0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(7):3182–3195

doi: 10.1210/en.2002-221121

Nuclear Translocation and Retention of Growth Hormone

HICHEM C. MERTANI, MIREILLE RACCURT, AUDE ABBATE, JENNY KINDBLOM, JAN TÖRNELL, NILS BILLESTRUP, YVES USSON, GÉRARD MOREL, AND PETER E. LOBIE

Centre National de la Recherche Scientifique Unité Mixte de Recherche 5123 Physiologie Energetique Cellulaire et Moléculaire (H.C.M., M.R., A.A., G.M.), Université Claude Bernard-Lyon-I, 69622 France; Department of Physiology (J.K., J.T.), University of Göteborg, 43183 Göteborg, Sweden; Hagedorn Research Laboratory (N.B.), 2820 Gentofte, Denmark; Laboratory DyOGen (Y.U.), Université Joseph Fourier, 38706 Grenoble, France; and Institute of Molecular and Cell Biology (P.E.L.), National University of Singapore, Singapore 117609, Republic of Singapore

We have previously demonstrated that GH is subject to rapid receptor-dependent nuclear translocation. Here, we examine the importance of ligand activation of the GH-receptor (GHR)-associated Janus kinase (JAK) 2 and receptor dimerization for hormone internalization and nuclear translocation by use of cells stably transfected with cDNA for the GHR. Staurosporine and herbimycin A treatment of cells did not affect the ability of GH to internalize but resulted in increased nuclear accumulation of hormone. Similarly, receptor mutations, which prevent the association and activation of JAK2, did not affect the ability of the hormone to internalize or translocate to the nucleus but resulted in increased nuclear accumulation of GH. These results were observed both by nuclear isolation and confocal laser scanning microscopy. Staurosporine treatment of cells in which human GH (hGH) was targeted to the cytoplasm (removal of secretion sequence) or to the nucleus (addition of the nuclear localization sequence of SV40 large T antigen) resulted in preferential accumulation of hGH in the nucleus. We further investigated the requirement of receptor dimerization for GH nuclear translocation using the non-receptor-dimerizing hGH antagonist, hGH-G120R, conjugated to fluorescein isothiocyanate. Confocal laser scanning microscopy demonstrated efficient internalization of both hGH and hGH-G120R but lack of nuclear translocation of hGH-G120R. Thus, we conclude that activation of JAK2 kinase and the subsequent tyrosine phosphorylation is not required for nuclear translocation of GH but is pivotal for the removal of the hormone from the nucleus, and that GH translocates into the nucleus in a GHR dimerized-dependent fashion. (*Endocrinology* 144: 3182–3195, 2003)

GH IS THOUGHT to initiate its biological actions, including induction of a number of RNA species in mammalian tissues (1), by interaction with a specific membrane-bound GH receptor (GHR) (2). The GHR lacks intrinsic tyrosine kinase activity; but, on ligand stimulation, receptor phosphorylation is induced by its physical association with the nonreceptor tyrosine kinase Janus kinase (JAK) 2 (3, 4). On binding the receptor at the cell surface, GH is internalized into the cell and redistributed to different subcellular compartments (5). The process of cytoplasmic internalization of GH has been recently under intense investigation and seems to involve the clathrin-coated pathway (6, 7), caveolae (8), and the ubiquitin conjugating system (9–12). The residue Phe 346 (13) and a β-turn endocytic code carried by the Asp 335 (14) were identified as structural requirements for efficient GHR internalization. Apart from down-regulation of cell surface receptor, the function of internalized GHR-ligand complex is not well understood but does not seem to be required for the transcriptional response to GH (13). Interestingly though, two recent reports demonstrated that endosomal GHR signal transduction continues after endocytosis and sustains signal transduction (15, 16). Thus, it is evident that the intracellular routing of internalized GH between lysosomes, mitochondria, and the Golgi apparatus (5) and its regulation by the proteasome system (17) are of biological importance. We have previously demonstrated that both GH (18, 19) and the GHR (18, 20) are subject to rapid nuclear translocation and that JAK2 is located constitutively in the nucleus (21). GH treatment of cells has previously been demonstrated to stimulate the tyrosine phosphorylation of nuclear JAK2 without an apparent change in its subcellular location (21). Recent work has also demonstrated that nuclear localized GH binding protein is required for the full transcriptional response to GH through the JAK-STAT (signal transducer and activator of transcription) pathway (21a).

The mechanism by which GH and or the GHR are translocated to the nucleus is not understood. The nuclear translocation of several other polypeptide hormones regulating growth responses has also been described (22). Tyrosine kinase activity seems necessary for the internalization of insulin, epidermal growth factor, and prolactin (PRL) (23); and therefore, as a consequence, is required for the nuclear translocation of exogenously applied polypeptide hormone and/or growth factor. The nuclear translocation of the related hormone PRL has also been demonstrated to require protein kinase C (PKC) activity (24).

We have taken complementary approaches to determine the role of tyrosine kinase activity and dimerization of the GHR in the processes of internalization and nuclear trans-

Abbreviations: BRL, Buffalo rat liver; CHO, Chinese hamster ovary; CLSM, confocal laser scanning microscopy; DIC, differential interferential contrast; FITC, fluorescein isothiocyanate; GHR, GH receptor; hGH, human GH; JAK, Janus kinase; NLS, nuclear localization signal; PKC, protein kinase C; PRL, prolactin; rGH, rat GH; STAT, signal transducer and activator of transcription; WT, wild-type.

location of GH: first, by inhibition with predetermined concentrations of tyrosine kinase inhibitor, which inhibit JAK2 activity; and second, by the use of receptor mutations, which are unable to bind JAK2 or induce tyrosine phosphorylation within the cell. We show that tyrosine phosphorylation is not required for internalization and nuclear translocation of GH but is pivotal for the removal of GH from the nucleus. In addition, we provide evidence that nuclear translocation of GH is dependent on cell surface receptor dimerization. This raises the possibility of a novel mechanism for nuclear translocation/accumulation used specifically by GH and not other peptide ligands.

Materials and Methods

Hormone iodination and labeling

Recombinant rat GH (rGH) and a guinea pig antirat GH antibody were obtained from Dr. A. F. Parlow (National Hormone and Pituitary program). Recombinant human GH (hGH) and hGH-G120R were a generous gift from Novo-Nordisk Asia Pacific Pte Ltd. (Singapore) and Pharmacia Pte Ltd. (Stockholm, Sweden). hGH was iodinated by the iodogen method (25) and fractionated on a Sepharose G-25 column. Specific activity of hGH was 80–150 μCi/μg protein. Fluorescein isothiocyanate (FITC)-hGH-G120R and FITC-hGH were prepared using a FluoroTag-FITC conjugation kit according to the supplier's instructions (Sigma, Singapore). Integrity of the labeled protein was verified on 15% PAGE followed by Western blot detection with anti-hGH (DAKO Corp., Singapore) according to standard procedures. Functional activity of FITC-hGH was determined in a reporter assay using the *c-fos*-SIE-CAT plasmid as described previously (8). The murine antirat GHR (mAb 263) was a generous gift from Dr. M. J. Waters (Queensland University, Brisbane).

Cellular transfection of GHR

Rat GHR cDNA was cloned into an expression plasmid containing an SV40 enhancer and a human metallothionein IIa promoter. The cDNAs were transfected into Chinese hamster ovary (CHO)-K1 cells with lipofectin together with the pIPB-1 plasmid that contains a neomycin resistance gene fused to the thymidine kinase promoter (26). Stable transfected cells were selected using 1 mg/ml G418. The complete rat GHR cDNA (27) coding for amino acids 1–638 was expressed in CHO4–638 or CHOA-638 cells (26). These cells expressed 40,000–80,000 receptor per cell and will be referred to as CHO-GHR_{1-638} (26). The construction of GHR cDNA expression plasmids containing a deletion of box 1 (Δ 297–311) and the individual substitution of proline residues 300, 301, 303, and 305 in box 1 for alanine has been described previously (28). These cDNAs were stably transfected into CHO-K1 cells; the Δ 297–311 mutation was expressed in CHO-GHR_{1-638}Δ 297–311 (20,000–60,000 per cell) cells, and P300,301,303,305A was expressed in CHO-GHR_{1-638} P300,301,303,305A cells (60–100 000 per cell) (28).

Construction of hGH expression plasmids and transient transfection

Three hGH expression plasmids were used for transient transfection experiments. The first contained the wild-type (WT)-hGH gene under the control of the metallothionein Ia promoter (see Ref. 32). Another construct contained the hGH gene but with removal of the gene sequence encoding the secretion sequence and addition of a methionine residue (XS-hGH), also under the control of the metallothionein Ia promoter. Last, a construct was made in which the nuclear localization signal (NLS) of the SV40 large T antigen, preceded by a methionin residue, was added to the hGH gene in which the secretion sequence had been removed (NLS-hGH), also under the control of the metallothionein Ia promoter.

Parental CHO cells were grown to confluence in 100-mm dishes and transiently transfected with 10 μg of the different hGH expression plasmid, using DOTAP according to the manufacturer instructions (Roche Pte Ltd., Singapore). After transfection for 12 h and a further incubation for 24 h ± 100 nM staurosporine, the cells were rinsed with cold PBS HCl (pH 3), and cytoplasmic and nuclear extracts were prepared (see below). The amount of hGH produced and secreted over a 24-h period into 7 ml serum-free media was then quantitated by ELISA (see below).

Hormone internalization, degradation, and nuclear uptake in transfected cells

For hormone internalization and degradation assays, CHO cells were grown to confluence in 6-well plates in Ham's F-12 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. For estimation of hormone nuclear uptake, cells were similarly grown to confluence in 100-mm-diameter dishes. When cell surface receptor expression and hormone internalization were compared with hormone nuclear uptake, both assays were performed in 100-mm-diameter dishes. Confluent cells were incubated in serum-free medium for 12 h before addition of ^{125}I-hGH (approximately 150,000 cpm/well) for the indicated time periods. Nonspecific or nonreceptor mediated uptake was estimated by the concomitant addition of 10 μg/ml unlabeled hGH to the media. For estimation of hormone internalization, the media was removed, the cells were washed once with PBS, and cell surface bound hormone was removed by washing the cells with 5 ml PBS-HCl (pH 3) for 1 min (5). The cells were then washed twice with 5 ml PBS, solubilized with 0.1 M NaOH-1% sodium dodecyl sulfate, scraped, and transferred to tubes for γ-spectrometry. Cell surface receptor was estimated by incubation of cells at 4 C for 4 h with ^{125}I-hGH, and nonspecific binding was determined by the addition of 10 μg/ml unlabeled hGH. Degraded GH is released into the media as iodotyrosine (29). Hormone degradation was estimated by trichloroacetic acid precipitation (10%) of media and quantitation of radioactivity in the supernatant. Rapid isolation of nuclei was performed as previously described (18, 30). Cells were washed 3 times in PBS before removal with a rubber policeman. Cells were centrifuged at 800 × *g*, the supernatant removed, then resuspended in a nuclear isolation buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM benzamidine hydrochloride, 3 mM aminoacetonitrile hydrochloride, and 0.5% Nonidet P-40. Extracts were vortexed for 10 sec, placed on ice for 5 min, centrifuged at 400 × *g*, the supernatant removed, and the process repeated twice more. The validity of this method and the characterization of the nuclear fraction have previously been reported (18). Pellets were counted on an LKB γ-spectrometer.

Treatment of cells with inhibitors of tyrosine kinase activity

Cells were cultured and processed as described above and exposed to staurosporine at the indicated concentrations, for 30 min, before the addition of ^{125}I-hGH (~150,000 cpm). For Herbimycin A, cells were exposed to the indicated concentrations for 18 h, before the addition of ^{125}I-hGH (31). The cells were in the continued presence of the drugs for the duration of the hormone processing experiments. Vehicle was added to control wells.

Immunofluorescence

Cells were grown to 30–50% confluence in 10% FBS-supplemented Ham's F-12 before being serum-deprived for 12 h. Cells were then incubated for the indicated time periods with 50 nM of rGH, washed in serum-free media, and subsequently fixed for 15 min in ice-cold 4% paraformaldehyde in PBS at the end of the respective time intervals. The cells were permeabilized in 0.1% TX-100-PBS for 5 min, washed extensively with PBS, and incubated for 1 h, with antirat GH antibody diluted to 1:1000 (1 μg /ml) and antirat GHR (mAb 263) diluted to 1:50 (25 μg /ml). Cy3 conjugated goat anti-guinea pig IgG, diluted to 1:500, and Rhodol green conjugated goat antimouse IgG (Molecular Probes, Inc., Leiden, The Netherlands), diluted to 1:100, were used, respectively, for detection. Controls were performed by: 1) omission of the primary antibody; 2) replacing the primary antibody with the same protein concentration of preimmune mouse or rabbit serum; and 3) preincubation (24 h at 4 C) of anti rGH or mAb 263 with 50 μg/ml recombinant hGH.

Trafficking of FITC-conjugated hormones

Because hGH-G120R exhibited partial agonist activity through the rat GHR, we used a cell line stably transfected with a chimeric receptor

containing the extracellular domain of the human receptor and the transmembrane and intracellular domains of the rat receptor [Buffalo rat liver (BRL)-hEC-GHR]. We have previously described that, in this cell line, the hGH-G120R did not exhibit agonist activity and antagonized the transcriptional activation induced by hGH (32). The cells were grown to 30–50% confluence on cover slips, starved for 12 h in Ham's F12 serum-free medium, and incubated for 0, 2, 5, 15, and 30 min with either FITC-hGH-G120R (50 nM) or FITC-hGH (50 nM). Cells were washed with PBS to remove unbound label and fixed for 30 min in 4% paraformaldehyde in PBS. After fixation, the cells were embedded in mowiol, and confocal laser scanning microscopy (CLSM) was performed using the LSM 410 system (Carl Zeiss, Göttingen, Germany). Controls of internalization and nuclear translocation included: 1) incubation at 4 C, followed by acid wash; 2) incubation in BRL parental cell line; 3) competition with 100-fold excess of unlabeled hGH or hGH-G120R; 4) preincubation with 5M excess of mAb 263; and 5) use of unrelated FITC-labeled BSA.

CLSM

All images were acquired with a Carl Zeiss LSM 410 confocal microscope equipped with laser sources, three separate detection channels with their own pinhole detector, and a fourth detection channel for differential interferential contrast (DIC) (Nomarski) transmitted light scanning microscopy. The fluorescence images of Cyanin3 and Rhodol green were collected sequentially on two different channels to avoid cross-talk between fluorescence signals. Cyanin 3 fluorescence was excited with a 543-nm HeNe laser source. A 560-nm dichroic beam splitter was used to separate laser excitation from emitted fluorescence; an additional 590-nm long-pass filter was used, after the pinhole, to reject nonspecific fluorescence. Rodhol green fluorescence was excited with a 488-nm Argon laser source. A 510-nm dichroic beam splitter was used to separate laser excitation from emitted fluorescence; an additional 515- to 545-nm band-pass filter was used, after the pinhole, to reject nonspecific fluorescence. The pinhole aperture was set to one Airy disk unit for each channel. An image of the same field was recorded in DIC transmitted light to facilitate the cell contour and nuclear contour delineation in the further image analysis stage. Image averaging through a recursive filter (Kalman filtering) was used for the acquisition of the fluorescence images to improve the signal *vs.* noise ratio.

Image analysis of CLSM images

The gain setting for the acquisition of the confocal microscopic images was not modified until examination of the entire experiment was completed. The parameters of image acquisition were carefully calibrated to the individual point that exhibited the highest level of immunoreactivity. Ten image triplets (Cy3-Rhodol green-DIC) were recorded per experimental slide. These images were stored on a computer disk and analyzed with a SAMBA 2005 image analysis system (SAMBA Technologies, Meylan, France). Dedicated software was developed for this particular application. Briefly, the DIC image of a triplet was used to interactively extract the contours of the cells and of the cell nuclei. From these contours, the program built the binary masks of the cytoplasm and of the nucleus of each cell. Then the Cy3 image was loaded. For each cell, the fluorescence intensities (arbitrary unit) were measured using the previously obtained binary mask: 1) inside the cytoplasm of each cell; and 2) inside the nucleus of each cell. Two parameters were obtained: the integrated fluorescence (that is, the sum of intensity values measured within the mask); and the average fluorescence intensity [that is, the integrated fluorescence divided by the area (in pixels) of the binary mask]. Next, the Rhodol green image was loaded, and the same measurements were made using the binary masks.

ELISA for hGH internalization and nuclear translocation

Subconfluent CHO-GHR_{1-638}, CHO-GHR_{1-638} Δ297–311, and CHO-GHR_{1-638} P300,301,303,305A cells were grown in 6-well plates. The cells were starved overnight; incubated with 50 nM hGH for 5, 10, 15, 30, and 60 min at 37 C; and rinsed with cold PBS HCl (pH 3). Cytoplasmic and nuclear extracts were prepared as described (24). The nuclear pellet was resuspended in 3 vol lysis buffer [20% glycerol, 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.1 mM Na_3VO_4] and incubated on ice for 30 min. Nuclear extracts were collected, and protein concentration was determined by the Bradford method. Before the assay, the nuclear fraction was diluted in water to reduce salt concentration. Nonspecific binding to the ELISA was estimated by preparation of nuclear/cytoplasmic extracts from cells unstimulated with hGH. The amount of cytoplasmic and nuclear hGH was calculated in triplicate, according to the manufacturer instruction and as previously described (32).

Statistics

Data are expressed as the mean of normalized values from triplicates ± SEM for a representative experiment. The degree of significance of differences between groups is calculated using Student's t test and ANOVA (Statview, Alsyd, France), where appropriate, and set at $P < 0.05$. Values are considered significantly different from control when a 95% confidence interval (calculated by multiplying the SEM by the t value for the relevant degree of freedom) excludes 100%. Experimental values are considered different from each other when their respective confidence intervals do not overlap, and *one asterisk* denotes that the mean is statistically different, at the 95% confidence level, from 100%.

Results

Effect of tyrosine kinase inhibitors on hormone internalization and nuclear uptake in GHR cDNA transfected cells

GH-stimulated tyrosine phosphorylation of cellular proteins is mediated by the receptor association and activation of JAK2 (3). The activity of this kinase and GH-stimulated tyrosine phosphorylation has previously been demonstrated to be inhibited by staurosporine (31). We used staurosporine at concentrations that inhibit the activity of the associated kinase (31), activation of STAT5 binding to SPI-GLE1 (33, 34), and GH-induced phosphorylation and functions within the cell (31, 35). We pretreated CHO-GHR_{1-638} cells for 30 min at 37 C with 100 nM staurosporine and subsequently studied the internalization, degradation, and nuclear translocation of ^{125}I-hGH. The cells were incubated for a further 90 min at 37 C with ^{125}I-hGH, in accordance with our previously published data showing maximal or near maximal internalization and nuclear translocation of hormone at this time when ^{125}I-hGH was used. As shown in Fig. 1A, staurosporine caused a slight, but not significant, decrease in cell surface receptor and a slight, but not significant, increase in hormone internalization. Interestingly, however, staurosporine caused a significant decrease in hormone degradation and a significant increase in hormone nuclear translocation/accumulation (Fig. 1A). The increase in nuclear uptake with staurosporine was dose-dependent, with increasing concentrations of staurosporine resulting in increased accumulation of ^{125}I-hGH in the nucleus (Fig. 1B). We also tested the effect of another agent that has been reported to inhibit JAK2 kinase activity (31). For this, the cells were pretreated with herbimycin A at 1 μg/ml for 18 h at 37 C, as previously described, before addition of ^{125}I-hGH. Such treatment with herbimycin A caused a large decrease in cell surface receptor expression and, as a consequence, also caused a decrease in internalization of hormone (Fig. 2). Degradation was slightly (but significantly) decreased. The nuclear uptake or accumulation of ^{125}I-hGH was not significantly different, compared with the vehicle-treated control. However, if the nuclear accumulation of ^{125}I-hGH is normalized to either the level of cell surface receptor or the level of internalized hormone, then

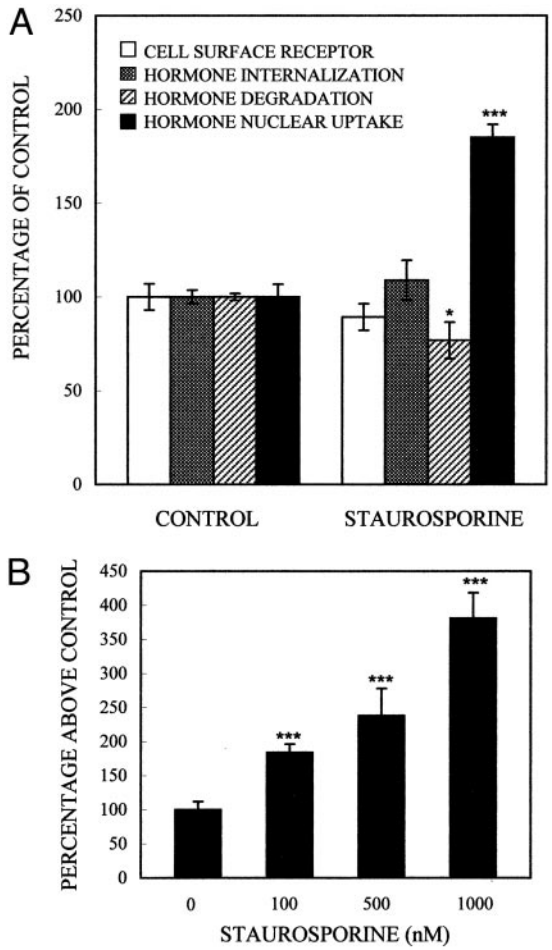

FIG. 1. A, Effect of staurosporine on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO-GHR_{1-638} cells. Estimation of cell surface receptor, internalized hormone, degraded hormone, and hormone nuclear uptake was performed as described in *Materials and Methods*. Staurosporine was used at a final concentration of 100 nM. Total added radioactivity was approximately 150,000 cpm/well. Staurosporine values were normalized to vehicle-treated control. In control experiments, total and nonspecific binding for cell surface receptor were 7206 ± 336 and 855 ± 16; for hormone internalization, 5179 ± 51 and 786 ± 58; for hormone degradation, 6932 ± 377 and 793 ± 41; and for nuclear uptake, 1195 ± 42 and 812 ± 56 cpm, respectively. In staurosporine experiments, total and nonspecific binding for cell surface receptor were 7204 ± 513 and 617 ± 103; for hormone internalization, 4958 ± 142 and 777 ± 22; for hormone degradation, 5349 ± 570 and 698 ± 37; and for nuclear uptake, 1290 ± 51 and 527 ± 104 cpm, respectively. Each point is the mean ± SEM of triplicate estimations. *, $P < 0.05$; ***, $P < 0.01$ *vs.* control. The results shown are representative of at least three experiments. B, Effect of staurosporine concentration on the nuclear accumulation of ^{125}I-hGH in CHO-GHR_{1-638} cells. Estimation of hormone nuclear uptake was performed as described in *Materials and Methods*. Staurosporine was used at the indicated concentrations. Total added radioactivity was approximately 150,000 cpm/well. Staurosporine values were normalized to the level of nuclear uptake of ^{125}I-hGH in vehicle-treated cells. In a control experiment, total and nonspecific binding was 1274 ± 49 and 823 ± 200 cpm, respectively. In staurosporine experiments, total and nonspecific binding were 1468 ± 47 and 906 ± 63 for 100 nM, 1536 ± 154 and 802 ± 133 for 500 nM, and 2131 ± 144 and 963 ± 97 for 1000 nM cpm, respectively. Each point is the mean ± SEM of triplicate estimations. ***, $P < 0.01$ *vs.* 0 nM staurosporine. The results shown are representative of at least three experiments.

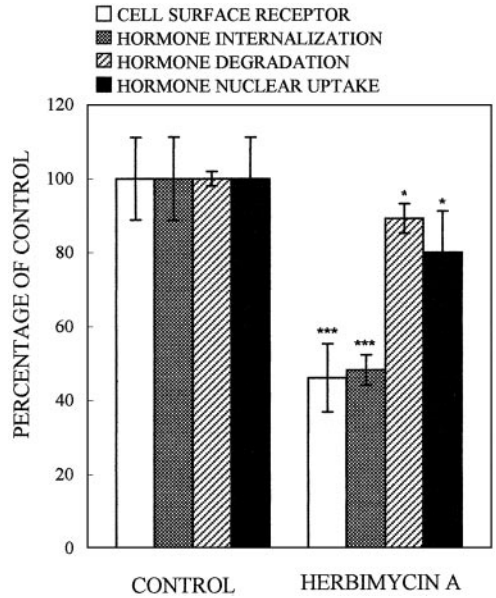

FIG. 2. Effect of 18-h incubation with Herbimycin A on cell surface receptor, hormone internalization, hormone degradation, and hormone nuclear uptake in CHO-GHR_{1-638} cells. Estimation of cell surface receptor, internalized hormone, degraded hormone, and hormone nuclear uptake was performed as described in *Materials and Methods*. Total added radioactivity was approximately 150,000 cpm/well. Herbimycin A values were normalized to the vehicle-treated control. In the control experiment, total and nonspecific binding for cell surface receptor were 3916 ± 366 and 673 ± 59; for hormone internalization, 2333 ± 482 and 683 ± 16; for hormone degradation, 4098 ± 65 and 517 ± 31; and for hormone nuclear uptake, 347 ± 96 and 100 ± 17 cpm, respectively. In herbimycin A experiments, total and nonspecific binding for cell surface receptor were 1822 ± 197 and 305 ± 43; for hormone internalization, 1106 ± 30 and 525 ± 50; for hormone degradation, 3667 ± 193 and 412 ± 37; and for hormone nuclear uptake, 284 ± 95 and 80 ± 17 cpm, respectively. Each *point* is the mean ± SEM of triplicate estimations. *, $P < 0.05$; ***, $P < 0.01$ *vs.* control. The results shown are representative of at least three experiments.

the nuclear accumulation of ^{125}I-hGH, as a percentage of internalized hormone, is higher in the herbimycin-A-treated cells (Fig. 2).

Hormone internalization and nuclear translocation in cells stably transfected with cDNA for GHR box 1 deletion and mutation

To verify that internalization and nuclear translocation of GH did not require tyrosine kinase activation, we examined cell clones stably transfected with cDNA for two kinase activation-deficient GHR mutations (26). The association and activation of JAK2 requires the membrane proximal proline-rich region of the receptor (28). GH is reported not to be able to stimulate tyrosine phosphorylation of any cellular protein in cells expressing either a deletion or mutation of this proline-rich region (28). One mutation involved the deletion of box 1 by removal of amino acids 297–311 of the receptor (GHR_{1-638} Δ297–311). These cells were able to internalize GH to the same extent as cells transfected with cDNA for WT receptor (CHO-GHR_{1-638}) (when normalized to total cell surface receptor) (Fig. 3A). However, significantly less degraded hormone was detected in the media from CHO-GHR_{1-638} Δ297–311 cells. The extent of nuclear translocation/accumu-

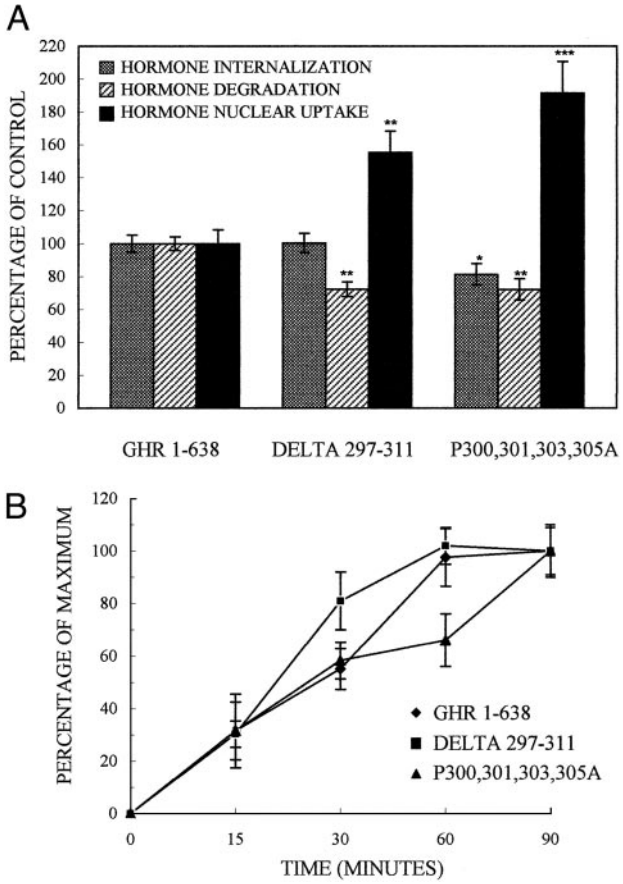

FIG. 3. A, Hormone internalization, hormone degradation, and hormone nuclear uptake in CHO-GHR_{1-638} cells and in cells lacking the box 1 region (CHO-GHR_{1-638} Δ297–311) or possessing a mutated box 1 region (CHO-GHR_{1-638} P300,301,303,305A). Estimation of internalized hormone, degraded hormone, and hormone nuclear uptake was performed as described in *Materials and Methods*. Hormone internalization degradation and nuclear uptake were normalized to the level of hormone internalization, degradation, and nuclear uptake in CHO-GHR1–638 cells. Total added radioactivity was approximately 150,000 cpm/well. In GHR 1–638 cell line, total and nonspecific binding for cell surface receptor were 3818 ± 169 and 507 ± 24; for hormone internalization, 3490 ± 93 and 788 ± 28; for hormone degradation, 6789 ± 238 and 612 ± 31; and for hormone nuclear uptake, 1550 ± 153 and 1054 ± 215 cpm, respectively. In DELTA 297–311 cell line, total and nonspecific binding for cell surface receptor were 3017 ± 166 and 411 ± 16; for hormone internalization, 2917 ± 189 and 325 ± 47; for hormone degradation, 5249 ± 153 and 756 ± 37; and for hormone nuclear uptake, 1443 ± 9 and 537 ± 17 cpm, respectively. In P300,301,303,305A cell line, total and nonspecific binding for cell surface receptor were 3081 ± 239 and 366 ± 59; for hormone internalization, 2528 ± 223 and 317 ± 16; for hormone degradation, 5127 ± 147 and 635 ± 29; and for hormone nuclear uptake, 1924 ± 196 and 811 ± 49 cpm, respectively. Each point is the mean ± SEM of triplicate estimations. *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$ *vs. control*. The results shown are representative of at least three experiments. B, Kinetics of hormone nuclear uptake in CHO-GHR_{1-638} cells and in cells lacking the box 1 region (CHO-GHR_{1-638} Δ297–311) or possessing a mutated box 1 region (CHO-GHR_{1-638} P300,301,303,305A). Estimation of hormone nuclear uptake was performed as described in *Materials and Methods*. Results were generated by allocating the maximal nuclear uptake for each individual cell line as 100%, and nuclear uptake was normalized to the maximal nuclear translocation observed for each individual cell line. Total added radioactivity was approximately 150,000 cpm/well Each *point* is the mean ± SEM of triplicate estimations. The results shown are representative of at least three experiments.

lation of ^{125}I-hGH in CHO-GHR_{1-638} Δ297–311 cells was greater than that observed for GHR_{1-638} cells, when normalized to total cell surface receptor (Fig. 3A). The untransfected parental cell line did not internalize nor nuclear translocate ^{125}I-hGH to any significant extent (18). We also examined internalization and nuclear translocation of ^{125}I-hGH in cells stably transfected with a receptor in which the individual proline residues of box 1 were substituted for alanine (GHR_{1-638} P300,301,303,305A). Cells stably transfected with cDNA for this receptor showed a significant deficit in internalization of ^{125}I-hGH, when compared with CHO-GHR_{1-638} (Fig. 3A). Degradation of the hormone was also significantly decreased in CHO-GHR_{1-638} P300,301,303,305A cells, compared with cells expressing the WT receptor. Nuclear translocation and accumulation were also significantly increased in CHO-GHR_{1-638} P300,301,303,305A cells, compared with CHO-GHR_{1-638} (Fig. 3A). Internalization and nuclear translocation of ^{125}I-hGH in these experiments were measured at a time point previously determined to represent maximal effect (18). We next sought to determine whether nuclear translocation in the box 1-deficient GHR was kinetically similar to the WT receptor. As is evident from Fig. 3B, all three cell lines display similar kinetics of ^{125}I-hGH nuclear translocation.

CLSM of GH nuclear translocation in cells stably transfected with cDNA for GHR and receptor box 1 deletion and mutation

We examined hormone internalization and nuclear translocation by quantitative analysis of CLSM in CHO-GHR_{1-638} (Figs. 4 and 5), CHO-GHR_{1-638} Δ297–311 (Figs. 6 and 7), and CHO-GHR_{1-638} P300,301,303,305A cells (Figs. 8 and 9). We have previously described that treatment of CHO-GHR_{1-638} cells with a more physiologically relevant dose of GH (50 nM is the dose of GH required to obtain a maximal stimulation of reporter activity in these cells) resulted in a more rapid and transient nuclear translocation of GH (18) than that observed with the low concentrations of ^{125}I-hGH (as above). As shown in Fig. 4 (A, D, G, J, M, and P), within 5 min of exposure of CHO-GHR_{1-638} cells to 50 nM rGH, the cytoplasm was intensely immunoreactive for GH, with some nuclear translocation already evident. Between 15 and 30 min, both the cytoplasm and nucleus were intensely labeled, indicative of a very prominent hormone nuclear translocation. Sixty minutes after exposure to hormone, GH immunoreactivity decreased dramatically, being barely detectable in either the cytoplasm or nucleus. This is presumably because the internalized hormone is rapidly degraded, or resecreted to the media via the Golgi apparatus, and the cell is unable to further internalize hormone because of acute receptor down-regulation at the cell surface. Accordingly, confocal analysis of GHR immunoreactivity in CHO-GHR_{1-638} cells (Fig. 4; B, E, H, K, N, and Q) demonstrates that, after GH exposure, immunoreactive GHR is redistributed over the Golgi area and accumulated in the nucleus as previously described (18). The nuclear translocation of the GHR was transient and was not observed 15 min after GH stimulation. Dual detection, showing colocalization of both GH and GHR immunoreactivity (Fig. 4; C, F, I, L, O, and R), indicated that

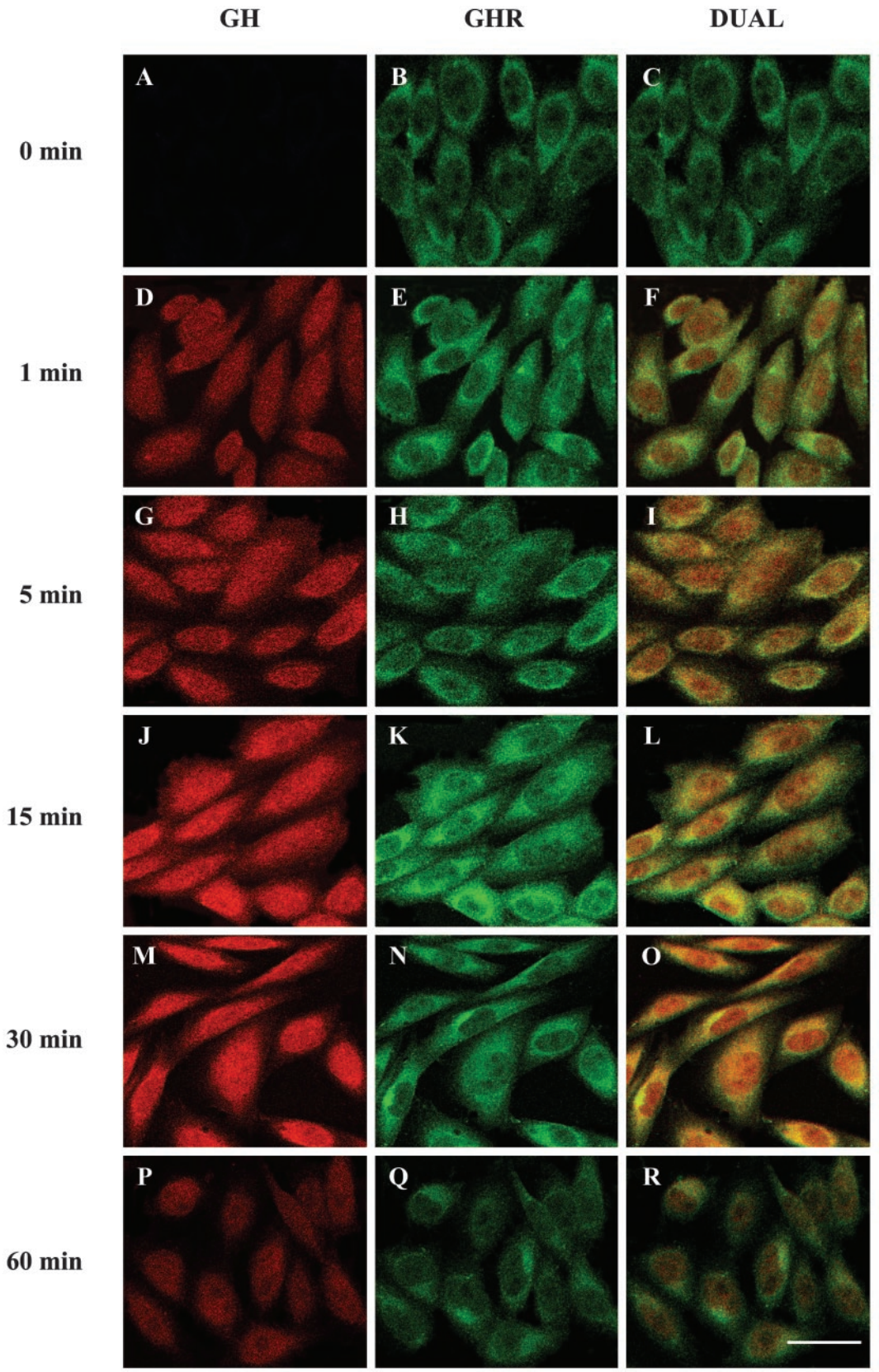

FIG. 4. Kinetics of GH and GHR internalization and nuclear translocation in CHO-GHR$_{1-638}$ cells. CLSM analysis at 0, 1, 5, 15, 30, and 60 min after GH stimulation was performed as described in *Materials and Methods*. The localization of GH is shown in the *left-hand column*, GHR in the *middle column*, and the dual localization (superimposed images) in the *right-hand column*. *Magnification bar*, 30 μm for all photographs.

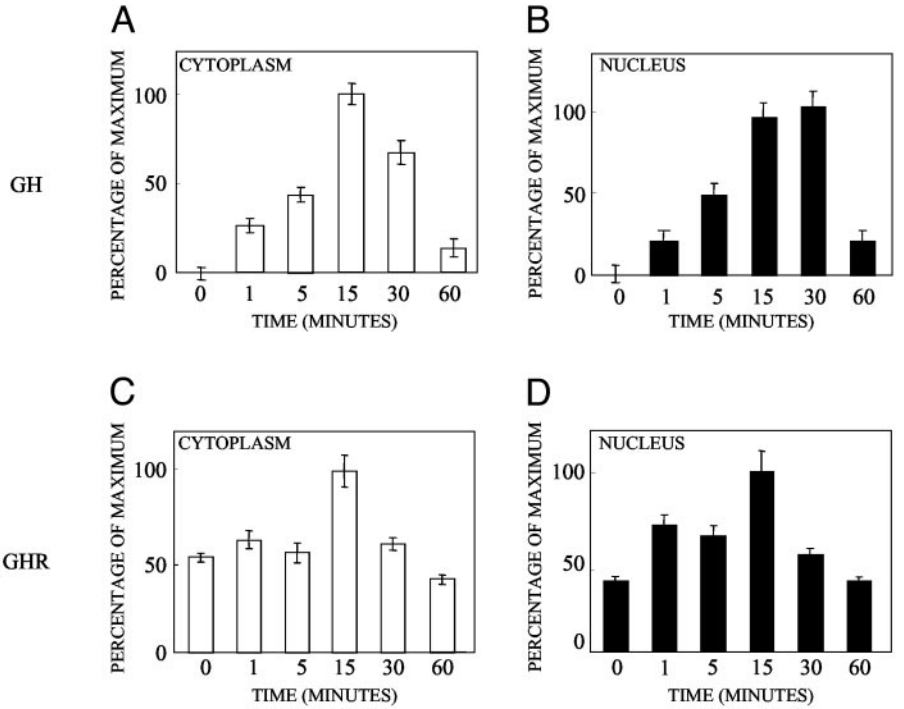

FIG. 5. Quantitative analysis of GH and GHR cytoplasmic internalization and nuclear translocation in CHO-GHR_{1-638} cells. GH and GHR immunoreactivity were quantified as described in *Materials and Methods*. The results shown are representative of at least three experiments.

only a portion of GH was imported in the nucleus in a complex form with its receptor. The time course of GH and GHR internalization and nuclear translocation in CHO-GHR_{1-638} was quantitatively analyzed in more detail. Figure 5 summarizes the data from three independent experiments. In agreement with the confocal images obtained in Fig. 4, GH immunoreactivity in the cytoplasm gradually increased during the first 15 min of exposure and then decreased to a very low level 60 min after exposure (Fig. 5A). GH was also subject to a rapid nuclear translocation in CHO-GHR_{1-638} cells that culminated 30 min after exposure and was undetectable 60 min after exposure (Fig. 5B). Quantitative analysis of GHR immunoreactivity distribution also demonstrated that, 15 min after GH exposure, GHR accumulated in the Golgi area (Fig. 5C) as well as in the nucleus (Fig. 5D). Nuclear and perinuclear GHR immunoreactivity progressively decreased after 15 min of GH exposure (Fig. 5D).

We next examined the kinetics of hormone internalization and nuclear translocation in both CHO-GHR_{1-638} Δ297–311 (Figs. 6 and 7) and CHO-GHR_{1-638} P300,301,303,305A (Figs. 8 and 9) cells to compare with the results obtained in CHO-GHR_{1-638} cells (Figs. 4 and 5). The basic pattern of GH internalization and nuclear translocation for CHO-GHR_{1-638} Δ297–311 (Fig. 6; A, D, G, J, M, and P) at 1, 5, and 15 min were identical to that observed for CHO-GHR_{1-638} cells. Thus, internalization of hormone was rapidly achieved within 5 min, with a considerable degree of nuclear translocation. By 15 min, the maximal accumulation of GH in the nucleus had been achieved. However, at 30 and 60 min, GH was still present in significant quantity in the nucleus of CHO-GHR_{1-638} Δ297–311, in contrast to CHO-GHR_{1-638}, where GH was almost totally absent from the nucleus at 60 min. Thus, deletion of box 1, which prevents the association of JAK2 to the GHR- and GH-induced tyrosine phosphorylation, resulted in increased nuclear accumulation of GH. Confocal analysis of immunoreactive GHR in GHR_{1-638} Δ297–311 cells (Fig. 6; B, E, H, K, N, and Q) demonstrated an accumulation in the cytoplasm, in the Golgi area, and in the nucleus between 5 and 15 min of stimulation. After 15 min of GH stimulation, nuclear GHR immunoreactivity slowly decreased, and cytoplasmic GHR immunoreactivity returned to basal levels (18). Interestingly, in GHR_{1-638} Δ297–311 cells, the receptor was partially retained in the nucleus 15 min after GH stimulation, as compared with CHO-GHR_{1-638} cells. Again, as indicated by the dual detection of both GH and GHR immunoreactivity (Fig. 6; C, F, I, L, O, and R), GH was partially imported into the nucleus in a complex form with its receptor. Internalization and nuclear translocation of GH and GHR in GHR_{1-638} Δ297–311 cells were also quantified (Fig. 7, A–D). Maximal internalization of GH occurred after 5 min (Fig. 7A), and was concomitant to the nuclear accumulation of the hormone that culminated at 15 min, and slowly decreased after that (Fig. 7B). However, compared with CHO-GHR_{1-638}, nuclear immunoreactivity of GH was still detected at 30 and 60 min. GHR immunoreactivity was rapidly redistributed over the cytoplasmic (Fig. 7C) and nuclear compartments (Fig. 7D). It is therefore apparent that GHR is rapidly translocated and partially retained in the nucleus after GH stimulation (Fig. 7D) of GHR_{1-638} Δ297–311 cells.

We also examined internalization and nuclear translocation of rGH in CHO-GHR_{1-638} P300,301,303,305A cells (Figs. 8 and 9). Basically, the pattern of rGH internalization and nuclear translocation in these cells (Fig. 8; A, D, G, J, M, and P) were very similar to that observed in CHO-GHR_{1-638} Δ297–311 (Fig. 6). The hormone was rapidly internalized, within 15 min, and progressively accumulated in the nucleus. Again in contrast to what was observed in CHO-

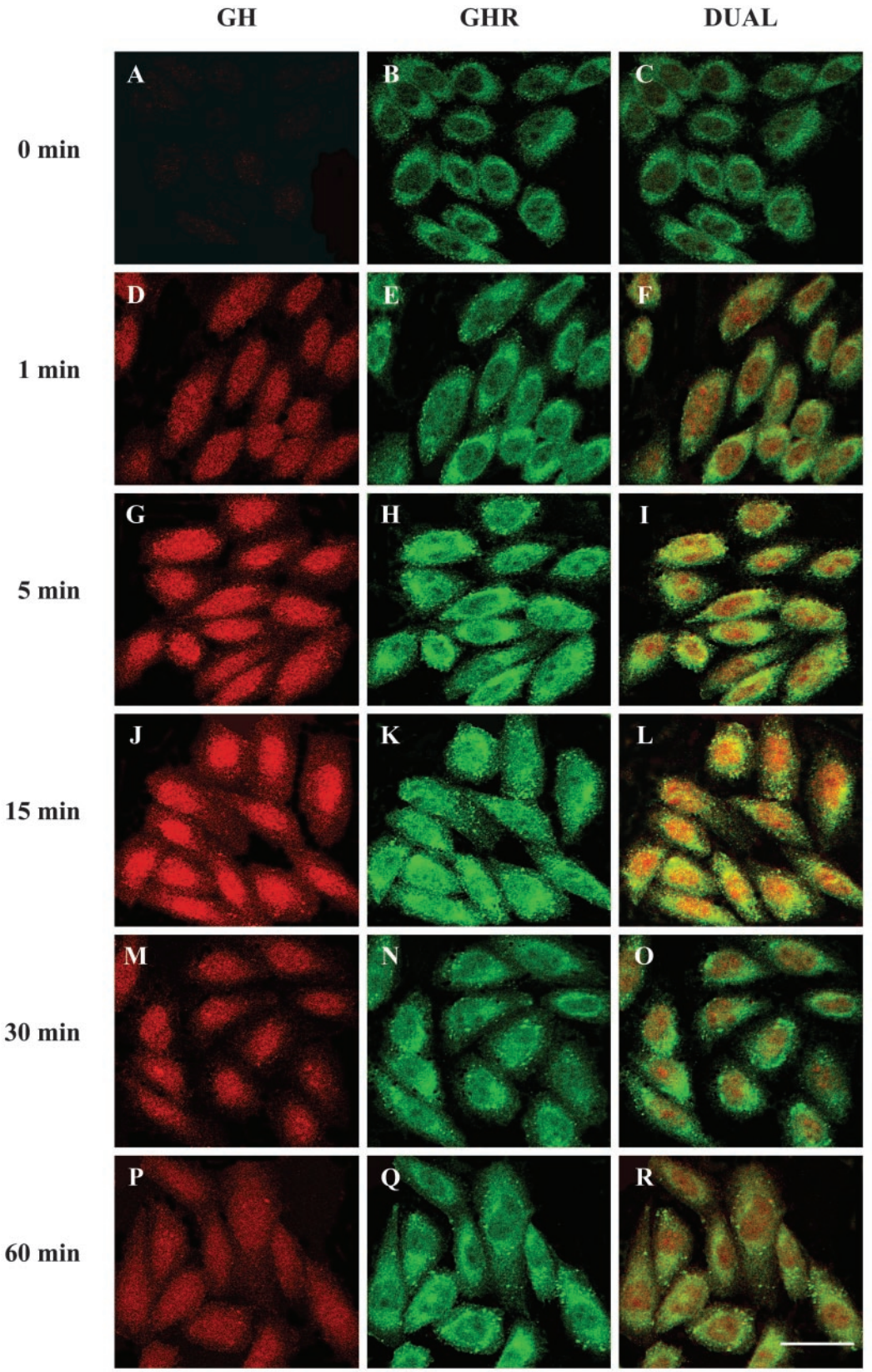

FIG. 6. Kinetics of GH and GHR internalization and nuclear translocation in cells lacking the box 1 region (CHO-GHR$_{1-638}$ Δ297–311). CLSM analysis at 0, 1, 5, 15, 30, and 60 min after GH stimulation was performed as described in *Materials and Methods*. The localization of GH is shown in the *left-hand column*, GHR in the *middle column*, and the dual localization (superimposed images) in the *right-hand column*. *Magnification bar*, 30 μm for all photographs.

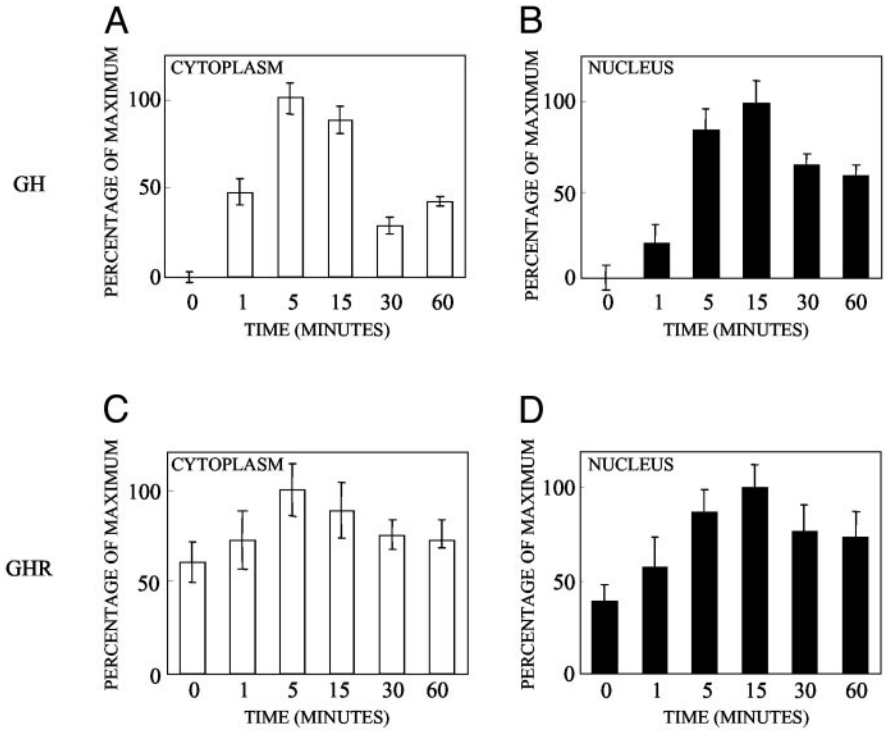

FIG. 7. Quantitative analysis of GH and GHR cytoplasmic internalization and nuclear translocation in cells lacking the box 1 region (CHO-GHR_{1-638} Δ297–311). GH and GHR immunoreactivity were quantified as described in *Materials and Methods*. The results shown are representative of at least three experiments.

GHR_{1-638}, GH immunoreactivity was still detected 60 min after stimulation (Fig. 8P). Thus, box 1 mutation of the GHR is accompanied by a prolonged presence of GH in the nucleus. GHR is also subject to a nuclear translocation in CHO-GHR_{1-638} P300,301,303,305A cells (Fig. 8; B, E, H, K, N, and Q). A portion of GH could possibly translocate to the nucleus, into a bound form with the receptor, as indicated by the double detection of both GH and GHR immunoreactivity (Fig. 8; C, F, I, L, O, and R). Quantitatively, analysis of GH and GHR internalization and nuclear translocation in GHR_{1-638} P300,301,303,305A cells (Fig. 9, A–D) was also very similar to that observed in CHO-GHR_{1-638} Δ297–311 (Fig. 7, A–D).

Measurement of internalized and nuclear translocated hormone by ELISA

The higher concentrations of rGH used for the confocal analysis resulted in different hormone internalization kinetics and accumulation of both cytoplasmic and nuclear GH. Thus, we also examined the internalization and nuclear uptake of 50 nM hGH in CHO-GHR_{1-638}, CHO-GHR_{1-638} Δ297–311, and CHO-GHR_{1-638} P300,301,303,305A cells by ELISA assay. Figure 10 summarizes the data from three independent experiments. In agreement with the confocal images obtained in Figs. 4, 6, and 8, the quantity of internalized hGH gradually increased, over 30 min of incubation, after which it declined (Fig. 10A) in the three cell lines. hGH ELISA was also performed on nuclear protein extracts from cells incubated with 50 nM of hGH. In CHO-GHR_{1-638}, the rapid increase of nuclear hGH accumulation was followed by a slow decrease, starting 15 min after stimulation (Fig. 10B). On the other hand, nuclear accumulation of hGH was continuous during the 60 min of incubation in CHO cells with the box 1 region mutated or deleted (Fig. 10B). Thus, we confirmed that nuclear retention of a physiological concentration of hGH is tyrosine-kinase-activity-dependent.

Staurosporine preferentially increases the nuclear accumulation of nuclear-targeted hGH

To further verify that tyrosine kinase activity was involved in the nuclear retention of GH, we examined the effect of staurosporine on the transient expression of three different hGH constructs (Fig. 11A) in parental CHO cells. Parental-nonreceptor-containing cells were chosen so as not to alter the desired location of the expressed hormones within the cell. Cells were transiently transfected with plasmids encoding WT hGH, hGH (but with the secretion sequence removed) (XS-hGH), and hGH [with the secretion sequence removed but with the addition of the nuclear localization sequence of the SV40 large T antigen (NLS-hGH)]. Characterization of these plasmids will be described in detail in conjunction with an extensive functional analysis [WT-hGH was detected by ELISA in media and cytoplasm but not nucleus; XS-hGH was detected only in cytoplasm; and NLS-hGH was detected predominantly in the nucleus, with small amounts occasionally detected in cytoplasm (presumably either nuclear leakage or newly synthesized NLS-hGH)]. Thus, we could selectively target hGH to either the cytoplasm or nucleus and examine, by ELISA, the effects of staurosporine on the accumulation of the hormone in the selected subcellular location (Fig. 11B). Staurosporine treatment of cells transiently transfected with the WT-hGH expression plasmid resulted in a 20% increase in hGH within the cell (cytoplasm), as measured by ELISA. No hGH was detected in the nucleus in the absence or presence of staurosporine. However, because

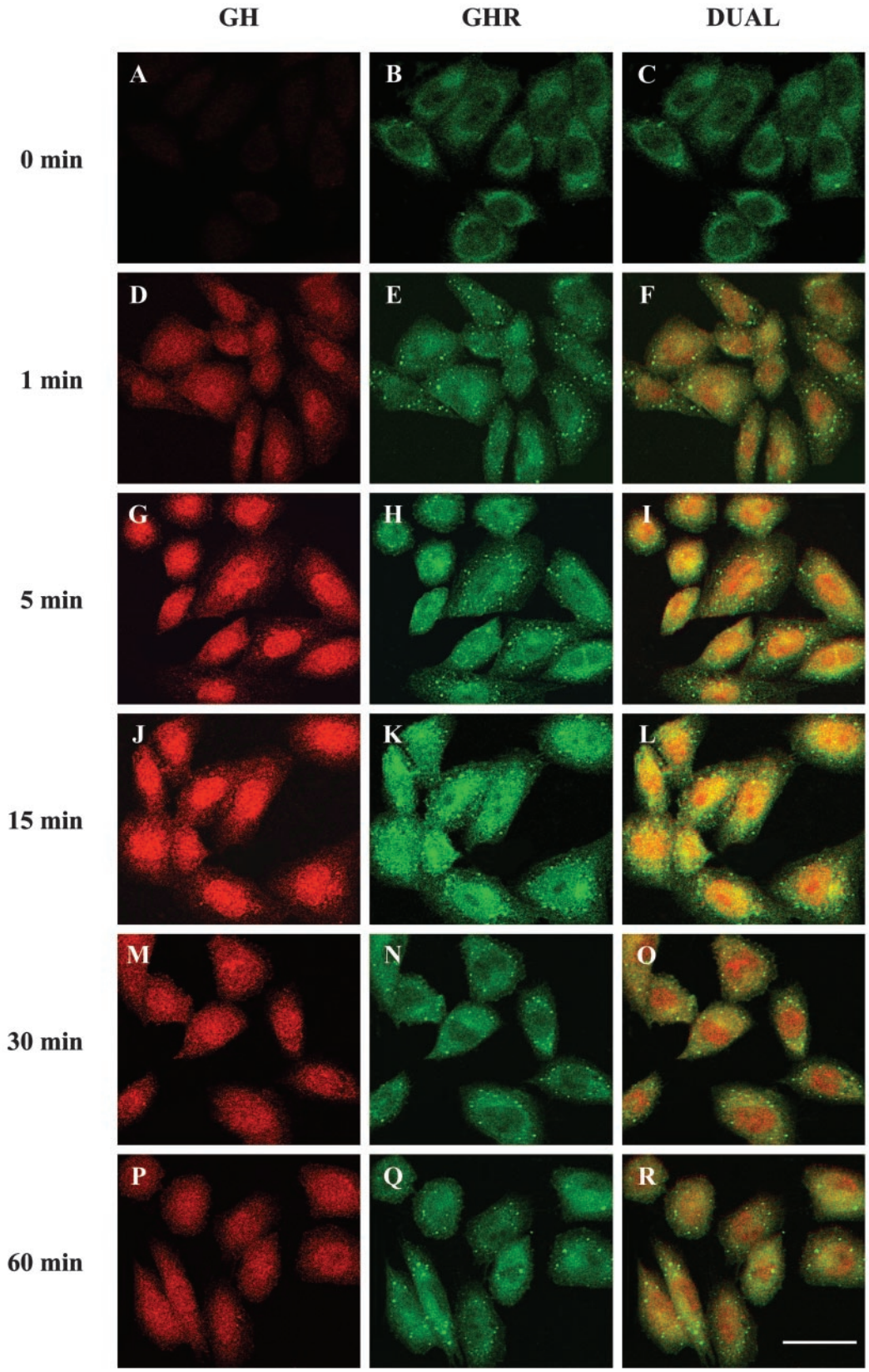

FIG. 8. Kinetics of GH and GHR internalization and nuclear translocation in CHO cells possessing a mutated box 1 region (CHO-GHR$_{1-638}$ P300,301,303,305A). CLSM analysis at 0, 1, 5, 15, 30, and 60 min after GH stimulation was performed as described in *Materials and Methods*. The localization of GH is shown in the *left-hand column*, GHR in the *middle column*, and the dual localization (superimposed images) in the *right-hand column*. *Magnification bar*, 30 μm for all photographs.

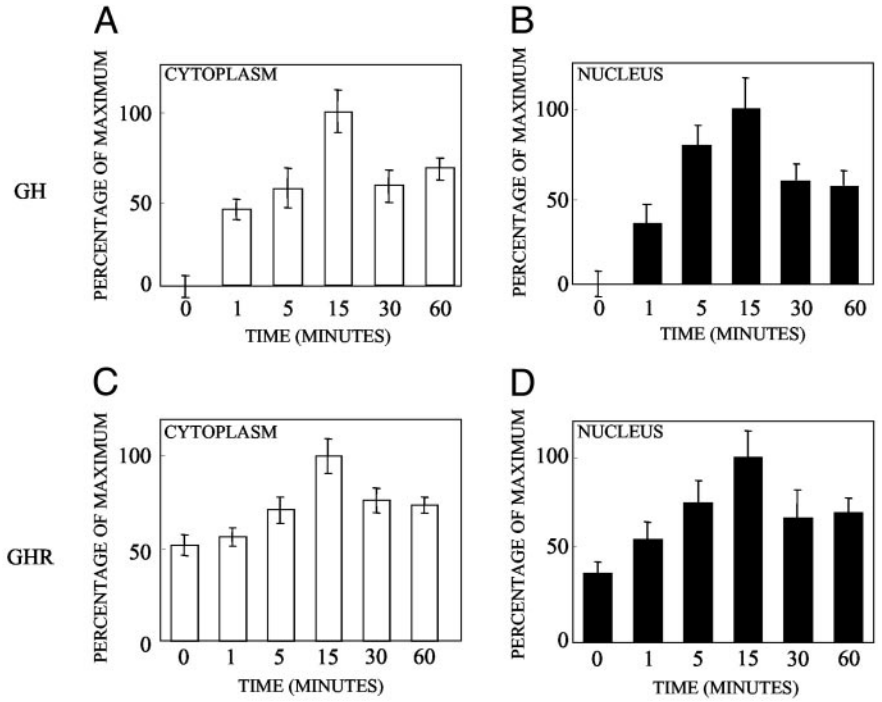

FIG. 9. Quantitative analysis of GH and GHR cytoplasmic internalization and nuclear translocation in cells possessing a mutated box 1 region (CHO-GHR$_{1-638}$ P300,301,303,305A). GH and GHR immunoreactivity were quantified as described in *Materials and Methods*. The results shown are representative of at least three experiments.

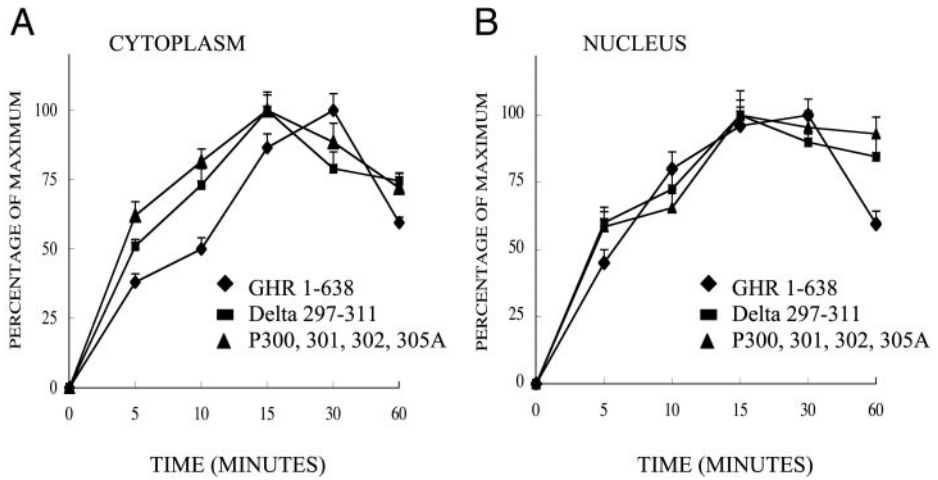

FIG. 10. Kinetic cytoplasmic and nuclear uptake of GH in CHO-GHR$_{1-638}$ cells (-◆-) and in cells lacking the box 1 region (-■-) (CHO-GHR$_{1-638}$ Δ297–311) or possessing a mutated box 1 region (-▲-) (CHO-GHR$_{1-638}$ P300,301,303,305A). Quantitative estimation of GH in each compartment was performed as described in *Materials and Methods*. Each *point* is the mean ± SEM of three ELISA estimations. The results shown are representative of at least three experiments.

staurosporine could potentially interfere with hGH secretion, we also examined the effect of staurosporine on the cytoplasmic accumulation of XS-hGH, which is not secreted from the cell. Again, staurosporine treatment of cells transiently transfected with the XS-hGH expression plasmid resulted in increased accumulation (32%) of XS-hGH within the cytoplasm. No XS-hGH was detected in the nucleus in the absence or presence of staurosporine. We therefore proceeded to examine the nuclear accumulation of NLS-hGH. Staurosporine treatment of cells transiently transfected with the NLS-hGH expression plasmid resulted in a dramatic increase in NLS-hGH in the nucleus (121%). NLS-hGH level in the cytoplasm was close to background. In a control experiment using an NLS-GHBP plasmid construct, we did not find an increase of nuclear GHBP retention under staurosporine treatment (data not shown). Thus, inhibition of tyrosine kinase activity in the cell results in preferential accumulation of nuclear localized GH.

Involvement of GHR dimerization in hGH nuclear translocation

To investigate the role of GHR dimerization in nuclear translocation of hGH, we used the non-receptor-dimerizing hGH antagonist, hGH-G120R, conjugated to FITC and compared it with hGH-FITC. Cells containing the extracellular domain of the human receptor and the transmembrane and intracellular domains of the rat receptor were stimulated with either hormone for 2 and 15 min. Confocal microscopic analysis revealed that cytoplasmic internalization followed the same spatial and temporal pattern for both hGH- and hGH-G120R-labeled hormones (Fig. 12). Incubation for 2 min in the presence of FITC-hGH or FITC-hGH-G120R resulted

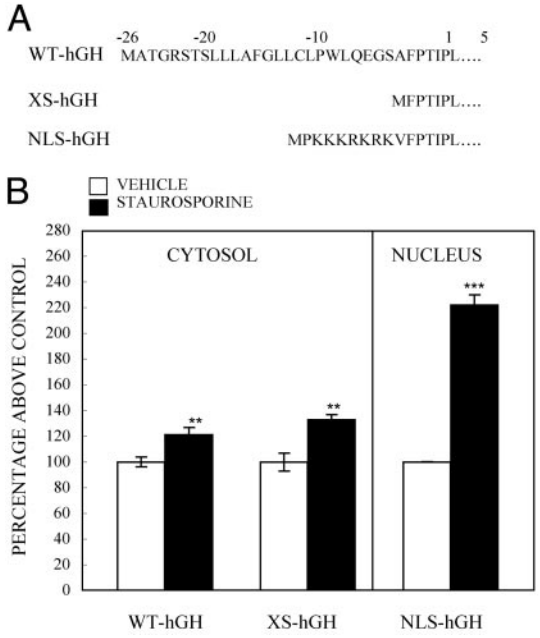

FIG. 11. Effect of staurosporine treatment of cells on the accumulation of WT-hGH, cytoplasmic targeted hGH, and nuclear targeted hGH. A, Structure of the NH2-terminal domain of hGH and the modified forms of hGH. Shown are the residues −26 to 5 of the precursor of the secreted form of hGH (WT-hGH), the sequence of hGH lacking the secretion sequence (XS-hGH), and the sequence of hGH where the NLS of the SV40 large T antigen has been substituted for the secretion sequence (NLS-hGH). B, The level of hGH expression in the presence or absence of 100 nM staurosporine in cells transiently transfected with the respective hGH expression plasmids was measured by ELISA as described in *Materials and Methods*. The level of expression of the hormone in the absence of staurosporine was designated to be 100% for each separate transiently transfected construct. Each *point* is the mean ± SEM of three estimations. The results shown are representative of at least three experiments.

in accumulation of fluorescent label in the cytoplasm (Fig. 12, A and B). At 15 min, we observed the maximal nuclear accumulation of FITC-hGH in BRL cells (Fig. 12C). In contrast, FITC-hGH-G120R did not accumulate in the nucleus 15 min after stimulation (Fig. 12D) nor at longer time periods (data not shown). The ELISA assay was also used to confirm that nuclear translocation of hGH-G120R was impaired (data not shown). Thus, we could demonstrate that cytoplasmic internalization of the GH/GHR complex is not dependent on receptor dimerization, whereas blocking GHR dimerization is sufficient to impair nuclear translocation of GH.

Discussion

We have demonstrated that JAK2 activity and/or tyrosine phosphorylation within the cell is not required for internalization and nuclear translocation of GH. Previous reports also lend credence that tyrosine kinase activity is not required for internalization of GH. Thus, it has been demonstrated that internalization of GH is not energy-dependent (Ref. 5; own unpublished observations), staurosporine does not inhibit internalization of the GHR (36), and GHR with the same box 1 mutations as used here still internalize hormone (13). We have confirmed and extended these observations to also demonstrate that tyrosine phosphorylation is not required for the nuclear translocation of GH. Thus, GH seems to use a distinct mechanism for internalization and nuclear translocation not shared by the polypeptide ligands thus far studied (37). Kinase activity has been reported to influence the nuclear translocation of proteins in two ways. First, phosphorylation of serine and threonine residues adjacent to the NLS significantly alters nuclear import rates (37, 38); and second, kinase activity (particularly PKC) increases the nuclear transport capacity (both import rate and pore size) (39). Although these parameters are necessary for nuclear translocation of growth factors like PRL (40, 41) and α-fibroblast growth factor (42), neither seems to be involved in the nuclear transport of GH. Indeed, no nuclear localization sequence has yet been experimentally identified in either GH or the GHR. Elucidation of the NLS of GH or its receptor should facilitate further study of the mechanism of nuclear translocation of GH.

We report here that tyrosine phosphorylation is a negative regulator of the accumulation of GH within the nucleus after translocation. The mechanism by which GH is removed from the nucleus and the role that tyrosine phosphorylation plays are not known. There exist two possibilities; either the hormone is degraded *in situ* or is reexported to the cytoplasm for degradation or resecretion from the cell. It has been reported that STAT1 is degraded within the nucleus as one mechanism for its removal (43). This degradation requires the phosphorylation and subsequent ubiquitinylation of STAT1 for its degradation by the proteasome. It has also been reported that after GH stimulation, STAT5b complexes with JAK2 in the nucleus and, subsequently, with the phosphotyrosine phosphatase SHP-1 (44). It is known that lysosomal degradation of GH is energy-dependent (45), and inhibition of lysosomal degradation of GH leads to increased nuclear accumulation of GH (18). Such proteolytic degradation in the nucleus may also be the case for the nuclear localized hGH targeted to the nucleus with the SV40 large T-antigen. Further experiments should determine whether nuclear extracts possess the capability to degrade GH and, in combination with microinjection studies in the presence of nuclear export inhibitors, would clarify the final fate of nuclear GH.

It is interesting that PRL (46) and GH (this study) seem to possess two different mechanisms for their internalization and nuclear translocation. This is actually surprising, considering the similarities in their receptor structure (4) and the fact that hGH can act as a ligand for both the GH and PRL receptors (47). The difference in the mechanisms must therefore lie with the receptor molecule. Indeed, previous studies have suggested considerable differences in the behavior of the receptor molecules in the nucleus. The PRL receptor seems to be constitutively localized in the nucleus (46, 48) (at least in some cells) and does not undergo ligand-dependent nuclear translocation (49), whereas the GHR undergoes a rapid and transient nuclear translocation upon ligand stimulation (18). This difference is further exemplified by the fact that PRL can activate both gene transcription (50) and PKC (51) directly in the nucleus, in contrast to GH (22). We have been unable, so far, to find a direct nuclear action for GH, presumably because the majority of the receptor is dependent on ligand for its nuclear localization. This would therefore preclude a direct GH action in nuclei isolated from serum-deprived cells because of the paucity of receptor. We have shown, however, that the majority of cellular JAK2 is

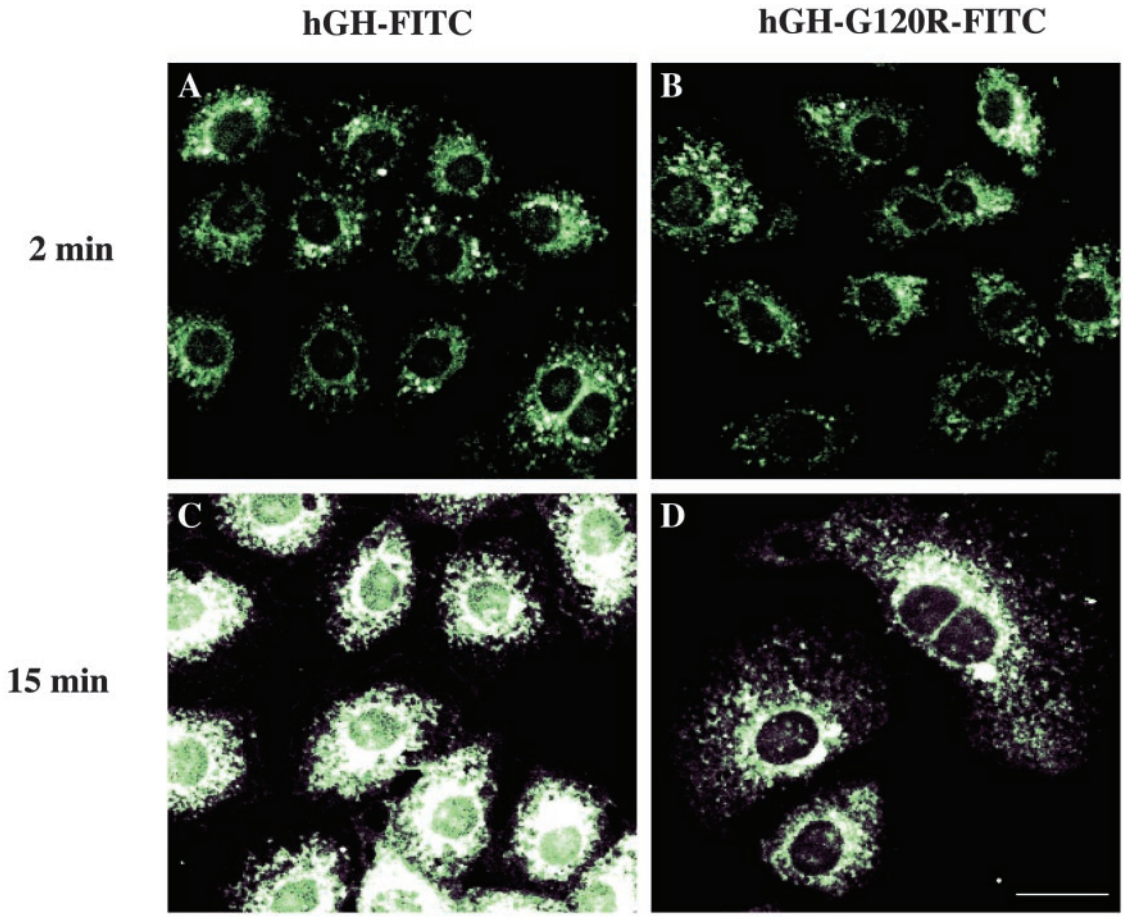

FIG. 12. Role of receptor dimerization in nuclear translocation of GH. Serum-deprived cells were treated with either 50 nM hGH-FITC (A and C) or 50 nM hGH-G120R-FITC (B and D) for 2 or 15 min. CLSM was performed as described in *Materials and Methods*. Rapid cytoplasmic internalization of both hGH-FITC and hGH-G120R-FITC occurred 2 min after stimulation (A and B). hGH-FITC is localized in the perinuclear region and in the nucleus (C), whereas hGH-G120R-FITC accumulated in the cytoplasm and is absent from the nucleus (D), 15 min after stimulation. The results shown are representative of at least three experiments. *Magnification bar*, 50 μm for all photographs.

localized to the nucleus and that GH stimulation of cells results in the appearance of tyrosine-phosphorylated JAK2 in the nucleus, without a change in the subcellular distribution of JAK2 (21). We (our manuscript in preparation) and others (52) have also previously demonstrated that STAT molecules involved in GH signal transduction possess a nucleocytoplasmic distribution in the absence of serum, suggesting that the JAK-STAT pathway can also function directly in the nucleus.

The importance of the receptor in GH nuclear translocation is demonstrated here by the use of the nonreceptor dimerizing hGH antagonist, hGH-G120R, and suggest, for the first time, that nuclear translocation of GH occurs in a complex with its receptor. Although not required for GH endocytosis, it seems that GHR dimerization is a prerequisite for GH nuclear translocation. Such a mechanism of control of translocation across the nuclear membrane has been described for transcription factors and protein kinases and correlates with changes in transcriptional activity (53). Whether GHR dimerization-mediated GH nuclear translocation has an impact on transcriptional activity is under investigation, and recent work demonstrates that nuclear localized GHBP is required for the full transcriptional response to GH through the JAK-STAT pathway (21a). The direct interaction between GH, GHR, and GHBP with transcription factors could provide a mechanistic basis for the function of GH in the nucleus. Indeed, retrotransport of PRL-cyclophilin B complexes in the nucleus of Nb2 cells and its interaction with STAT5 have recently been demonstrated to act as a strong transcriptional inducer of STAT5 activity (50). Identification of GH nuclear targets clearly represents an important challenge for future studies, as recently demonstrated in chicken, where the direct interaction between a herpes virus protein and GH was correlated to T cell cancer resistance (54).

In conclusion, we have demonstrated that tyrosine phosphorylation is not required for internalization and nuclear translocation of growth hormone but is pivotal for the removal of GH from the nucleus, and that GHR dimerization promotes GH nuclear translocation. This raises the possibility of a novel mechanism for nuclear translocation/accumulation used specifically by GH and not other polypeptide ligands.

Acknowledgments

Received October 28, 2002. Accepted March 17, 2003.

Address all correspondence and requests for reprints to: Peter E. Lobie, Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609, Republic of Singapore. E-mail: mcbpel@imcb.nus.edu.sg.

References

1. **Flores-Morales A, Stahlberg N, Tollet-Egnell P, Lundeberg J, Malek RL, Quackenbush J, Lee NH, Norstedt G** 2001 Microarray analysis of the *in vivo* effects of hypophysectomy and growth hormone treatment on gene expression in the rat. Endocrinology 142:3163–3176
2. **Carter-Su C, Schwartz J, Smit LS** 1996 Molecular mechanisms of growth hormone action. Annu Rev Physiol 58:187–207
3. **Argetsinger LS, Carter-Su C** 1996 Mechanism of signaling by growth hormone receptor. Physiol Rev 76:1089–1107
4. **Moutoussamy S, Kelly PA, Finidori J** 1998 Growth-hormone-receptor and cytokine-receptor-family signaling. Eur J Biochem 255:1–11
5. **Roupas P, Herington AC** 1989 Cellular mechanisms in the processing of growth hormone and its receptor. Mol Cell Endocrinol 61:1–12
6. **Ilondo MM, Vanderschueren-Lodeweyckx M, Courtoy PJ, De Meyts P** 1992 Cellular processing of growth hormone in IM-9 cells: evidence for exocytosis of internalized hormone. Endocrinology 130:2037–2044
7. **van Kerkhof P, Sachse M, Klumperman J, Strous GJ** 2001 Growth hormone receptor ubiquitination coincides with recruitment to clathrin-coated membrane domains. J Biol Chem 276:3778–3784
8. **Lobie PE, Sadir R, Graichen R, Mertani HC, Morel G** 1999 Caveolar internalization of growth hormone. Exp Cell Res 246:47–55
9. **Strous GJ, van Kerkhof P, Govers R, Ciechanover A, Schwartz AL** 1996 The ubiquitin conjugation system is required for ligand-induced endocytosis and degradation of the growth hormone receptor. EMBO J 15:3806–3812
10. **Govers R, van Kerkhof P, Schwartz AL, Strous GJ** 1997 Linkage of the ubiquitin-conjugating system and the endocytic pathway in ligand-induced internalization of the growth hormone receptor. EMBO J 16:4851–4858
11. **Govers R, van Kerkhof P, Schwartz AL, Strous GJ** 1998 Di-leucine-mediated internalization of ligand by a truncated growth hormone receptor is independent of the ubiquitin conjugation system. J Biol Chem 273:16426–16433
12. **Govers R, ten Broeke T, van Kerkhof P, Schwartz AL, Strous GJ** 1999 Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor. EMBO J 18:28–36
13. **Allevato G, Billestrup N, Goujon L, Galsgaard ED, Norstedt G, Postel-Vinay MC, Kelly PA, Nielsen JH** 1995 Identification of phenylalanine 346 in the rat growth hormone receptor as being critical for ligand-mediated internalization and down-regulation. J Biol Chem 270:17210–17214
14. **Vleurick L, Pezet A, Kuhn ER, Decuypere E, Edery M** 1999 A beta-turn endocytic code is required for optimal internalization of the growth hormone receptor but not for alpha-adaptin association. Mol Endocrinol 13:1823–1831
15. **Alves dos Santos CM, van Kerkhof P, Strous GJ** 2001 The signal transduction of the growth hormone receptor is regulated by the ubiquitin/proteasome system and continues after endocytosis. J Biol Chem 276:10839–10846
16. **Alves Dos Santos CM, ten Broeke T, Strous GJ** 2001 Growth hormone receptor ubiquitination, endocytosis and degradation are independent of signal transduction via JAK2. J Biol Chem 276:32635–32641
17. **van Kerkhof P, Govers R, Alves dos Santos CM, Strous GJ** 2000 Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. J Biol Chem 275:1575–1580
18. **Lobie PE, Wood TJ, Chen CM, Waters MJ, Norstedt G** 1994 Nuclear translocation and anchorage of the growth hormone receptor. J Biol Chem 269: 31735–31746
19. **Mertani HC, Waters MJ, Morel G** 1996 Cellular trafficking of exogenous growth hormone in dwarf rat pituitary. Neuroendocrinology 63:257–268
20. **Lobie PE., Barnard R, Waters MJ** 1991 The nuclear growth hormone receptor/binding protein: antigenic and physicochemical characterization. J Biol Chem 266:22645–22652
21. **Lobie PE, Ronsin B, Silvennoinen O, Haldosen LA, Norstedt G, Morel G** 1996 Constitutive nuclear localization of Janus kinases 1 and 2. Endocrinology 137:4037–4045

21a. **Graichen R, Sandstedt J, Goh EL, Isaksson OG, Tornell J, Lobie PE** 2003 The growth hormone-binding protein is a location-dependent cytokine receptor transcriptional enhancer. J Biol Chem 278:6346–6354

22. **Lobie PE, Morel G, Mertani HC, Morales-Bustos O, Wood TJJ, Waters MJ, Norstedt G** 1995 The growth hormone receptor, growth hormone and the nucleus. Endocrinol Metab 2:61–72
23. **Mertani HC, Morel G, Lobie PE** 1999 Cytoplasmic and nuclear cytokine complexes. Vitam Horm 57:79–121
24. **Rao YP, Buckley DJ, Olson MD, Buckley AR** 1995 Nuclear translocation of prolactin: collaboration of tyrosine kinase and protein kinase C activation in rat Nb2 node lymphoma cells. J Cell Physiol 163:266–276
25. **Salacinski PRP, McLean C, Sykes JEC, Clement-Jones VV, Lowry PJ** 1981 Iodination of proteins, glycoproteins and peptides using a solid phase oxidizing agent 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril (Iodogen). Anal Biochem 117:136–146
26. **Möller C, Hansson A, Enberg B, Lobie PE, Norstedt G** 1992 Growth hormone induction of tyrosine phosphorylation and activation of mitogen activated protein kinases in cells transfected with rat GH receptor cDNA. J Biol Chem 267:23403–23408
27. **Mathews LS, Enberg B, Norstedt G** 1989 Regulation of rat growth hormone receptor gene expression. J Biol Chem 264:9905–9910
28. **VanderKuur JA, Wang X, Zhang L, Campbell GS, Allevato G, Billestrup N, Norstedt G, Carter-Su C** 1994 Domains of the growth hormone receptor required for association and activation of JAK2 tyrosine kinase. J Biol Chem 269:21709–21717
29. **Gorin E, Gritching G, Goodman HM** 1984 Binding and degradation of ^{125}I-human growth hormone in rat adipocytes. Endocrinology 115:467–475
30. **Horiuchi R, Cheng S, Willingham M, Pastan I** 1982 Inhibition of nuclear uptake of 3–3′,5-triiodo-L-thyronine by monodansylcadaverine in GH3 cells. J Biol Chem 257:3139–3144
31. **Campbell GS, Christian LJ, Carter-Su C** 1993 Evidence for involvement of the growth hormone receptor-associated tyrosine kinase in actions of growth hormone. J Biol Chem 268:7427–7434
32. **Liu N, Mertani HC, Norstedt G, Tornell J, Lobie PE** 1997 Mode of the autocrine/paracrine mechanism of growth hormone action. Exp Cell Res 237:196–206
33. **Sliva D, Wood TJ, Schindler C, Lobie PE, Norstedt G** 1994 Growth hormone specifically regulates serine protease inhibitor gene transcription via gamma-activated sequence-like DNA elements. J Biol Chem 269:26208–26214
34. **Wood TJ, Sliva D, Lobie PE, Pircher TJ, Gouilleux F, Wakao H, Gustafsson JA, Groner B, Norstedt G, Haldosen LA** 1995 Mediation of growth hormone-dependent transcriptional activation by mammary gland factor/Stat 5. J Biol Chem 270:9448–9453
35. **Moller C, Emtner M, Arner P, Norstedt G** 1994 Growth hormone regulation of lipid metabolism in cells transfected with growth hormone receptor cDNA. Mol Cell Endocrinol 99:111–117
36. **Saito Y, Teshima R, Yamazaki T, Ikebuchi H, Sawada J** 1994 Ligand-induced internalization and phosphorylation-dependent degradation of growth hormone receptor in human IM-9 cells. Mol Cell Endocrinol 106:67–74
37. **Rihs H, Peters R** 1989 Nuclear transport kinetics depend on phosphorylation site containing sequences flanking the karyophilic signal of the simian virus 40 T antigen. EMBO J 8:1479–1484
38. **Rihs H, Jans D, Fan H, Peters R** 1991 The rate of nuclear cytoplasmic transport is determined by the casein kinase II site flanking the nuclear localization sequence of the SV 40 T antigen. EMBO J 10:633–639
39. **Feldherr C, Akin D** 1995 Stimulation of nuclear import by simian virus 40-transformed cell extracts is dependent on protein kinase activity. Mol Cell Biol 15:7043–7049
40. **Rao YP, Buckley DJ, Olson MD, Buckley AR** 1995 Nuclear translocation of prolactin: collaboration of tyrosine kinase and protein kinase C activation in rat Nb2 node lymphoma cells. J Cell Physiol 163:266–276
41. **Belkowski SM, Levine JE, Prystowsky MB** 1999 Requirement of PI3-kinase activity for the nuclear transport of prolactin in cloned murine T lymphocytes. J Neuroimmunol 94:40–47
42. **Klingenberg O, Wiedocha A, Citores L, Olsnes S** 2000 Requirement of phosphatidylinositol 3-kinase activity for translocation of exogenous aFGF to the cytosol and nucleus. J Biol Chem 275:11972–11980
43. **Kim TK, Maniatis T** 1996 Regulation of interferon-gamma-activated STAT1 by the ubiquitin-proteasome pathway. Science 273:1717–1719
44. **Ram PA, Waxman DJ** 1997 Interaction of growth hormone-activated STATs with SH2-containing phosphotyrosine phosphatase SHP-1 and nuclear JAK2 tyrosine kinase. J Biol Chem 272:17694–17702
45. **Roupas P, Herington AC** 1987 Processing of growth hormone by rat adipocytes in primary culture: differentiation between release of intact hormone and degradative processing. Endocrinology 121:1521–1530
46. **Rao YP, Olson MD, Buckley DJ, Buckley AR** 1993 Nuclear co-localization of prolactin and the prolactin receptor in rat Nb2 node lymphoma cells. Endocrinology 133:3062–3065
47. **Waters MJ, Friesen HG** 1979 Purification and partial characterization of a nonprimate growth hormone receptor. J Biol Chem 254:6815–6825
48. **Buckley AR, Montgomery DW, Hendrix MJ, Zukoski CF, Putnam CW** 1992 Identification of prolactin receptors in hepatic nuclei. Arch Biochem Biophys 296:198–206
49. **Clevenger CV, Russell DH, Appasamy PM, Prystowsky MB** 1990 Regulation of IL-2 driven T-lymphocyte proliferation by prolactin. Proc Natl Acad Sci USA 87:6460–6464
50. **Rycyzyn MA, Clevenger CV** 2002 The intranuclear prolactin/cyclophilin B complex as a transcriptional inducer. Proc Natl Acad Sci USA 99:6790–6795
51. **Buckley AR, Crowe PD, Russell DH** 1988 Rapid activation of protein kinase C in isolated rat liver nuclei by prolactin, a known hepatic mitogen. Proc Natl Acad Sci USA 85:8649–8653
52. **Wang YF, Yu-Lee LY** 1996 Multiple stat complexes interact at the interferon regulatory factor-1 interferon-gamma activation sequence in prolactin-stimulated Nb2 T cells. Mol Cell Endocrinol 121:19–28
53. **Cyert MS** 2001 Regulation of nuclear localization during signaling. J Biol Chem 276:20805–20808
54. **Liu HC, Kung HJ, Fulton JE, Morgan RW, Cheng HH** 2001 Growth hormone interacts with the Marek's disease virus SORF2 protein and is associated with disease resistance in chicken. Proc Natl Acad Sci USA 98:9203–9208