Growth hormone induces detergent insolubility of GH receptors in IM-9 cells

Jeffrey F. Goldsmith, Sung Joong Lee, Jing Jiang, and Stuart J. Frank. Growth hormone induces detergent insolubility of GH receptors in IM-9 cells. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E932–E941, 1997.—In this study, we examined human growth hormone (hGH)-induced changes in nonionic detergent solubility characteristics of its receptor (hGHR). Exposure of IM-9 cells to hGH caused a time- and concentration-dependent loss of immunoblottable detergent-extractable hGHRs and a corresponding accumulation of receptors in a detergent-insoluble pool. At 37°C, the loss of detergent-soluble and the accumulation of detergent-insoluble hGHRs both preceded hGH-induced loss of total cell hGHRs. The detergent-insoluble receptor pool was progressively enriched in an apparent disulfide-linked form of the hGHR. Exposure to hGH at 4°C allowed hGH-induced hGHR disulfide linkage but did not promote changes in receptor detergent solubility, indicating that hGHR detergent insolubility cannot be explained solely by the formation of that linkage. Experiments carried out with hGH at 20°C and with the phorbol ester, phorbol-12,13-myristate acetate, at 37°C indicated that loss of detergent-soluble hGHRs can be uncoupled from accumulation of detergent-insoluble receptors. From these data, we envision at least two related, but coupled from accumulation of detergent-insoluble receptors. From these data, we envision at least two related, but coupled from accumulation of detergent-insoluble receptors. From these data, we envision at least two related, but coupled from accumulation of detergent-insoluble receptors. From these data, we envision at least two related, but coupled from accumulation of detergent-insoluble receptors. From these data, we envision at least two related, but coupled from accumulation of detergent-insoluble receptors. From these data, we envision at least two related, but

downregulation; receptor trafficking; protein sorting; signal

BINDING OF A POLYPEPTIDE ligand to its cell surface receptor typically sets into motion a series of ligand-specific events. These include signal initiation, ligand-receptor internalization, signal desensitization, and intracellular routing and disposition (degradation and/or recycling) of ligand and receptor (28). Interaction of growth hormone (GH), a 22-kDa peptide hormone, with its receptor (GHR) leads to rapid dimerization of GHRs with formation of a 1:2 GH-GHR complex; this dimerization is believed obligated for GH signal transduction (7, 16). GH-induced GHR dimerization acutely leads to activation and tyrosine autophosphorylation of the GHR-associated cytoplasmic tyrosine kinase, JAK2, which promotes transient tyrosine phosphorylation of the GHR (2). These events initiate signal cascades that involve enzymatic activation of ras, mitogen-activated protein kinase, and phosphatidylinositol-3′ kinase, as well as the recruitment of the latent cytoplasmic transcription factors, STATs 1, 3, and 5, to the GHR-JAK2 complex, where they are tyrosine phosphorylated and activated (2, 30). Shortly after initiation of this signaling, surface GHRs undergo internalization, a process dependent on the presence of a phenylalanine residue in the proximal one-third of the receptor cytoplasmic domain (1) and an intact cellular ubiquitination system (25). Although events that desensitize GH-induced signaling are incompletely understood, it is presumed that GHR internalization as well as GH-induced activation of protein tyrosine phosphatase(s) (12) contributes to this desensitization.

Multiple observations have been made regarding the fate of bound GH after the processes referred to above are initiated. Bound GH has been shown to be 1) internalized and degraded in lysosomes (4, 14), 2) exocytosed, either intact or degraded, from the cell (15), or 3) targeted to the nucleus, where its associations and functions are as yet unclear (20). The fate of GH-activated GHRs is even less clear, owing in large part to the paucity of direct-reacting reagents for use in tracking the receptor. Downregulation of GHRs (14, 18), defined as a decrease in cell surface GHR concentration after chronic prior exposure to GH, is presumed related to GHR internalization (1) and perhaps receptor degradation (24) or sequestration, but these phenomena have not been elucidated. Transient GH-induced association of the GHR with the nucleus has been observed and may have either a direct role in signaling or serve in this respect merely to transport GH to this subcellular destination (21).

In our previous studies, we have detected in human (h) IM-9 lymphoblasts the hGH-induced appearance of a high-M₄ form of the hGHR when detergent-soluble cellular proteins are electrophoretically resolved under nonreduced conditions and anti-hGHR immunoblotted (10). Roughly one-half of the hGHRs that become acutely tyrosine phosphorylated in response to hGH treatment undergo this apparent disulfide linkage, although the role played by disulfide-linked hGHRs with regard to signaling and/or receptor trafficking is unclear. In this study we further examined the fate of activated receptors in IM-9 cells by monitoring the detergent extractability of hGHRs after stimulation of cells with hGH. We observed a quantitatively significant and progressive hGH-induced insolubility of hGHRs in Triton X-100 that cannot be accounted for exclusively by formation of the apparent disulfide-linked form of the receptor. Our results indicate the existence of a hGH-induced pool of long-lived hGHRs not previously detected biochemically in these cells.
METHODS

Materials. Recombinant hGH was kindly provided by Eli Lilly (Indianapolis, IN). Phorbol-12,13-myristate acetate (PMA), staurosporine, cytochalasin B, nocodazole, and nystatin were purchased from Sigma Chemical (St. Louis, MO), as were routine reagents, unless otherwise noted.

IM-9 cell culture, stimulation, and protein extraction. IM-9 cells were maintained as described previously and stimulated with hGH or PMA after serum starvation for 16–20 h (10). Serum starvation was accomplished by substituting 0.5% (wt/vol) bovine serum albumin (BSA; fraction V, Boehringer Lilly, Indianapolis, IN) for fetal calf serum in the culture medium. Serum-starved cells were resuspended at 25–50 × 10⁶ cells/ml in Binding buffer (BB; consisting of 25 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 0.1% (wt/vol) BSA, and 1 mM dextrose). After a 15-min preincubation at the specified temperature for equilibration, hGH (added at a 1:40 dilution), PMA (added at a 1:100 dilution), or vehicle (BB or dimethyl sulfoxide, depending on the stimulus being controlled) was added for the indicated durations. For time course experiments, hGH, PMA, or BB was added such that hGH and PMA are expressed as 30 min into hGH treatment [45.4 ± 3.8% (n = 5) of total hGHRs remaining vs. 63.1 ± 9.6% (n = 4) of total hGHRs remaining at 30 min (P = 0.0001)]. The difference became more progressive with increasing duration of hGH incubation and was more notable after 120 min [22.8 ± 3.2% (n = 7) of detergent-soluble hGHRs remaining compared with unstimulated controls, n = 2]. In contrast, the relative hGH-induced loss of detergent-soluble hGHRs significantly differed from that of total hGHRs as early as 30 min into hGH treatment [69.4 ± 5.0% (n = 4) of detergent-soluble hGHRs remaining compared with 102.7 ± 3.8% (n = 2) of total hGHRs remaining at that time point (P = 0.01)].

The time courses of hGH-induced loss of detergent-soluble and total hGHRs are qualitatively compared in Fig. 1C, in which the pooled results of multiple experiments like those in Fig. 1, A and B, are graphically displayed. Significant hGH-induced loss of total hGHRs was observed only after 180 min of hormone exposure (38.1 ± 5.8% of hGHRs remaining compared with unstimulated controls, n = 2). In contrast, the relative hGH-induced loss of detergent-soluble hGHRs significantly differed from that of total hGHRs as early as 30 min into hGH treatment [69.4 ± 5.0% (n = 4) of detergent-soluble hGHRs remaining compared with 102.7 ± 3.8% (n = 2) of total hGHRs remaining at that time point (P = 0.01)]. This difference became more progressive with increasing duration of hGH incubation and was more notable after 120 min [22.8 ± 3.2% (n = 7) of detergent-soluble hGHRs remaining compared with 83.1 ± 9.6% (n = 4) of total hGHRs remaining (P = 0.0001)].

RESULTS

hGH induces detergent insolubility of the IM-9 hGH receptor. To examine the influence of hGH on the cellular level of hGHRs, IM-9 cells were exposed to the hormone at 37°C and evaluated by specific anti-hGHR immunoblotting. When cells were lysed in a buffer containing 0.2% Triton X-100, the level of immunoblottable hGHRs in the detergent-soluble cell extract was observed to progressively decline after exposure to hGH for 30 min or longer (Fig. 1A). To determine whether this hGH-induced loss of detergent-soluble hGHRs reflected a loss of total cell hGHRs, cells were solubilized directly in SDS (1%) sample buffer, and the resulting total cell extract was analyzed by anti-hGHR immunoblotting. Very little change in the level of total hGHRs was detected after even 120 min of hGH exposure (Fig. 1B).

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The finding that hGH induced a more rapid decline in detergent-soluble hGHRs than in total hGHRs led us to consider that another pool of receptors might be accounted for when only the detergent-soluble fraction of the cell was analyzed. Therefore, the insoluble pellet remaining after detergent extraction was resuspended in SDS sample buffer and solubilized. After separation of proteins by SDS-PAGE, hGHRs present in this detergent-insoluble extract were detected by anti-hGHR immunoblotting. As seen in Fig. 1D, treatment with hGH for periods of 30 or more min promoted

and a 28-mm MicroNikkor lens over a lightbox of variable intensity (Northern Light Precision 890, Imaging Research, Toronto, Canada). Quantification was performed using a Macintosh II-based image analysis program (Image 1.49, developed by W. S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). The fraction of hGHRs remaining in extracts (detergent-soluble or total) from hGH-treated cells was estimated by measuring by densitometry the intensity of the specifically detected hGHR signal relative to that signal present within the same experiment in extracts from unstimulated cells. As indicated when graphically shown, pooled data from numerous experiments are displayed as the means ± SE. The significances of differences of pooled results are estimated by unpaired t-tests.

Electrophoresis and immunoblotting. Resolution of proteins in the detergent-soluble, detergent-insoluble, and total cell extracts (prepared as above) under reduced or nonreduced conditions by SDS-polyacrylamide gel electrophoresis (PAGE), Western transfer of proteins, and blocking of nitrocellulose membranes [Hybond-enhanced chemiluminescence (ECL); Amersham] with 2% BSA were performed as previously described (10, 30). Anti-hGHR serum (directed at the residue 317–620 or 271–620 regions of the cytoplasmic domain) was prepared using a solid-state video camera (Sony-77, Sony) and was used for detection of specifically recognized hGHRs, according to the manufacturer’s suggestions.

Data analysis. Densitometry of ECL immunoblots was performed using a solid-state video camera (Sony-77, Sony) and a 28-mm MicroNikkor lens over a lightbox of variable intensity (Northern Light Precision 890, Imaging Research, Toronto, Canada). Quantification was performed using a Macintosh II-based image analysis program (Image 1.49, developed by W. S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). The fraction of hGHRs remaining in extracts (detergent-soluble or total) from hGH-treated cells was estimated by measuring by densitometry the intensity of the specifically detected hGHR signal relative to that signal present within the same experiment in extracts from unstimulated cells. As indicated when graphically shown, pooled data from numerous experiments are displayed as the means ± SE. The significances of differences of pooled results are estimated by unpaired t-tests.
accumulation of hGHRs in the detergent-insoluble fraction. hGH-induced detergent-insoluble hGHRs were most detectable at 60–120 min and were somewhat diminished after 180 min of hGH exposure (Fig. 1D and data not shown). These findings suggested that, at 37°C, a significant redistribution of hGHRs from detergent-soluble to detergent-insoluble subcellular fractions was induced during 30–120 min of exposure to hGH.

Alterations in hGHR detergent solubility were hGH concentration dependent (Fig. 2). Both loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs were observed after 60 min of exposure to as little as 5 ng/ml of hGH, a concentration of GH readily achieved in the circulation of rodents and primates. The most demonstrable effects were seen at hGH concentrations of 50–500 ng/ml; further experiments were therefore carried out with 500 ng/ml hGH. When cells were exposed to this concentration of hGH for only 10 min, followed by washout of the hormone and resuspension in buffer alone for 90 min, both the loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs proceeded despite the lack of continued presence of the hormone (not shown).

The same general effect of hGH on the detergent solubility of the hGHR was noted over a range of conditions used during cell lysis and protein extraction. Loss of detergent-soluble hGHRs and accumulation of the receptor in the detergent-insoluble fraction in re-
response to hGH were not significantly affected by varying the concentrations of Triton X-100 (0.2–4%) or NaCl (0–300 mM) or by use of Nonidet P-40 instead of Triton X-100 in the extraction buffer (not shown).

The pool of hGH-induced detergent-insoluble hGHRs includes disulfide-linked hGHRs. We investigated the distribution of the hGH-induced apparent disulfide-linked hGHR form in detergent-soluble and -insoluble fractions (Fig. 3). As previously observed (10), the level of detergent-soluble disulfide-linked hGHRs declined after 60 min of hGH exposure. In contrast, detergent-insoluble hGHRs progressively accumulated as the duration of hGH treatment increased to 90 min. Notably, the accumulation of the disulfide-linked hGHR form in the detergent-insoluble fraction was more progressive than that of the non-disulfide-linked hGHR. Thus the disulfide-linked hGHR, a form associated with hGHR activation (10), comprises a significant component of the detergent-insoluble hGHRs that appear in response to hGH. Interestingly, as we found for hGH-induced disulfide-linked hGHR formation (10), tyrosine kinase activation was apparently not required for hGH-induced changes in hGHR detergent solubility. Pretreatment with staurosporine [1.25 µM, a concentration that inhibits detectable hGH-induced tyrosine phosphorylation in IM-9 cells (10)] did not affect hGH-induced loss of detergent-soluble hGHRs or their accumulation in the detergent-insoluble fraction (not shown).

hGH-induced accumulation of detergent-insoluble hGHRs is temperature sensitive. To address whether hGH-induced detergent insolubility of hGHRs and hGH-induced formation of apparent disulfide-linked hGHRs were related phenomena, we considered whether disulfide linkage might be sufficient to confer detergent insolubility to the hGHR. To approach this, the abilities of hGH to promote hGHR disulfide linkage and alterations in hGHR detergent solubility were examined at low temperatures.

As shown in Fig. 4A, stimulation of IM-9 cells at 4°C, a temperature that prevents receptor trafficking other than that associated with lateral movement within the membrane, allowed continued detectability of the hGH-induced disulfide-linked detergent-soluble hGHR form. Formation of disulfide-linked hGHRs was somewhat slower at 4°C than at 37°C but was nonetheless easily detectable at this lower temperature. In contrast, no hGH-induced loss of detergent-soluble hGHRs (Fig. 4B) or accumulation of detergent-insoluble hGHRs [either under reduced conditions, as in Fig. 4C, or under nonreduced conditions (data not shown)] was noted after as long as 120 or 240 min, respectively, of hGH exposure. This inability to detect changes in hGHR detergent solubility under conditions that allow hGH-induced formation of disulfide-linked hGHRs indicates that hGH-induced accumulation of detergent-insoluble hGHRs cannot be explained solely by diminished detergent solubility of the disulfide-linked form.

We also examined the ability of hGH to induce hGHR detergent solubility changes when stimulation was performed at 20°C, a temperature at which some postendocytic trafficking within cells is markedly reduced (8). Incubation of cells at 20°C leads to an increase in detergent-soluble immunoblottable hGHRs in comparison with 37°C (not shown), a finding that may relate to alteration of a basal (hGH-independent) hGHR redistributive process at the lower temperature. However, despite the increased basal detergent-soluble hGHR levels detected at 20°C, hGH induced a similar degree of loss of hGHRs from this fraction at both temperatures (Fig. 5A). In contrast, although observed
at 37°C, hGH-induced accumulation of hGHRs in detergent-insoluble extracts was not detectable when cells were stimulated at 20°C (Fig. 5B). Correspondingly, the loss of total hGHRs in response to hGH more closely paralleled the loss of detergent-soluble receptors at 20°C than at 37°C [after 90 min of hGH exposure at 20°C, remaining total hGHRs = 56.9 ± 2.3% (n = 2) vs. remaining detergent-soluble receptors = 47.5 ± 6.8% (n = 2), P = 0.32; after 90 min of hGH exposure at 37°C, remaining total hGHRs = 81.9 ± 9.4% (n = 3) vs. remaining detergent-soluble receptors = 44.8 ± 5.2% (n = 9), P = 0.004 (as in Fig. 1C)]. These findings
indicate that the hGH-induced phenomena of loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs can be uncoupled at 20°C and may imply that the acquisition of hGHR detergent insolubility requires access to a postendocytic trafficking pathway that is not operational at that temperature.

We note that pretreatment with cytoskeletal disrupting agents did not clearly prevent hGH-induced redistribution of hGHRs. Treatment with the actin-depolymerizing agent, cytochalasin B (50 µg/ml for 4 h before and then during 60 min of hGH treatment), slightly lessened hGH-induced accumulation of hGHRs in the detergent-insoluble fraction, but the level of detergent-soluble hGHRs was also similarly diminished by this treatment (data not shown). Treatment with the microtubular disrupting agent, nocodazole (10–50 µg/ml for 120 min before and then during 120 min of hGH treatment), had no effect on hGH-induced accumulation of detergent-insoluble hGHRs (data not shown). Thus the trafficking pathway leading to hGH-induced hGHR detergent insolubility does not necessarily require a fully intact cytoskeleton.

Phorbol ester treatment induces loss of detergent-soluble hGHRs by a mechanism apparently distinct from that of hGH. Both hGH and phorbol ester treatments of IM-9 cells are known to cause downregulation of hGHRs (18, 26). In IM-9 cells, roughly one-half of surface hGHRs are lost by treatment of cells at 37°C with 440 ng/ml hGH for 45 min (18) or with the phorbol ester PMA (greater than or equal to 1 µM) for 30 min (26). The mechanisms of these downregulations are unclear, but the hGH-induced downregulation is believed to be related more to acceleration of disposition of hGHRs than to diminution in their synthesis (18).

Given their similarity in decreasing IM-9 cell surface hGHR levels, we examined whether hGH and PMA also exerted similar effects on hGHR detergent solubility. Both PMA and hGH induced a loss of detergent-soluble hGHRs (Fig. 6A). As shown earlier, the time course of the hGH-induced effect was characterized by significant and progressive loss of soluble hGHRs after 30 min. The PMA-induced loss of detergent-soluble hGHRs was quantitatively similar to that caused by hGH but was nearly maximal by 30 min (Fig. 6A and B). In contrast to hGH, PMA treatment induced no accumulation of full-length hGHRs in the detergent-insoluble fraction. Instead, the low level of hGHRs present in this fraction basally was further reduced by PMA in a fashion parallel to the effect of PMA on detergent-soluble hGHRs (Fig. 6B). In other experiments, the acute loss of total cell full-length hGHRs in response to PMA was further demonstrated by direct solubilization of the cells in hot SDS sample buffer before electrophoresis and immunoblotting (J. Alele, J. F. Goldsmith, J. Jiang, and S. J. Frank, unpublished observations).

Notably, the loss of detergent-soluble hGHRs induced by PMA, but not that induced by hGH, was accompanied by the corresponding appearance of a tightly spaced set of bands at 65–68 kDa (a doublet resolved in

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**Fig. 5. Uncoupling of hGH-induced loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs when cells are stimulated at 20°C.**

A: quantitative comparison of hGH-induced loss of detergent-soluble hGHRs at 37 and 20°C. Fractions of immunoblottable hGHRs remaining in detergent-soluble extracts of cells exposed to hGH (500 ng/ml) for 90 and 120 min at 37 and 20°C were determined densitometrically as in Fig. 1C. Pooled data at each time point are presented as mean values ± SE. Data for 37°C stimulations are same data summarized in Fig. 1C. Numbers of independent observations averaged for each condition are indicated. P values are, between 90-min points at 37 and 20°C, P = 0.81; between 120-min points at 37 and 20°C, P = 0.82 (i.e., no statistically significant difference between 37 and 20°C values at either 90 or 120 min).

B: hGH does not promote accumulation of detergent-insoluble hGHRs when cells are stimulated at 20°C. Cells were exposed at 37 or 20°C to hGH (500 ng/ml) or vehicle for indicated durations. Reduced detergent-insoluble extracts (5 million cell equivalents/sample) were resolved by SDS-PAGE and hGHRs were detected by immunoblotting. Experiment shown is representative of 2 such experiments.
Fig. 6. Phorbol ester treatment of IM-9 cells promotes loss of detergent-soluble hGHRs without accumulation of detergent-insoluble hGHRs. A: loss of detergent-soluble hGHRs in response to phorbol-12,13-myristate acetate (PMA) and hGH. Cells were exposed at 37°C to PMA (100 µg/ml), hGH (500 ng/ml), or vehicle (control) for indicated durations. Reduced detergent-soluble extracts (5 million cell equivalents/sample) were resolved by SDS-PAGE, and hGHR-reactive proteins were detected by immunoblotting. Arrows indicate closely spaced bands at 65–68 kDa, appearance of which is enhanced by PMA but not hGH treatment. Band observed in each lane below 65- to 68-kDa doublet is nonspecific, being detectable even without incubation in a primary blotting antibody (not shown). B: lack of accumulation of detergent-insoluble hGHRs in response to PMA. Cells were exposed at 37°C to PMA (1 µg/ml) or vehicle (0 min point) for indicated durations. Reduced detergent-soluble and detergent-insoluble extracts (5 million cell equivalents/sample) were resolved by SDS-PAGE, and hGHR-reactive proteins were detected by immunoblotting. Arrows indicate resolution of 3 closely spaced bands at 65–68 kDa in response to PMA. Note that, despite loss of full-length hGHRs from detergent-soluble extract, there is no accumulation of hGHRs in detergent-insoluble extract.

DISCUSSION

By anti-hGHR immunoblotting, we observe in the IM-9 B-lymphoblastoid cell line that hGH causes time- and concentration-dependent loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs. The time courses of the two changes are roughly parallel, with the loss of detergent-soluble receptors likely slightly preceding their appearance in the detergent-insoluble fraction. Both changes are progressive and most readily detectable after 30 min of hGH stimulation, and both precede the hGH-induced loss of SDS-extractable total cell immunoblatable hGHRs. We find that the detergent-insoluble pool of hGHRs that arises in response to hGH is markedly and progressively populated with the apparent disulfide-linked hGHR form. Because this form is detectable as early as 1 min into stimulation and is acutely tyrosine phosphorylated (10), we conclude that a significant fraction of detergent-insoluble receptors arises from a pool of activated hGHRs. Thus our findings indicate that, in IM-9 cells at 37°C, it is likely that hGH-induced disposition of detergent-soluble hGHRs is not entirely via ligand-induced receptor degradation but rather is also contributed to by redistribution of previously activated receptors to a detergent-insoluble subcellular fraction. We note that if hGH stimulation is performed at 20°C rather than at 37°C, there is also loss of detergent-soluble hGHR (Fig. 5A); however, there is no accompanying accumulation of detergent-insoluble hGHRs (Fig. 5B). Thus the phenomena of hGH-induced loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs can be uncoupled at 20°C.

Furthermore, treatment of cells with PMA at 37°C promotes rapid and dramatic loss of detergent-soluble hGHRs that is also not accompanied by accumulation of detergent-insoluble hGHRs. However, this loss of detergent-soluble receptors appears to be mechanistically
different from that promoted by hGH. Although we are as yet uncertain as to their exact identification, the size and immunoreactivity of the 65- to 68-kDa bands (Fig.
6, A and B), the detection of which is increased in
response to PMA but not in response to hGH, are
consistent with their being “remnants” (fragments
that include the transmembrane and cytoplasmic domains)
generated after selective proteolysis of mature GHRs at
sites(s) at or near the membrane. Such selective
proteolysis in response to PMA has been observed for
other membrane proteins, the extracellular domains of
which are “shed” as water-soluble proteins (3). We
speculate that in IM-9 cells PMA causes loss of deter-
gen sulfide hGHRs (and likely hGH downregulation)
in part by inducing shedding of the extracellular
domain of the membrane hGHR [thereby generating the
water-soluble hGH binding protein (5, 13, 27)] without
redistributing hGHRs to another (i.e., detergent-
insoluble) subcellular fraction. In contrast, it is clear
that in IM-9 cells hGH-induced loss of detergent-
soluble receptors appears not to be accompanied by
such a selective hGHR proteolysis.

We note the recent findings of King et al. (17) in
which PMA-induced acute loss of cell surface GHRs in
murine 3T3-F442A fibroblasts was apparently not ac-
companied by loss of total cell GHRs and was therefore
attributed to an intracellular redistribution of recep-
tors. We cannot yet explain the differences between our
findings and those of King et al. on this matter, but we
note the marked differences between species in mecha-
nisms of generation of the GH binding protein (5).
Given that rodents are not thought to generate the GH
binding protein by cleavage of the transmembrane
GHR as much as by alternative splicing of GHR mRNA
(5), we speculate that the sensitivity to PMA for the
elicitation of such a cleavage might differ between
rodent and human cell lines. We are currently testing
this hypothesis.

Although the hGH-induced detergent-insoluble pool
of hGHRs is well populated with the disulfide-linked
hGHR, the stimulations performed at 4°C result in
hGH disulfide linkage without acquisition of deter-
gen sulfide insolvibility, indicating that disulfide linkage is not
sufficient for hGH-induced hGHR detergent insolubility.
Whether hGHR disulfide linkage, which occurs in
response to hGH apparently at the cell surface, is
required for disposition of hGHRs to a detergent-
insoluble pool is as yet uncertain.

Detergent insolubility of receptors with potential
relevance to signaling has been observed in other
systems (6, 11). In these cases, detergent insolubility is
thought to be related to association of the receptors
with the cytoskeleton. Our studies in IM-9 cells have
not yet conclusively indicated such an association for
the hGH in response to hGH. The mild diminution
that we observed in the levels of both basal detergent-
soluble hGHRs and hGH-induced detergent-insoluble
hGHRs in the presence of cytochalasin B is consistent
with previous data indicating that cytochalasins by
themselves decrease cell surface hGHR expression in
IM-9 cells by undefined mechanisms (29). These data,
coupled with the lack of effect of nocodazole on the
process, lead us to conclude that hGH-induced hGHR
detergent insolubility in IM-9 cells is independent of the
integrity of microtubules and is, at most, only
partially dependent on an intact actin cytoskeleton.

Our biochemical observation of hGH-induced hGHR
detergent insolubility in IM-9 cells raises two major
questions that will be the focus of future studies: 1) Do
detergent-insoluble hGHRs, which appear to in part
derive from an activated pool of hGHRs, reside prefer-
entially in a particular subcellular location or organ-
elle? and 2) Does the acquisition of detergent insolubil-
ity allow this pool of hGHRs to perform a particular
role, for example, in hGHR signaling or trafficking, in
response to hGH?

In regard to the first question, GHRs have been
observed in certain cell types to undergo translocation
to the nucleus in a phenomenon thought to be related to
the appearance of GH in that organelle (20, 21). We
think it unlikely on several grounds that the hGH-
induced accumulation of detergent-insoluble hGHRs
that we observe reflects such a nuclear translocation.
In previous radioautographic ultrastructural studies of
the uptake of [125I]-hGH by IM-9 cells, no nuclear localiza-
tion of the labeled hormone was detected over a time
course of 180 min of stimulation (4), leading us to
believe that IM-9 cells may not behave similarly to
other cell types studied in this respect. Second, when
observed, GHR nuclear localization in Chinese hamster
ovary cell-rat GHR transfectants was acute (within 10
min of GH treatment) and transient (redistributed to
nonnuclear areas of the cell in a pattern identical to
untreated cells by 60 min into GH exposure) (21). This
is in contrast to our observed time course of hGH-
induced hGHR detergent insolubility in IM-9 cells
(most easily detectable after 30 min and progressive for
at least 2 h). Finally, in our experiments, even when
extraction was performed with up to 4% Triton X-100,
hGHRs still became detergent insoluble in response to
hGH. This concentration of Triton X-100 would be
expected to solubilize nuclear membrane-associated
GHRs (21).

Other potential subcellular localizations have been
described that could, in principle, include detergent-
insoluble GHRs. Caveolae, for example, are detergent-
insoluble submembranous vesicles that have been noted
to be enriched in signaling molecules such as growth
factor receptors and other tyrosine kinases and sub-
strates (19). Typically, treatment of cells with the
antibiotic nystatin disrupts caveolae formation (23).
Our preliminary experiments indicate that incubation
of cells with nystatin (50 µg/ml) does not prevent the
hGH-induced acquisition of hGHR detergent insolubil-
ity (data not shown), leading us to tentatively conclude
that detergent-insoluble hGHRs are not associated
with caveolae.

“Capping” of hGH (and therefore presumably of
hGHRs as well) has been described in IM-9 cells (9).
Whereas their detergent solubility characteristics are
unknown, these hGH caps form within 30 min of
stimulation at one pole of the cell; their function and
disposition are also unknown. Studies of the capping of hormones other than GH and of other ligands by lymphocytes indicate that caps are intimately associated with the actin cytoskeleton, although they can be at least partially solubilized in nonionic detergents (22). Morphological studies of hGHR distribution in response to hGH may allow us to discern whether capping of hGHRs correlates with hGH detergent insolubility in our system.

Regarding the second question raised by our observations (is there a particular function to be ascribed to the detergent-insoluble hGHR?), we note that the time course of its formation is similar to the time courses observed in IM-9 cells for the phenomena of 1) hGH-induced hGHR downregulation (14, 18), 2) the appearance of a nondissociative component of 125I-hGH binding (14), and 3) the exocytic release of previously internalized hGH from the cell (15). These three phenomena may be related. Notably, exocytosis of internalized hGH nearly ceased at 20°C, whereas endocytosis continued (15). This uncoupling may be related to the uncoupling of hGH-induced loss of detergent-soluble hGHRs and accumulation of detergent-insoluble hGHRs that we have observed at that temperature.

In summary, our data indicate that IM-9 cells hGHRs undergo at least two related, but separable, intracellular trafficking routes in response to hGH. We envision that the loss of detergent-soluble hGHRs likely reflects in part their endocytosis and degradation, presumably similar to a pathway followed by many receptors. Additionally, another hGH routing pathway in these cells that involves the accumulation of detergent-insoluble receptors is activated by hGH at 37°C. hGHRs routed to this pathway are long-lived and are enriched in the apparent disulfide-linked receptor form. We speculate that the second pathway, by serving either as a delivery route for hGH exocytosis or as a vehicle for receptor sequestration, may be involved in receptor downregulation and may therefore be important in modulation of hormonal responses. Furthermore, as a practical matter, our findings sound a cautionary note that the evaluation of the fate of hGHRs undergo at least partially solubilized in nonionic detergents (22).

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In summary, our data indicate that in IM-9 cells hGHRs undergo at least two related, but separable, intracellular trafficking routes in response to hGH. We envision that the loss of detergent-soluble hGHRs likely reflects in part their endocytosis and degradation, presumably similar to a pathway followed by many receptors. Additionally, another hGH routing pathway in these cells that involves the accumulation of detergent-insoluble receptors is activated by hGH at 37°C. hGHRs routed to this pathway are long-lived and are enriched in the apparent disulfide-linked receptor form. We speculate that the second pathway, by serving either as a delivery route for hGH exocytosis or as a vehicle for receptor sequestration, may be involved in receptor downregulation and may therefore be important in modulation of hormonal responses. Furthermore, as a practical matter, our findings sound a cautionary note that the evaluation of the fate of hGHRs, in general, may not be.


