Studies with a Growth Hormone Antagonist and Dual-fluorescent Confocal Microscopy Demonstrate that the Full-length Human Growth Hormone Receptor, but Not the Truncated Isoform, Is Very Rapidly Internalized Independent of Jak2-Stat5 Signaling*

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We have investigated trafficking of two negative regulators of growth hormone receptor (GHR) signaling: a human, truncated receptor, GHR1–279, and a GH antagonist, B2036. Fluorescent-labeled growth hormone (GH) was rapidly internalized by the full-length GHR, with >80% of the hormone internalized within 5 min of exposure to GH. In contrast, <5% of labeled GH was internalized by cells expressing truncated GHR1–279. Using another truncated receptor, GHR1–317 fused to enhanced green fluorescent protein (EGFP), we have exploited fluorescence energy transfer to monitor the trafficking of ligand-receptor complexes. The data confirmed that internalization of this truncated receptor is very inefficient. It was possible to visualize the truncated GHR1–317-EGFP packaged in the endoplasmic reticulum, its rapid movement in membrane bound vesicles to the Golgi apparatus, and subsequent transport to the cell membrane. The GH antagonist, B2036, blocked Jak2-Stat5-mediated GHR signaling but was internalized with a similar time course to native GH. The results: 1) demonstrate the rapid internalization of GH when studied under physiological conditions; 2) confirm the hypothesis that internalization of cytoplasmic domain truncated human GHRs is very inefficient, which explains their dominant negative action; and 3) show that the antagonist action of B2036 is independent of receptor internalization.

Growth hormone (GH)1 has diverse biological actions, including promoting cellular growth and differentiation, that are mediated by binding to a specific, high affinity cell surface receptor (GHR). The GHR is a member of the type I cytokine family of receptors, which possess in common a single trans-membrane domain and lack intrinsic kinase activity (1). Binding of a single molecule of GH results in receptor dimerization and signaling through a phosphorylation cascade that includes the Jak-Stat pathway. Activation of the Jak-Stat pathway is dependent on an intact cytoplasmic domain of the receptor forming multisubunit complexes with associated tyrosine kinases. Recently, we (2) and others (3) identified a truncated form of the receptor, GHR1–279, in normal human liver and certain human cell lines. This truncated receptor lacks 97% of the cytoplasmic domain of the receptor and has a dominant negative action on Jak-Stat signaling (2). In human tissues the truncated receptor is expressed at a low level compared with the full-length receptor (2, 4). However, in cell transfection experiments, the truncated receptor always demonstrates a greater level of receptor expression at the cell surface compared with the full-length receptor (2). It has been hypothesized that the truncated receptor lacks essential internalization signals in the cytoplasmic domain and therefore remains at the cell surface available to heterodimerize with the full-length receptor and inhibit signaling (2, 5). In addition, the truncated receptor generates large amounts of GH-binding protein (GHBP) (2, 3). Confirmation that these truncated receptors have a dominant negative action on GH signaling comes from patients who are heterozygous for a mutation that encodes a very similar truncated receptor, GHR1–277 (6, 7). These patients have high levels of GHBP and GH insensitivity, presumably because of increased levels of truncated receptor at the cell surface, which fails to internalize and heterodimerizes with the full-length receptor to inhibit signaling (6).

The GHR is internalized via clathrin-coated pits (8, 9). This internalization is dependent upon an intact ubiquitination system, and GHR ubiquitination is dependent upon an intact endocytic pathway (9). It has been suggested that a small fraction of GHR is constitutively ubiquitinated and internalized but that GH-induced dimerization results in an increase in ubiquitination and internalization (9). The Phe327 residue within the cytosolic tail of the GHR is involved in both GHR ubiquitination and ligand-induced receptor endocytosis (9, 10). In addition, it has been reported that there is an ubiquitin-independent internalization signal, based on a di-leucine repeat (Leu347,348), which is inactive in the full-length receptor but is activated in a truncated receptor, GHR1–349 (11). It has been suggested that this motif may function in the internalization of an as yet unidentified GHR isoforms (11).

Internalization is not required for Jak-Stat signal transduction, as GHR mutated at Phe327 signals but is not internalized

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‡ The abbreviations used are: GH, growth hormone; GHR, GH receptor; GHBP, GH-binding protein; hGH, human GH; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; LHRE, lactogen response element; ER, endoplasmic reticulum; TK, tyrosine kinase; FRET, fluorescence energy transfer; Jak, Janus kinase; Stat, signal transducer and activator of transcription.
Texas Red labeling was performed using Texas Red-X succinimidyl ester (Molecular Probes, Leiden, Netherlands). GH or antagonist were dialyzed overnight against borate-buffered saline (pH 8.5). 100 μg of GH or antagonist were incubated with Texas Red at a 1:1 molar ratio in a final volume of 200 μl for 1 h at room temperature and away from light. The reaction mixtures were separated on a 1-ml-bed volume Bio-Gel P10 5-kDa polyacrylamide gel.

Transcription assays were performed in 293 cells expressing the GHR using either a reporter gene containing a Stat5-binding element (LHRE) fused to a minimal TK promoter and luciferase or the LHRE-TK fused to EGFP. Luciferase activity was measured as previously reported (21). In studies using the LHRE-TK-EGFP, 293 cells were plated on 4-well coverglass slides, precoated with fibronectin at a density of 3 × 10^5 cells per well (ml), transfected by the calcium phosphate method with LHRE-TK-EGFP (550 ng/slide), and incubated overnight in complete medium before transfer into serum-free medium containing either 500 ng/ml GH and 100 ng/ml dexamethasone (stimulated) or dexamethasone alone (unstimulated). Fluorescence was detected 24 h later on the stage incubator of the confocal microscope, an analysis of images was performed using the public domain NIH Image (http://rsb.info.nih.gov/nih.image).

**Fluorescence Imaging**—For experiments on living cells, fluorescence was detected using a Molecular Dynamics CLSM2010 confocal fluorescent microscope equipped with dual fluorescence and transmission detection. Cells were transfected from the Silicon Graphics ImageStation (SGI) to the CSLM 2010, converted to Macintosh 8-bit Tagged Image File Format, and analyzed with NIH Image, and the data were exported to EXCEL for further analysis. All experiments were performed at 37 °C using a stage incubator enclosure fitted to the microscope. For the detection of Texas Red, cells were excited at 585 nm, and detection was through a 610 nm long pass filter (red channel). For EGFP, cells were excited at 488 nm and detection was through a 530 nm band pass filter (green channel). In all experiments used for quantitative analysis, the laser power was kept below the level of saturation for the fluor and the photomultiplier voltage set so that the brightest pixels were <250 8-bit units. In the fluorescence energy transfer (FRET) experiments cells were excited with the 488 nm line, and data were collected on the red channel.

**Calculation of Internalization**—Using NIH Image the fluorescence within the cell membrane was divided by the total fluorescence of the cell after subtracting the background. This number was multiplied by 100 to give a percentage, and the mean of two measurements for each cell was used in the analysis.

**Western Ligand Blotting**—293 cells were starved overnight in serum-free medium and then stimulated for 15 min at 37 °C with GH or antagonist. Cells were lysed in PBS-TDS (phosphate-buffered saline, 1% Triton X-100, 12 mM sodium deoxycholate, 3.5 mM SDS, and 4.7 mM sodium orthovandate), and 1 mg of protein was precipitated with Jakt2 antibody (Upstate Biotechnology, Buckingham, UK) at 1:250 dilution with 20 μl of protein A-agarose (Sigma). Precipitated proteins were separated on a 10% SDS-polyacrylamide gel and after blotting onto polyvinylidene difluoride, phosphorylation was detected with an antiphosphotyrosine antibody (1:2500) (4G10, Upstate Biotechnology, Buckingham, UK) and the ECL system (Amersham Pharmacia Biotech).

**RESULTS**

To compare internalization of the truncated GHR1–279 with the full-length human GHR, we generated stable clones in a human kidney cell line, 293 cells. Cells were transfected with either receptor and selected under G418. Clones were picked that expressed a high level of GH binding. In our experimental conditions (125I-GH, 1 × 10^3 cpm, on 3 × 10^5 cells), the selected full-length human GHR expressing clone showed 16% specific binding, and the GHR1–279 clone showed 26% specific binding.

**Characterization of Stable Clone 293 Cells Expressing the Full-length and Truncated GHR and Affinity of the GH Antagonist**—In cells expressing the full-length receptor, competition studies with 125I-GH and 125I-B2036 showed a single class of receptors (Fig. 1). The antagonist B2036 has 8 mutations in the GH binding site 1, which would be expected to increase its affinity for the extracellular domain of the GHR (18). In an assay based on GHBP immobilized on an enzyme-linked immunosorbent assay plate, the antagonist had an affinity five...
times greater than native GH. We compared the binding of GH and the antagonist, B2036, on intact cells expressing the full-length GHR. In displacement studies using either labeled GH or antagonist, the apparent affinities calculated by Scatchard analysis for GH \( (K_a = 1.00 \times 10^9 \text{ M}^{-1}) \) and B2036 \( (K_a = 0.96 \times 10^9 \text{ M}^{-1}) \) were comparable (Fig. 1). Thus, in intact cells, the antagonist appears to have a similar affinity to native GH.

Functional activity of the full-length receptor was measured in transient transfections with Stat5 reporter constructs, consisting of Stat5 binding sites (LHRE) fused to either EGFP or luciferase. In the luciferase assay test, GH stimulation induced a bell-shaped curve with maximal stimulation (5-fold) occurring between 50 and 500 ng/ml and returning to basal values for GH levels not shown.).

Labeling of GH and Antagonist with Texas Red—For internalization studies, GH and the antagonist B2036 were labeled with Texas Red. We first examined the effect of labeling on GH binding to its receptor and then incubated Texas Red with GH at different molar ratios from 1:1 to 1:10 (GH:Texas Red). A ratio greater than 1:3 resulted in a GH molecule with a reduced affinity for the GHR. Therefore, for all of the studies we elected to label GH and antagonist with a 1:1 molar ratio of protein to Texas Red.

Internalization of Texas Red-labeled GH in 293 Cells Expressing the Full-length and Truncated GHR—To study internalization, 293 cells were plated on coverglass and incubated overnight in serum-free medium. Analysis was performed by confocal microscopy at 37 °C. 10 nM Texas Red-labeled GH was added in new starvation medium, and the cells were incubated for 5 min at 37 °C before washing and the addition of phenol red-free medium. Confocal images were recorded sequentially over time. Images were analyzed with NIH Image, and internalization was assessed by measuring the fluorescence within the cell membrane expressed as a percentage of the total fluorescence associated with the cell. No binding or internalization was seen in 293 cells not transfected with the GHR, and in all experiments binding and internalization were specifically displaced by the simultaneous addition of 2 μg of unlabeled GH.

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\(^2\) William Bennett, personal communication.
the full-length human GHR and transiently transfected with a Stat5
fluorescence.

Confocal images (hGH was rapid with more than 80% of labeled GH internalized
at 5 min (Fig. 3A), and membrane binding was difficult to
detect at 20 min (Fig. 3B). After 15 min, most GH was internalized (mean ± S.E., 92 ± 1.3%; n = 8 cells). In contrast, cells
expressing the truncated GHR1–279, which lacks 97% of the
cytoplasmic domain of the receptor, showed little internalization. At 5 min there was well defined surface binding with <5%
internalization of Texas Red-labeled GH (Fig. 3C), and at 20

min there was <30% internalization (Fig. 3D). After 15 min, internalization of Texas Red-labeled GH was 6.7 ± 1.8%
(mean ± S.E.; n = 14 cells). The difference in internalization
for the full-length and truncated receptor over time is shown in
Fig. 3E.

Previous publications (19) had reported inhibition of internal-
ization by cooling cells to 4 °C. We attempted to repeat these
studies. However, cells incubated for 10 min at 4 °C on cover-
glass behaved abnormally, showing delayed internalization
and frequently separating from the coverglass.

Studies with GHR-EGFP Fusion Proteins—To further study
the trafficking of the GHR, we generated fusion proteins with
EGFP. Transient expression of the EGFP expression vector
alone (pEGFP-N2, CLONTECH) in 293 cells resulted in a dif-
fuse distribution of fluorescence throughout the cytoplasm as
well as in the nucleus of some cells. In contrast, transient and
stable transfection of the truncated GHR1–317 fused to EGFP
resulted in a cell surface distribution as shown by radioligand
binding and laser scanning confocal microscopy (Fig. 4). In
addition, the GHR1–317-EGFP was distributed within the cell
consistent with an endoplasmic reticulum (ER) localization as
well as in a large juxtanuclear fluorescent area representing
the Golgi complex (Fig. 4A). The Golgi localization of the GHR
represented a large proportion of the total fluorescent GHR in
the cell. Control experiments with classical immunofluores-
cence on fixed cells were performed to verify that the presence
of the GFP moiety did not significantly alter the distribution
of the receptor. Labeling was done on 293 cells expressing either
the full-length or the 279 truncated receptor permeabilized or
not with Triton X-100 (Fig. 5). Labeling was present both at the
cell surface and in intracellular compartments with a jux-

Fig. 2. Stimulation of the LHRE-TK-EGFP reporter gene by hGH. Confocal images (×20) are shown of 293 cells stably expressing
the full-length human GHR and transiently transfected with a Stat5
reporter construct with the LHRE-TK fused to EGFP. A, cells incubated
in the absence of GH showing occasional cells with background fluores-
cence. B, cells incubated with GH at 500 ng showing induction of
fluorescence. C, graph showing intensity of fluorescence plotted against
cell size for cells stimulated with GH or unstimulated. The images
analyzed were all under the level of saturation for the detector. How-
ever, to provide a clear representation and show the backgrounds, the
contrast has been enhanced in both A and B.

For cells expressing the full-length GHR, internalization of
GH was rapid with more than 80% of labeled GH internalized
at 5 min (Fig. 3A), and membrane binding was difficult to
detect at 20 min (Fig. 3B). After 15 min, most GH was internalized (mean ± S.E., 92 ± 1.3%; n = 8 cells). In contrast, cells
expressing the truncated GHR1–279, which lacks 97% of the
cytoplasmic domain of the receptor, showed little internalization. At 5 min there was well defined surface binding with <5%
internalization of Texas Red-labeled GH (Fig. 3C), and at 20

channel.
The truncated GHR-EGFP fusion would not be expected to internalize based on previous reports (10) suggesting that F327 is required for this process. Cells were incubated with Texas Red-GH as described above and imaged as described previously (Fig. 6). After 30 min at 37 °C, 24% of the cell-associated Texas Red-GH was internalized, whereas 85% of the truncated receptor was within the cell. The FRET image shows that 21% of the GH-GHR complexes were internalized and that the blue to red and the orange to red images are superimposed, confirming that ligand associated with the cell is receptor-bound and internalization of the truncated receptor-ligand complexes is very inefficient compared with that seen with full-length GHR.

Visualization of GHR1–317-EGFP Trafficking—The EGFP fusion provided us with the opportunity for real time analysis of protein trafficking events in individual cells (Fig. 4). Packaging and movement of the receptor in vesicles was easily visualized. Budding of vesicles and there transport to the Golgi was rapid, occurring within seconds, and at this stage a large proportion of the GHR remained within the Golgi. Vesicles budding from the Golgi apparatus appeared to move in a random fashion toward the cell surface membrane and fused with the membrane (Fig. 4). A small proportion of the vesicles were moving from the cell surface membrane into the cell, presumably representing membrane turnover.

Studies on the GH Antagonist—The GH antagonist, B2036, inhibited GH stimulation of the Stat5 reporter construct in a dose-dependent manner with complete inhibition at a 1:5 molar ratio of GH to antagonist (Fig. 7A). Western blotting confirmed that the antagonist fails to induce Jak2 phosphorylation (Fig. 7B). Internalization of the antagonist was studied in a manner similar to native GH. Texas Red-labeled antagonist was incubated with 293 cells stably expressing the full-length GHR for 5 min and then washed; the cells were then imaged by confocal
microscopy. The Texas Red-labeled antagonist showed identical internalization dynamics to the native GH with the majority of antagonist internalized by 5 min after exposure to the antagonist (Fig. 7C).

**DISCUSSION**

The studies reported here demonstrate that internalization of GH with its receptor is a much more rapid process than previously appreciated. Studies using iodinated GH have generally been performed at 4 °C to prevent internalization and allow equilibrium of bound and unbound GH to occur before warming the cells and studying internalization by acid stripping the surface bound ligand (9, 15, 19). In these studies internalization occurred over a period of 1 h with 30 (9) or 65% (15) internalized at 30 min and 85% at 60 min (15). In our experiments using confocal microscopy and Texas Red-labeled GH, surface binding could be demonstrated by briefly incubating the cells at 4 °C and then scanning. However, when cells were incubated with fluorescent GH at 37 °C and immediately scanned, the majority of labeled GH was internalized by 5 min, and after this time point it became difficult to see the cell surface binding as almost all GH bound to its receptor was internalized. It is likely that the previously reported slower course for internalization, as well as the lack of complete internalization, was due to the chilling of cells, as we were able to delay internalization by this procedure. However, in our experiments the chilling of cells was also associated with other changes. In particular, the cells tended to lift from the coverslip after 10 min at 4 °C, and it is probable that these non-physiological conditions may cause other changes in cell function.

We studied the internalization of a truncated GHR1–279 that is found in normal human tissues (2, 3). This truncated GHR, lacking the major part of the cytoplasmic domain of the receptor, acts as a dominant negative inhibitor of GHR signaling (2) and generates large amounts of GH-binding protein (2, 3). Using confocal microscopy and Texas Red-labeled GH, there was a great difference between internalization of the full-length and of the truncated receptor. At 5 min almost all labeled GH was internalized by the full-length GHR, whereas for the truncated receptor <5% was internalized. A similar but less dramatic difference in internalization between the full-length and truncated receptor (70 versus 10% at 1 h) has been shown by acid stripping (5). This failure of internalization of the truncated human receptor could be predicted from previous studies with the rat and rabbit receptor. In these studies, Phe327, which is deleted in the truncated receptor, proved to be essential for normal ubiquitination and internalization of the receptor (9, 10). In our experiments we also show a great difference in the kinetics of receptor internalization. After 1 h of exposure, we observed some internalization of the truncated receptor (between 5 and 30%). This degree of receptor internalization is similar to that reported for other truncated receptors (5, 9, 10). This internalization may represent cell surface membrane turnover or an uncharacterized internalization signal such as the di-leucine-mediated internalization reported for another truncated GHR (11). Experiments using acid stripping to monitor the cell surface bound GH after 1 h of exposure also reported similar differences in internalization between the full-length and truncated receptor (70 versus 10%) (5). The demonstration that the truncated receptor fails to internalize confirms the hypothesis that a lack of internalization underlies the mechanism for its dominant negative action (2, 6).

Transfection of 293 cells with the EGFP expression vector
alone showed diffuse fluorescence throughout the cell including the nucleus, demonstrating that the EGFP protein alone moves between all compartments of the cell. In contrast, the truncated GHR1–317-EGFP fusion protein showed a specific cellular localization, similar to that seen by immunofluorescence in cells expressing full-length and truncated receptor. The GHR-EGFP fusion was visualized within vesicles generated at the ER and transported to the Golgi. The fluorescence seen within the cell represents packaging of the GHR and translocation to the cell surface, which could be observed in real time and was a rapid process. The truncated receptor emerged from the ER at multiple peripheral sites resulting in the accumulation of chimeric protein in numerous peripheral membrane structures. These units, once established, appeared to rapidly (within seconds) translocate to the Golgi apparatus by a process resembling a random walk. From the Golgi apparatus vesicles budded off to then transfer and fuse with the cell membrane. The dynamics of this vesicular transport were very similar to that shown for ER-to-Golgi transport elucidated with a viral glycoprotein tagged to GFP (21). To date, there has been no report analyzing the cellular distribution and dynamic trafficking of a cytokine receptor linked to GFP. The truncated receptor that we studied allowed us mainly to visualize translocation to the cell surface membrane, as the receptor lacked the essential internalization domain (9). The lack of internalization was confirmed by the studies with FRET. However, a proportion of fluorescence was
internalized and was presumed to be the result of nonspecific membrane turnover as was the case for the other truncated receptors. Previous receptor studies have reported visualization of the glucocorticoid receptor (22) and the G protein-coupled cholecystokinin and β2-adrenergic receptors (23, 24). The internalization of the β2-adrenergic receptor is slower than we have demonstrated with the GHR, with only 30% internalized 20 min after exposure to ligand in HeLa cells (24). However, in 293 cells a β2-adrenergic-GFP fusion protein was at least partially internalized within 5 min of exposure to agonist (28). The kinetics of internalization are different for these G protein-coupled receptors, which are recycled, and for the cholecystokinin receptor, for which the recycling time is 20 to 60 min (23).

B2036 is an analogue of human GH in which 8 amino acids in binding site 1 are mutated to increase the binding affinity to the receptor, and 1 amino acid in binding site 2 is mutated to prevent binding to a second receptor. Preliminary reports in normal subjects and acromegalic patients suggest that B2036 can reduce IGF-I levels (16, 17). However, to date there are no reports on the effect of this antagonist at the level of receptor internalization and signaling. The mutations selected for site 1 were based on observations made using the extracellular domain of the receptor to select for high affinity variants of hGH (18). Using an assay based on displacement of hGH from immobilized hGHBP, B2036 had five times the affinity to hGH.

However, in our assay, based on full-length GHR in intact cells, B2036 had an affinity comparable to hGH. This assay presumably measures the affinity of GH for the GHR dimer. It may be that as the GH antagonist (GHa) is thought to dimerize in the complex GHR-GHa-GHa-GHR (15), this could give an affinity similar to GH, which forms a dimer in the complex GHR-GHR-GHR. The GH antagonist G120K (B2036) has site 2 for binding GHR mutated, similar to the previously reported G120R, which blocks GH-stimulated cell proliferation (14). Our functional studies demonstrated that the antagonist G120K completely blocked GHR signaling through Stat5 at a 1:5 molar ratio. This was associated with inhibition of Jak2 phosphorylation. Our studies confirm and extend the previous reports that the GH antagonist G120R inhibits signaling (15) by demonstrating that G120R has a similar action, blocks Jak2 phosphorylation, and also completely abolishes Stat5 signaling. The GH antagonist G120R has previously been shown to internalize, suggesting that the abilities of GH to stimulate tyrosine phosphorylation and internalization are separate functions (15). The time course for internalization of the antagonist was similar to that reported for native GH with approximately 75% internalized within 40 min. We considered the possibility that the time course for internalization may differ between the antagonist and native hormone, but this time lag may have been missed in previous experiments. Under physiological conditions we found an identical level of internalization for the antagonist and native GH, with the major part of both of them internalized within 5 min of exposure to labeled hormone. These results demonstrate the potent antagonist action of G120K on Jak-Stat signaling, indicating that this antagonist action is unrelated to any change in internalization.

Together our results demonstrate that GHR trafficking can be studied by dual-fluorescent confocal microscopy. Using chimeric receptors fused to EGFP, we have visualized the translocation of the GHR from the ER to the cell surface. The studies of truncated GHR, which acts as an antagonist to GHR signaling, confirm that these receptors are unable to internalize rapidly, which explains their dominant negative action and also accounts for the accumulation to high levels of such mutants, a process that would enhance their dominant negative activity. The GH antagonist G120K behaves in a manner similar to G120R, blocking GHR signaling through Jak2-Stat5. Fluorescent labeling of the antagonist G120K confirms that its antagonist action does not effect internalization, which occurs despite inhibition of receptor signaling.

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