ubiquitinated in NIH3T3-c-Cbl cells compared with NIH3T3-vector cells (Fig. 7). To demonstrate that ubiquitination of STAT5 would result in proteosomal degradation of STAT5, we used the proteosomal inhibitor MG132. Use of a proteosomal inhibitor would result in accumulation of the ubiquitinated protein destined for proteosomal degradation. Pretreatment with MG132 enhanced the level of ubiquitinated STAT5 in both cell lines, particularly in NIH3T3-c-Cbl cells, indicative that MG132 inhibited the degradation of ubiquitinated STAT5. Thus, forced expression of c-Cbl enhanced the ubiquitination and subsequent proteosomal degradation of STAT5.

Proteosomal inhibitor results in sustained presence of phosphorylated STAT5

We therefore examined if overexpression of c-Cbl would indeed result in increased degradation of STAT5 at extended times after GH stimulation. Both NIH3T3-vector and NIH3T3-c-Cbl cells were pretreated with the proteosome inhibitor, MG132, before stimulation with hGH for 2 and 6 h and compared with the respective cell line not treated with proteosome inhibitor (Fig. 8A). We reasoned that if the level of tyrosine-phosphorylated STAT5 (and hence STAT5 transactivation) is indeed declining due to enhanced c-Cbl-mediated ubiquitination and degradation, then pretreatment with MG132 would reverse this effect. As shown in Fig. 8A (left panel), the level of tyrosine-phosphorylated STAT5 after 2 or 6 h of hGH stimulation in NIH3T3-c-Cbl cells was significantly less compared with NIH3T3-vector cells. This suggested enhanced degradation of tyrosine phosphorylated STAT5 in NIH3T3-c-Cbl cells as a consequence of c-Cbl overexpression. One hour of pretreatment of both cell lines with 50 μM MG132 before hGH stimulation resulted in significantly higher levels of phosphorylated STAT5 in both cell lines (Fig. 8A, right panel) and a restoration of the level of tyrosine phosphorylated STAT5 in NIH3T3-c-Cbl cells to that observed in untreated NIH3T3-vector cells. Thus, inhibition of the proteosomal degradation of STAT5 resulted in higher sustained levels of phosphorylated STAT5 in the cell and countered the effect of c-Cbl overexpression on the ability of GH to stimulate sustained tyrosine phosphorylation of STAT5.

The proteosome inhibitor MG132 reversed c-Cbl inhibition of GH-stimulated STAT5-mediated transcription

To examine if the inhibition of GH-stimulated STAT5-mediated transcription as a consequence of c-Cbl overexpression was indeed due to proteosomal degradation, we therefore determined the effect of the proteosome inhibitor MG132 on GH-stimulated STAT5-mediated transcription. One hour pre-treatment of NIH3T3-vector cells with 50 μM MG132 before GH stimulation greatly enhanced GH-stimulated STAT5-mediated transcriptional activation in NIH3T3-vector cells (Fig. 9). Importantly, use of MG132 could reverse the inhibition of GH-stimulated STAT5-mediated transcription consequent to overexpression of c-Cbl. Indeed, MG132 treatment of NIH3T3-c-Cbl cells resulted in significantly higher GH-stimulated STAT5-mediated transcription than that observed in NIH3T3-vector cells without addition of MG132. Thus, inhibition of GH-stimulated STAT5-mediated tran-scription by c-Cbl could be reversed by proteosomal inhibition, indicating that enhanced c-Cbl overexpression results in increased ubiquitination and subsequent proteosomal degradation of STAT5, leading to decreased STAT5-mediated transcription.

![Fig. 4. Effect of c-Cbl overexpression on hGH-stimulated PI3K activity. A](https://example.com/fig4a.png) B](https://example.com/fig4b.png)
scription is due to enhanced proteosomal degradation of STAT5.

Discussion

The GHR has been found to be polyubiquitinated upon GH stimulation, and it occurs via the ubiquitin-dependent endocytosis motif of the GHR after dimerization of the receptor (63, 64). This ubiquitination of the GHR has been linked to the endocytic pathway in ligand-induced internalization and subsequent degradation of the receptor (63–65). Here we have demonstrated that c-Cbl overexpression does not affect the level of cell surface GHR expression. This is not
surprising since the GHR itself is a nontyrosine kinase receptor, does not possess a tyrosine kinase domain and therefore is not able to interact with the tyrosine kinase binding (TKB) domain of c-Cbl. If c-Cbl would regulate GH signaling pathways, one would expect the regulation by c-Cbl to be on the receptor associated JAK2 kinase. JAK2 has intrinsic tyrosine kinase activity (66) and has a TK domain that is able to interact with the TKB domain of c-Cbl (14, 67–71). However, we have demonstrated that the level of GH-stimulated tyrosine phosphorylated JAK2 is not altered by overexpression of c-Cbl. Furthermore, JAK2 activity is required for the further activation of p44/42 MAPK (27), and this is also not altered by c-Cbl overexpression in NIH3T3-c-Cbl cells. Studies on murine embryonic fibroblasts derived from c-Cbl-deficient mice also demonstrated that the level of activity of JAK1 and Tyk2 stimulated by interferon-γ appear to be regulated independently of c-Cbl (72). This is concordant with our observation here that GH-stimulated JAK2 tyrosine phosphorylation and subsequent activation of particular JAK2 downstream effector pathways are not affected by c-Cbl overexpression.

The evidence for a regulatory function of c-Cbl in receptor tyrosine kinase signal transduction came from genetic studies in C. elegans (10, 73). c-Cbl was later found to be able to down-regulate signaling pathways including those used by EGF, PDGF, and colony stimulating factor-1 by promoting multubiquitination of the respective receptor molecule and thus targeting these molecules for degradation (11–13). This negative regulation is dependent on the integrity of both the TKB and the RING finger domain of c-Cbl (70, 74). The c-Cbl RING finger has intrinsic E3 ligase activity and can independently recruit ubiquitin-conjugating enzymes and direct ubiquitin transfer to substrates (48, 75, 76). Many nonreceptor tyrosine kinases are also negatively regulated by c-Cbl. However, the mechanism of regulation is not as straightforward as those for receptor tyrosine kinases. Evidence has shown that c-Cbl overexpression down-regulated c-Fyn by targeting c-Fyn for degradation (17), but the protein levels of spleen tyrosine kinase (Syk) and 70-kDa ζ-associated protein (ZAP-70) are not altered although their activity is suppressed (14, 16). Moreover, the regulation of Src by c-Cbl is independent of the E3 ligase activity of c-Cbl.

Genetic studies in C. elegans indicate that Sli-1, a p120cbl homolog, plays a negative regulatory role in control of the Ras signaling pathway initiated by the C. elegans EGF receptor homolog (10). However, other studies in NIH3T3 cells overexpressing c-Cbl have shown that c-Cbl suppresses EGF stimulated activation of the JAK/STAT pathway but has no effect on the Ras pathway in EGF receptor signaling (45). Our data also support this observation in as much that overexpression of c-Cbl in NIH3T3 cells does not affect the activation of p44/42 MAPK by GH. These data partially contradict the results expected from the role of Sli-1 protein in the let-23-mediated vulval induction pathway (10). One possibility is that the EGF receptor-mediated signaling pathways are differently regulated by c-Cbl between nematodes and mammals. In mammalian cells, only the EGF receptor (EGFR) (product of oncogene of avian erythroblastosis virus) family kinases is able to bind c-Cbl (77), indicating that c-Cbl only regulates the kinase activity of EGFR receptor but not that of the other c-ErbB proteins. This redundancy may not exist in nematodes; therefore, Sli-1 can directly regulate the let-23-mediated vulval induction pathway (10).

We have demonstrated that overexpression of c-Cbl in NIH-3T3 cells resulted in enhanced GH stimulation of PI3K activity. This is concordant with previous studies which have demonstrated that c-Cbl is involved in the regulation of cell morphology and functional organization of the actin cytoskeleton (78–82). Cytoskeletal reorganization is generally PI3K dependent, including actin cytoskeletal reorganization stimulated by GH (83), and c-Cbl has been demonstrated to

A

B

![Fig. 6. Effect of c-Cbl overexpression on hGH-stimulated STAT5-mediated transcription. A, Effect of GH on STAT5 (Spi2.1-GLE1-CAT)-mediated transcription in NIH3T3-c-Cbl and NIH3T3-vector cells. NIH3T3 cells were grown to 60–80% confluence and transiently transfected with Spi2.1-GLE1-CAT. Cells were treated with 50 nM hGH and processed for CAT assay as described in Materials and Methods. The CAT activities were normalized on the basis of protein contents well as on the luciferase activity of pCMV-LUC vector. The data are presented as the mean ± SD of triplicate determinations. The data presented are from at least three independently performed experiments. B, Effect of c-Cbl overexpression on hGH-stimulated STAT5 (Spi2.1-GLE1-CAT)-mediated transcription. NIH3T3-vector cells were grown to 60–80% confluence and transiently transfected with pUC-GAGGS and Spi2.1-GLE1-CAT or pUC-GAGGS-c-Cbl and Spi2.1-GLE1-CAT. Cells were treated with 50 nM hGH and processed for CAT assay as described in Materials and Methods. The CAT activities were normalized on the basis of protein contents well as on the luciferase activity of pCMV-LUC vector. The data are presented as the mean ± SD of triplicate determinations.](https://academic.oup.com/endo/article-abstract/143/9/3590/2880985/3598-Endocrinology-September-2002-143-9-3590)
possess PI3K interacting regions. c-Cbl is able to associate with both the SH2 and SH3 domains of p85 (53–55, 57). Separate studies have identified a specific tyrosine residue on c-Cbl (tyrosine 731) that is essential for direct interaction with the SH2 domain of p85 (56, 84), and this interaction has been demonstrated to be required for c-Cbl-mediated PI3K activation (54, 56, 58). Members of the cytokine receptor superfamily such as the IL-4 receptor and the prolactin receptor activate the PI3K pathway through stimulation of the tyrosine phosphorylation of c-Cbl and its association with the p85 subunit of PI3K (54, 58). We have also demonstrated here that c-Cbl overexpression enhances GH-stimulated activation of PI3K activity and GH may therefore use a similar c-Cbl-dependent mechanism for activation of PI3K as reported for other cytokine receptor superfamily members (54, 56, 58, 69). We have also previously demonstrated that GH stimulates actin cytoskeletal reorganization and this reorganization is PI3K dependent (83). We have also observed that c-Cbl overexpressing cells exhibit altered actin cytoskeletal morphology compared with NIH3T3-vector 3T3 cells (Goh, E. L. K., and P. E. Lobie, unpublished observations) consistent with the higher basal and GH-stimulated levels of PI3K activity in c-Cbl overexpressing cells.

We have demonstrated here that both the initial activation of STAT5 and the ability of STAT5 to bind to its DNA-responsive element are not affected by the overexpression of c-Cbl. However, GH-dependent STAT5-mediated transcriptional activation is diminished by c-Cbl overexpression which we demonstrated was due to enhanced proteosomal degradation of STAT5 in NIH3T3-c-Cbl cells. Separate studies have shown that the proteosome inhibitors, MG132 and lactacystin, affected STAT4, STAT5, and STAT6 turnover but not that of STAT1, STAT2, and STAT3 by significantly stabilizing the tyrosine phosphorylated form (85). Recent studies have also demonstrated that murine embryonic fibroblasts derived from c-Cbl-deficient mice exhibit significantly increased levels of STAT1 and STAT5 protein (72). The increased levels of STAT1 in c-Cbl-deficient mice was not due to decreased degradation but rather due to increased synthesis (72). As such, the proteosomal inhibitor MG132 had no effect on the level of STAT1 in the c-Cbl replete or c-Cbl-deficient cells. This is apparently not the case with STAT5 as equal levels of STAT5 were observed here in the unstimulated state and also STAT5 was equipotently activated by GH in the vector and c-Cbl transected cells. In contrast, STAT2 and STAT3 expression and activation appear to be regulated independently of c-Cbl (72). Further, we could prevent the c-Cbl enhanced degradation of tyrosine phosphorylated STAT5 and inhibition of GH-stimulated STAT5-mediated transcription by use of the proteosomal inhibitor MG132. Thus c-Cbl regulates STAT5 levels, and therefore activity, by promotion of its degradation. We have also demonstrated in this study that STAT5 is more heavily ubiquitinated in the cell line overexpressing c-Cbl and marked accumulation of ubiquitinated STAT5 is observed in c-Cbl overexpressing cells treated with MG132. To our knowledge, this is the first demonstration that a transcription factor (level and activity) is being directly regulated by c-Cbl through ubiquitination and proteosomal degradation. c-Cbl mediated mult ubiquitination of STAT5 would therefore result in degradation and subsequent diminished STAT5-mediated transcriptional activity. We demonstrated that the proteosomal inhibitor MG132 greatly enhanced the GH-stimulated STAT5-mediated transcriptional activation in both NIH3T3 cells as well as the NIH3T3 cells overexpressing c-Cbl. Therefore, by reducing the degradation of ubiquitin-conjugated STAT5, MG132 is able to enhance STAT5-mediated transcriptional activation, either directly by the consequent increased pool of phosphorylated STAT5, or through other unidentified mechanisms. Use of the proteosome inhibitor MG132 has previously been demonstrated to prolong signaling from JAK2 to STAT5b in the rat liver cell line (CWSV-1) (84). In this study, the mechanism of the MG132 effect was not investigated and was postulated to be an indirect stabilizing effect on the GHR-JAK2 complex (86). We have, however, demonstrated the normal activation of JAK2 and certain JAK2 activated downstream signaling pathways. Thus, the effect of c-Cbl on the level of STAT5-mediated transcription is presumably due to direct c-Cbl mediated ubiquitination and subsequent proteosomal degradation. We have therefore
identified a novel mechanism for regulation of STAT5-mediated transcription. Cellular stimuli resulting in increased levels of c-Cbl in the cell would therefore preferentially direct the GH response to be mediated by transcriptional pathways other than STAT5, such as p44/42 MAPK stimulated Elk-1-mediated transcription.

Fig. 8. The level of GH-stimulated tyrosyl-phosphorylated STAT5B is increased by pretreatment with the proteasome inhibitor, MG132. A, Western blot analysis of the GH-stimulated tyrosine phosphorylation of STAT5 in NIH3T3-vector and NIH3T3-c-Cbl cells. Cells were either untreated or pretreated with 50 μM MG132 for 1 h and then stimulated with 50 nM hGH for 0, 2, and 6 h and processed for Western blotting with polyclonal antibody recognizing tyrosine-phosphorylated STAT5α/β protein as described in Materials and Methods. The data presented are representative of at least three independently performed experiments. B, Quantitative analysis by densitometry of the level of tyrosine-phosphorylated STAT5α/β upon hGH stimulation of NIH3T3-vector and NIH3T3-c-Cbl cells. The graph is representative of the experiment in A. The level of tyrosine-phosphorylated STAT5α/β are plotted as arbitrary units relative to the arbitrary units of untreated NIH3T3-vector cells at 0 h, thus resulting in the bar chart.

Fig. 9. The proteasomal inhibitor MG132 prevented c-Cbl inhibition of GH-stimulated STAT5-mediated transcriptional activation. Effect of pretreatment with MG132 on GH-stimulated STAT5-mediated transcription in NIH3T3-vector and NIH3T3-c-Cbl cells. NIH3T3 cells were grown and transiently transfected with Spi2.1-GLE1-CAT. Cells were pretreated 1 h with 50 μM MG132 prior stimulation with 50 nM hGH and processed for CAT assay as described in Materials and Methods. The CAT activities were normalized on the basis of protein contents well as on the luciferase activity of pCMV-LUC vector. The data are presented as the mean ± SD of triplicate determinations. The data presented are from at least three independently performed experiments.

Proteasomal degradation of STATs may be a common mechanism employed by members of the cytokine receptor superfamily. It has been reported that IL-2-induced DNA-binding activity and tyrosine phosphorylation of STAT5 are stabilized by MG132 (87). However, no detectable ubiquitination of the STAT proteins was observed (87). Interpretation of their results suggested that proteasome-mediated protein...
degradation modulated the protein-tyrosine phosphatase activity that negatively regulated the JAK/STAT pathway stimulated by IL-2 (87). Similarly, IL-6 stimulation of Hep3B hepatoma cells stably expressing p53 demonstrated that IL-6 caused a rapid and marked loss of cellular immunostaining for STAT3 and STAT5 (88). However, the loss was blocked by MG132 and the level of both proteins subsequently remained unchanged, suggesting that IL-6 triggered proteosomal degradation and/or p53-dependent masking but not degradation of STAT3 and STAT5 (88).

The mechanism by which c-Cbl promotes ubiquitination of STAT5 remains to be determined. Recent evidence derived from studies performed in Cbl-b overexpressing cells have demonstrated that the c-Cbl regulation of protein levels by its E3 ligase activity is not solely restricted to receptor tyrosine kinase or nonreceptor tyrosine kinases (89). Multiubiquitination of the p85 subunit of PI3K can also occur through interaction of the proline rich domain in Cbl-b with the SH3 domain of p85 (89). STAT5 does not possess a tyrosine kinase domain for binding to the TKB domain of c-Cbl, nor does it possess any SH3 domain for binding to the proline-rich region of c-Cbl. It is possible that STAT5 either interacts with c-Cbl directly or indirectly through another adapter protein. This would be possible upon formation of the multiprotein signaling complex stimulated by GH centered around CrkII (22). Indeed, we have previously demonstrated that overexpression of CrkII, which would enhance the formation of the multiprotein signaling complex that includes c-Cbl, also results in inhibition of GH-stimulated STAT5-mediated transcription (39).

c-Cbl is expressed in multiple GH-responsive tissues (40–44), suggestive that the level of c-Cbl in these different cell types will regulate the STAT5-mediated response to GH or to other cytokines using STAT5. For example, c-Cbl deficient (c-Cbl–/–) mice exhibit lymphoid hyperplasia with changes in T-cell behavior, marked splenomegaly due to splenic extramedullary hematopoiesis and the mammary gland of c-Cbl–/– female mice exhibits increased ductal density and branching (44). These features are also shared by GH transgenic mice that exhibit altered thymocytic responses (90), marked splenomegaly (e.g. Ref. 91) and mammary gland hyperplasia (92). Interestingly, these cellular responses are also presumably mediated by STAT5 as indicated by recent studies in STAT5 deficient animals (93–96). Thus, physiological or pathological conditions which result in decreased cellular c-Cbl would allow for increased STAT5-mediated proliferation resulting in tissue hyperplasia and potential oncogenic transformation. Given the demonstration that STAT5 can be both necessary and sufficient for malignant transformation (97), and that v-Cbl exerts its transforming effects by acting as a dominant negative inhibitor of c-Cbl, then regulated control of STAT5 activity by c-Cbl may constitute a major mechanism for tissue homeostasis.

In summary, we have demonstrated here that c-Cbl is a negative regulator of GH-stimulated STAT5-mediated transcription by the direction of STAT5 for proteosomal degradation. We therefore provide the first evidence that c-Cbl directly regulates the level and activity of a transcription factor through ubiquitination and proteosomal degradation. Thus, the extent to which GH stimulates STAT5-mediated transcription in any particular cell type would depend on the cellular level of c-Cbl and the factors regulating such. c-Cbl, together with other identified negative regulators of GH-stimulated STAT-mediated transcription such as the suppressor of cytokine signaling proteins (98), therefore constitute an array of mechanisms by which the cell can limit or prevent STAT-mediated transcriptional events in response to GH or other stimuli.

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