Caveolar and Lipid Raft Localization of the Growth Hormone Receptor and Its Signaling Elements

IMPACT ON GROWTH HORMONE SIGNALING*

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The growth hormone receptor (GHR) is a transmembrane glycoprotein member of the cytokine receptor superfamily that mediates the somatogenic and metabolic effects of the growth hormone (GH). GH signaling is transduced via the receptor-associated cytoplasmic tyrosine kinase called Janus protein kinase 2 (JAK2). The major intracellular signaling systems activated by JAK2 in response to GH include the signal transducer and activator of transcription (STAT) 5 and extracellular signal-regulated kinase (ERK)-1 and -2 pathways. In this report, we investigate the role of cholesterol-rich plasma membrane microdomains (caveolae and lipid rafts) in GH signaling. By subcellular fractionation of the GH-responsive 3T3-F442A murine preadipocyte, we found dramatic enrichment (6.7-fold) of plasma membrane GHR in the caveolae membranes (CM). JAK2 was also represented in the CM fraction, but was less enriched (2.5-fold) than GHR. ERK1/2 and the important ERK pathway upstream small adaptor protein, Grb2 (growth factor receptor-bound protein 2), were also enriched in caveolae (2.3- and 8.3-fold, respectively), but STAT5 was barely detected in the same fraction. Correspondingly, GH-induced tyrosine-phosphorylated GHR, JAK2, and ERK1/2 were highly represented in the CM fraction, whereas tyrosine-phosphorylated STAT5 was enriched in the non-membrane fraction of the post-nuclear supernatant. Additionally, GH induced further accumulation of GHR, Grb2, and SHC proteins in the CM fraction. Interestingly, treatment of the cells with the caveolae-disrupting agent, methyl-β-cyclodextrin (mβCD), selectively inhibited GH-induced ERK1/2 activation but not STAT5 phosphorylation; repletion of cholesterol in mβCD-treated cells restored GH-induced ERK activation. Comparison of 3T3-F442A cells with the GHR-expressing human IM-9 lymphoblasts revealed similar enrichment of GHR in the lipid raft fraction of IM-9 as in the CM fraction of 3T3-F442A, but there were dramatic differences in the ERKs and Grb2. The IM-9 cells, in which ERKs are not activated by GH, displayed no enrichment of ERKs and Grb2 in the lipid raft fraction. Our results suggest that localization of GHRs in the CM fraction of the plasma membrane plays important roles in signaling.

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The growth hormone receptor (GHR) is a transmembrane glycoprotein member of the cytokine receptor superfamily that mediates the somatogenic and metabolic effects of GH. GH binding to the GHR extracellular domain causes conformational changes that result in enhanced receptor cytoplasmic domain association with, and activation of, the non-receptor tyrosine kinase JAK2. Among the major signaling systems engaged after GH-induced JAK2 activation are the STATs (in particular STAT5b) and the mitogen-activated protein (MAP) kinases (most notably ERK1 and ERK2). STAT5b activation is critical for GH-induced transcription of ghrelin, including insulin-like growth factor-1, serine protease inhibitor 2A, and certain hepatic cytochrome P450 enzymes 4 and 7. Depending on the cellular context, GH-induced ERK activation has been linked to c-fos gene expression, cellular proliferation, and crosstalk between the GH and EGF signaling systems 8 and 12. The structural and functional requirements for GH-induced activation of STAT5 are different from those governing ERK activation. Full STAT5 activation requires GH-induced tyrosine phosphorylation of the GHR cytoplasmic tail, whereas GH-induced ERK activation requires only the membrane proximal GHR cytoplasmic domain (the region required for JAK2 activation) to be present 13 and 23. Additionally, in contrast to activation of STAT5, the ability to couple GH stimulation to ERK activation varies among cell types 24. Cavolae are small plasma membrane invaginations (50–100 nm diameter) characterized by a high cholesterol content, low buoyant density, and the presence of a caveolin coat that exist in a wide range of cell types, including epithelial cells, muscle cells, endothelial cells, adipocytes, and fibroblasts 25 and 27. Cavolae share similarities with lipid rafts by being enriched in cholesterol and sphingolipids and are likely a special subset of rafts 28 and 29. Multiple functions have been ascribed to cavolae, including endocytosis, transcytosis, cholesterol transport, and signal transduction 27 and 29. However, the role of cavolae and rafts in signaling appears to vary among different systems. For example, both the insulin and EGF receptors have been shown to be localized to cavolae, but this

1 The abbreviations used are: GHR, growth hormone receptor; BB, binding buffer; CM, cavolae membrane; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GH, growth hormone; Grb2, growth factor receptor-bound protein 2; JAK2, Janus protein kinase 2; LR, lipid raft; mβCD, methyl-β-cyclodextrin; NCM, non-cavolae membrane; NLR, non-lipid raft; PBS, phosphate-buffered saline; PM, plasma membrane; PNS, post-nuclear supernatant; SHC, Src homologous and collagen protein; STAT, signal transducer and activator of transcription.

2 Twenty-four hours of free access to Ref. 1 can be obtained at www.academicpress.com/cytokinereference.
localization is believed to affect signaling by these two receptors differentially. Caveolin-1 can be tyrosine phosphorylated directly by insulin receptors, and caveolae positively regulate insulin signaling (30–32). In contrast, EGF signaling may be negatively regulated by association of the EGFR with caveolae (33, 34).

The role, if any, of caveolae in GH signaling is poorly understood. In the only previous study to address this issue, Lobie et al. (35) found that GHRs, exogenously expressed in Chinese hamster ovary cells, could be detected in caveolae by electron microscopy. Transient overexpression of caveolin-1 in that system led to enhanced GH-induced STAT-dependent activation of a reporter gene. In the current study, we examined the subcellular localization of endogenous GHRs. Using both murine 3T3-F442A preadipocyte fibroblasts and human IM-9 B-lymphoblasts, we found that GHRs were enriched in caveolae/lipid rafts and that, in 3T3-F442A cells, signaling via the ERK and STAT5 pathways was differentially dependent on the integrity of caveolae.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human GH was kindly provided by Eli Lilly. Methyl-p-cyclodextrin (mCD), cholesterol, Percoll, and routine reagents were obtained from Sigma. Optiprep™ was purchased from Accurate Chemical and Scientific Corp. (Westbury, NY).

Antibodies—Polyclonal anti-caveolin and anti-SHC were purchased from BD Transduction Laboratories, as were monoclonal anti-clathrin heavy chain and anti-Grb2. Monoclonal anti-STAT5α (G2) and polyclonal anti-EGFR were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-STAT5 affinity-purified rabbit polyclonal antibody (recognizing the tyrosine-phosphorylated form Tyr-694) of STAT5α and STAT5b was purchased from Zymed Laboratories Inc.. Anti-active mitogen-activated protein kinase homodimer affinity-purified rabbit antibody (anti-active ERK, recognizing the dually phosphorylated Thr-183 and Tyr-185 residues corresponding to the active forms of ERK1 and ERK2) was from Promega (Madison, WI). Monoclonal anti-phosphotyrosine antibody 4G10, anti-mitogen-activated protein kinase affinity-purified rabbit antibody (anti-ERK, recognizing both ERK1 and ERK2), and polyclonal anti-phospho-JAK2 (recognizing phosphorylated Tyr-1007 and Tyr-1018) were purchased from Upstate Biotechnology (Lake Placid, NY).

Anti-JAK2(1–233) polyclonal serum (directed at residues 746–1129 of murine JAK2) has been described previously (36). The rabbit polyclonal antisera, anti-GHR Tyr-314, raised against a bacterially expressed N-terminus His-tagged fusion protein incorporating human GHR resistant to anti-GHRcyt-AL47, raised against a bacterially expressed N-

GH Receptor Caveola/Lipid Raft Localization

Tricine, pH 7.8), including inhibitors, and Dounce homogenized (20 strokes). The lysate was centrifuged at 10,000 ×g for 10 min to remove nuclei and unbroken cells. The resulting pellet was resuspended in buffer A, homogenized, and centrifuged. Supernatants from both centrifugations were combined (referred to as the post-nuclear supernatant (PNS)) and loaded onto 23 ml of 30% (w/v) Percoll in buffer A prior to centrifugation at 26,000 rpm for 35 min in a Beckman SW28 rotor. The plasma membrane (PM) fraction, a visible band roughly 5.5 cm from the bottom of the centrifuge tube, was collected and sonicated (six bursts of 10 s each). This sonicate was mixed with 50% (w/v) Opti-Prep in buffer B (0.25 M sucrose, 6 mM EDTA, and 120 mM Tricine, pH 7.8) to yield 4 ml of 23% Opti-Prep and loaded at the bottom of a centrifuge tube, on top of which 6 ml of a 10–20% (w/v) gradient was layered. The sample was centrifuged at 22,000 rpm for 90 min in a Beckman SW41 rotor. The resulting non-floating fraction (the bottom 3.5 ml of the tube) was collected and is referred to as the non-caveolae membrane/non-lipid raft (NCM/NLR) fraction. The top 5 ml (the floating fraction) was collected, mixed with 4 ml of 50% (w/v) Opti-Prep, and loaded at the bottom of another tube. On top of this was layered 2 ml of 5% (w/v) Opti-Prep, and the sample was centrifuged at 22,000 rpm for 90 min in a Beckman SW41 rotor. The caveolae membrane/lipid raft (CM/LR) fraction was collected just above the interface of these two gradients.

Treatment with mCD or Cholesterol-mCD Complexes—For treatment with mCD to deplete cholesterol, serum-starved cells were transferred to Dulbecco’s modified Eagle’s medium containing 50 mM HEPES, pH 7.2, and 0.1% (w/v) bovine serum albumin with or without 10 mM mCD and incubated at 37 °C for 30 min, after which the cells were washed twice with PBS. Cells were either collected and directly solubilized in boiling Laemmli sample buffer or treated continuously for cholesterol repletion. To replete cholesterol, cells were incubated for 15 min at 37 °C in Dulbecco’s modified Eagle’s medium containing 50 mM HEPES, pH 7.2, and 0.1% (w/v) bovine serum albumin with or without 10 mM cholesterol-mCD complexes. Synthesis of the cholesterol-mCD complexes was carried out as described (41).

Protein Extraction, Electrophoresis, and Immunoblotting—Laemmli sample buffer was added to samples from caveolae/lipid raft preparations after protein content was determined. For cholesterol depletion experiments, samples were sonicated immediately before fractionation and collected cell pellets. Extracted proteins were then resolved by SDS-PAGE and immunoblotted as indicated below. Proteins resolved by SDS-PAGE were transferred to Hybrid ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with TBST buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% (w/v) Tween 20 containing 2% (w/v) bovine serum albumin) and incubated with primary antibodies (0.5–1 μg/ml) as specified in each experiment. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies (1:10,000 dilution) and washed. The bond antibodys were detected with SuperSignal chemiluminescent substrate (Pierce). Membrane stripping was performed according to the manufacturer’s suggestions.

Densitometric Analysis—Densitometric quantitation of enhanced chemiluminescence immunoblots was performed using a high resolution scanner and the ImageJ 1.30 program (developed by W. S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data from several experiments are displayed as mean ± S.E. The significance (p value) of differences of pooled results was estimated by t tests.

RESULTS

GHR Is Enriched in Caveolae in 3T3-F442A Cells—To begin to examine the subcellular distribution of endogenous GHRs and relevant signaling molecules, we first used the 3T3-F442A preadipocyte fibroblast. This murine cell line is well characterized for its GH responsiveness and for the presence of immunologically detectable GHRs (2, 8, 11, 12, 38, 42, 44–46). As described under “Experimental Procedures,” cell extracts were prepared under detergent-free conditions using the methods of Smart et al. (40), and fractionation was accomplished by density gradient centrifugation (diagrammed in Fig. 1). Such a fractionation exploits the low density characteristics of the cholesterol-rich caveolar membrane to allow its isolation. Samples of equal total protein content (5 μg) from four separate fractions, namely PNS, PM, NCM, CM, were resolved by SDSPAGE and immunoblotted to determine the relative abundance of several proteins in the fractions (Fig. 2).
GH Receptor Caveolae/Lipid Raft Localization

GHR was detected with our anti-GHR\textsubscript{3-AL47}, a rabbit serum against the receptor cytoplasmic domain (37) that, as expected, was found to be enriched in the plasma membrane fraction relative to the post-nuclear supernatant (Fig. 2A, lane 2 versus lane 1). Interestingly, although the NCM and CM were both derived from the PM, the GHR was greatly enriched in the CM in comparison to the NCM, in which very little receptor was detected (Fig. 2A, lane 4 versus lane 3). When quantitated densitometrically in multiple experiments, the GHR content (normalized for total protein content) was roughly 6.7 ± 1.7-fold greater in the CM versus the PM (Fig. 2A, lane 4 versus lane 2 and Table I), suggesting that GHR associated preferentially with caveolar microdomains within the plasma membrane. Caveolin-1, a resident caveolar protein, showed a similar pattern of subcellular distribution as did GHR (Fig. 2A), being 9.5 ± 1.8-fold enriched in CM versus PM (Table I). The EGF receptor has been shown previously to be enriched in cholesterol-rich plasma membrane microdomains, possibly including caveolae (47–49). Indeed, immunoblotting of the 3T3-F442A fractions with anti-EGFR and densitometric quantitation revealed a CM enrichment of EGFR (6.8 ± 1.0-fold versus PM), similar in degree to that observed for GHR (Fig. 2A and Table I). In contrast, clathrin heavy chain was detected in all four fractions (Fig. 2A) and, in particular, showed no enrichment (1.1 ± 0.3-fold versus PM; Table I) in the CM fraction. This important control indicates that the paucity of GHR in the NCM fraction is not due to a lack of membrane components in that fraction.

We next examined the subcellular distribution of three signaling molecules relevant for GH signal transduction (Fig. 2B). JAK2 is a cytoplasmic tyrosine kinase that is devoid of a transmembrane domain (50); nevertheless, it associates with the GHR and other cytokine receptors, largely by virtue of its N-terminal FERM domain (23, 51, 52). Immunoblotting with anti-JAK2\textsubscript{NLS} (36) showed a pattern of distribution similar to that of GHR; but with a less dramatic enrichment in CM versus PM (2.5 ± 0.6-fold; Table I). ERKs 1 and 2, despite being thought of as cytosolic proteins, were distributed in all fractions in 3T3-F442A cells. Within the plasma membrane fractions, ERK1 and ERK2 were enriched in CM versus NCM (lane 4 versus lanes 2 and 3). In contrast to the ERKs, STAT5 was found almost exclusively in the post-nuclear supernatant, with only minimal anti-STAT5 signal detectable in membranous fractions (lane 1 versus lanes 2–4).

GH-induced GHR Translocation and ERK Activation in Caveolae in 3T3-F442A Cells—Having documented the enrichment of GHR and JAK2 in caveolae, we next investigated the effects of GH treatment on the tyrosine phosphorylation of the GHR and signaling molecules in the CM fraction. Cells were stimulated with GH or vehicle for 15 min prior to extraction and fractionation, and the four fractions were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine (Fig. 3A, anti-pTyr). In the region of the gel containing the GHR and JAK2, GH-dependent tyrosine phosphorylation was most notable in the CM and PM fractions, consistent with the distribution of the GHR and JAK2. The same cell extracts were immunoblotted sequentially with anti-phosphotyrosine-JAK2 (Fig. 3B, anti-pTyr-JAK2), anti-phosphotyrosine-STAT5 (Fig. 3C, anti-pTyr-STAT5), and anti-phospho (active) ERK (Fig. 3D, anti-active ERK 1/2) to detect GH-induced activation of JAK2 tyrosine phosphorylation, STAT5 tyrosine phosphorylation, and ERK 1/2 phosphorylation, respectively. JAK2 activation, in concert with the results of the anti-phosphotyrosine blot in Fig.

Table I

| Molecule       | Fold enrichment CM vs. PM |
|----------------|---------------------------|
| GHR            | 6.7 ± 1.7                 |
| Caveolin-1     | 9.5 ± 1.8                 |
| EGFR           | 6.8 ± 1.0                 |
| Clathrin (HC)  | 1.1 ± 0.3                 |
| JAK2           | 2.5 ± 0.6                 |

Fig. 1. Schematic representation of the subcellular fractionation scheme utilized. See “Experimental Procedures” for details.

Fig. 2. Subcellular distributions of GHR and downstream signaling elements in 3T3-F442A cells. PNS, PM, NCM, and CM fractions were isolated from serum-starved 3T3-F442A cells using the method developed by Smart et al. (40) (as in Fig. 1 and under “Experimental Procedures”). Equal amounts of protein (5 μg) from each fraction were resolved by SDS-PAGE and sequentially immunoblotted for GHR, caveolin-1, EGFR, and clathrin heavy chain (A), and for JAK2, ERK1/2, and STAT5 (B). The experiments shown are representative of 6–9 such experiments.

Fig. 3. GH-induced activation of JAK2 tyrosine phosphorylation, STAT5 tyrosine phosphorylation, and ERK 1/2 phosphorylation, respectively. JAK2 activation, in concert with the results of the anti-phosphotyrosine blot in Fig.
3A, was most pronounced in the CM and PM fractions. Consistent with their dichotomous subcellular distribution, the phosphorylated forms of STAT5 and ERK1/2 induced in response to GH were detected in distinctly different fractions, with most of the (non-nuclear) phosphorylated STAT5 excluded from the membrane fractions and most of the activated ERKS concentrated in the caveolae membrane.

As these data suggested substantial involvement of the CM fraction in GH signaling, we investigated further the effects of GH on the abundance of signaling molecules within this fraction. In the experiments in Fig. 4, the GHR and JAK2 were examined. Within the CM fraction, we observed that GH treatment caused an increase in the abundance of the GHR detected by immunoblotting with anti-GHRcyt-AL47 (Fig. 4A, upper panel). When several such experiments were analyzed densitometrically, GH was found to increase GHR abundance by roughly 40% in the CM fraction (p < 0.05; Fig. 4B). In contrast to the GHR, the abundance of JAK2 in the CM fraction was not changed by GH treatment (Fig. 4, A (middle panels) and B). As a control, the abundance of clathrin heavy chain was assessed by immunoblotting and also did not differ between cells exposed to GH or vehicle (Fig. 4, A (lower panel) and B).

GH-induced ERK activation in 3T3-F442A cells has been shown to involve SHC and Grb2 as upstream mediators (53, 54). The EGFR has also been shown to participate in GH-induced ERK activation by virtue of providing a binding site for Grb2 in response to GH (55). We also examined the status of these signaling molecules within the CM fraction of 3T3-F442A cells in response to GH. Both Grb2 and SHC were found in the CM fraction of 3T3-F442A cells (Fig. 5A) (as seen below in Fig. 8B, SHC was widely distributed among the subcellular fractions in these cells, whereas Grb2 was substantially enriched in the CM fraction). Furthermore, SHC localized to the CM fraction was inducibly tyrosine phosphorylated in response to GH treatment (not shown). Interestingly, GH treatment of 3T3-F442A cells resulted in further accumulation of both Grb2 and the three SHC isoforms into the CM fraction (>50% increase of both Grb2 and SHC in response to GH) (p < 0.01 for each; Figs. 5, A and B). In contrast to Grb2 and SHC, the abundance of EGFR in the CM fraction did not change in response to GH (Fig. 5, A and B).

Cholesterol Depletion Selectively Affects GH-induced ERK Signaling in 3T3-F442A Cells—Cholesterol is crucial for structural and functional integrity of caveolae and lipid rafts; the proper cholesterol concentration is required to preserve these microdomains in the liquid-ordered state (56). Experimentally, the lowering of plasma membrane cholesterol levels by use of cholesterol depleting reagents has been shown to alter caveolar structure and function (57). In light of the data on caveolae and GH signaling in Figs. 3–5, we investigated the impact of treatment with the cholesterol-depleting drug mβCD on GH-induced ERK and STAT5 activation (Fig. 6).

3T3-F442A cells were pretreated for 30 min with or without 10 mM mβCD prior to stimulation with GH (+) or its vehicle (−) for 5 min. Cells were then solubilized directly in hot SDS-containing Laemmli sample buffer (as described under “Experimental Procedures”), and total cell extracts were resolved by SDS-PAGE and immunoblotted with anti-active ERK (Fig. 6A, left panel). Pretreatment with mβCD substantially reduced subsequent GH-induced ERK activation. This effect was quantitated for a group of similar experiments by densitometrically comparing the anti-active ERK signals normalized for ERK abundance (by reprobing with anti-ERK; not shown). As seen in Fig. 6A (right panel), pretreatment with mβCD reduced GH-induced ERK activation by roughly 45% on average (n = 4; p < 0.01), suggesting that cholesterol depletion markedly affects this aspect of GH signaling. To address this matter further, we tested the effect of pretreatment with mβCD followed by washing and reincubation with either cholesterol (designated mβCD + cholest in Fig. 6B) or medium (mβCD in Fig. 6B).
phorylation was unaffected by mERK activation (Fig. 6A) revealed the enhancement to be significant in the absence of GH treatment (considered 100%). (n = 6 for EGFR; n = 4 for SHC; and n = 8 for Grb2). p values are indicated (NS = not significant).

We also tested the effect of mCD treatment on acute activation of STAT5 phosphorylation (Fig. 6C). Cells were pretreated with mCD or vehicle and then stimulated for 5 min with GH prior to total cell extraction, SDS-PAGE, and immunoblotting with anti-phosphotyrosine-STAT5 (designated p-STAT5 in Fig. 6C, left panel). In contrast to the findings for ERK activation (Fig. 6A), GH-induced STAT5 tyrosine phosphorylation was unaffected by mCD pretreatment, with quantitation revealing no statistical difference in the levels of phosphotyrosine-STAT5 detected under the two conditions (Fig. 6C, right panel). These results suggest that cholesterol depletion and the resultant disruption of caveolae rafts selectively impacts GH-induced ERK signaling as compared with STAT5 signaling. This is consistent with the notion that signaling downstream of the GHR is compartmentalized based on the subcellular localization of the signaling elements.

Comparison of Localization of GHR and Signaling Molecules in IM-9 and 3T3-F442A Cells—The IM-9 cell is a human B-lymphoblast that endogenously expresses the GHR and responds to the GH with activation of JAK2 and tyrosine phosphorylation of the GHR (39, 58-61). Interestingly, although GH-induced JAK2 activation in these cells leads to STAT5 activation, ERKs are not tyrosine phosphorylated or activated in IM-9 cells in response to GH (24, 39, 62). Immune cells do not express caveolins and lack caveolar invaginations on the plasma membrane (26). However, they do have lipid rafts, and these are considered important in immune cell signaling (63). Rafts can be co-isolated with caveolae by using the same fractionation scheme employed above (64). We sought to compare our findings in 3T3-F442A cells with another endogenous GHR-expressing cell type, and, thus, we investigated IM-9 cells.

Analogous to the experiments with 3T3-F442A cells in Fig. 2, serum-starved IM-9 cells were first examined for the distribution of GHR and JAK2 (Fig. 7A). Equal amounts of total protein (5 μg) from the PNS, PM, NLR, and LR fractions were resolved by SDS-PAGE and immunoblotted with anti-GHR. As in 3T3-F442A, the receptor in IM-9 cells was enriched in the PM fraction, and within that fraction it was highly localized to the LR membrane (26). We also probed for the subcellular localization of JAK2 (Fig. 7B). JAK2 was enriched in the LR fraction of IM-9 cells, similar to that seen in 3T3-F442A cells (Fig. 2B), with an apparent shift of nearly 2-fold on average (n = 4; Fig. 7C, left panel). The ratio of the LR to the PM was nearly 2-fold on average (n = 4; Fig. 7C, right panel). We also tested the effect of mCD treatment on acute activation of STAT5 phosphorylation (Fig. 7C). Cells were treated as described for A and evaluated by anti-phosphotyrosine-STAT5 (anti-pTyr-STAT5) immunoblotting (left panel) and quantitated by densitometry for anti-phosphotyrosine-STAT5 abundance, expressed as a percentage of the GH-stimulated level in the absence of mCD (mean ± S.E., n = 4) (right panel). C, effect of treatment with mCD on GH-induced STAT5 tyrosine phosphorylation. Cells were treated as described for A and evaluated by anti-phosphotyrosine-STAT5 (anti-pTyr-STAT5) immunoblotting (left panel) and quantitated by densitometry for anti-phosphotyrosine-STAT5 abundance, expressed as a percentage of the GH-stimulated level in the absence of mCD (mean ± S.E., n = 4; NS = not significant) (right panel).
brane. Reprobing with anti-JAK2\textsubscript{AL3} revealed that JAK2 was detected in each fraction. However, JAK2 was markedly enriched in the LR, much as it was enriched in the CM fraction in 3T3-F442A cells.

We next examined the distribution and activation profiles of STAT5 and ERKs in IM-9 cells (Fig. 7B). In these experiments, serum-starved cells were treated with GH or vehicle prior to preparation of the subcellular fractions and sequential immunoblotting with anti-phosphotyrosine STAT5 (Fig. 7B, top row) and anti-STAT5 (Fig. 7B, second row from the top). As in 3T3-F442A, non-nuclear STAT5 in IM-9 cells was largely found in the PNS rather than in the PM or its subfractions. Upon GH stimulation, STAT5 resolved as two bands, one co-migrating with the STAT5 from unstimulated cells, and the other exhibiting the retarded migration expected of the tyrosine-phosphorylated STAT5 (19). Anti-phosphotyrosine STAT5 blotting verified this finding and demonstrated the marked paucity of activated STAT5 in the membranous fractions. Unlike 3T3-F442A cells, ERKs in IM-9 cells were found by anti-ERK blotting (Fig. 7B, bottom row) to be distributed broadly, but with marked enrichment in the PNS fraction and relative depletions from the LR fraction. As has been observed previously in non-fractionated IM-9 extracts (24), no GH-induced ERK activation was detected by anti-phosphotyrosine ERK blotting in any of the subcellular fractions (Fig. 7B, third row from the top).

The differences in ERK distribution between 3T3-F442A and IM-9 cells were more clearly illustrated by resolving the PM and CM/LR fractions side-by-side by SDS-PAGE prior to anti-ERK blotting (Fig. 8A). In this display, the relative enrichments of IM-9 ERKs in the PNS and 3T3-F442A ERKs in caveolae are evident. Data from several such experiments were subjected to densitometric quantitation to estimate the relative enrichment of ERKs in the CM fraction of 3T3-F442A and the LR fraction of IM-9 (in each case, either versus the PM fraction or versus the PNS fraction). As displayed in Table II, in 3T3-F442A ERKs were enriched 2.3 ± 0.5-fold in the CM fraction relative to the PM fraction and 1.8 ± 0.4-fold in the CM fraction relative to the PNS. In IM-9, the enrichment of ERKs in the LR fraction was 0.49 ± 0.27-fold relative to the PM fraction and 0.19 ± 0.06-fold relative to the PNS, reflecting the relatively low abundance of ERKs in the LR fraction in these cells.

These findings prompted us to also compare the two cells with regard to the distributions of other proteins known to be involved in GH-induced ERK signaling. In both 3T3-F442A and IM-9 cells, SHC was detected in all four fractions. There was no statistically significant enrichment of SHC in the CM/LR fraction in either cell (Fig. 8B, upper panel, and data not shown). However, the small adaptor protein, Grb2, differed from SHC in this regard (Fig. 8B, lower panel). In 3T3-F442A cells, Grb2 was heavily concentrated in the caveolar membranes (8.3 ± 2.1-fold versus the PM fraction and 8.9 ± 1.5-fold versus the PNS) (Table II). In contrast, Grb2 in IM-9 cells was not enriched in the LR fraction versus the PM fraction (1.3 ± 0.7-fold) and was relatively depleted from the LR fraction versus the PNS (0.52 ± 0.26-fold). These results indicate that the distribution of ERKs and proteins linking GH with ERK activation varied among these two GH-responsive cell types, such that ERKs and Grb2 were substantially more concentrated in the caveolar fraction in 3T3-F442A cells (in which GH-induced ERK activation is robust) than in the lipid raft fraction in IM-9 cells (in which GH fails to induce ERKs).

**DISCUSSION**

It is increasingly apparent that an important determinant governing signal transduction from cell surface receptors is the...
spatial distribution of the receptors within the plasma membrane and their regulated interactions with signaling molecules variably present within subcellular regions. In particular, several notable signaling receptors are enriched in cholesterol-rich microdomains within the plasma membrane, and this localization is relevant for their signaling potential (30–34, 65–73). In this report, we have utilized a well validated multistep fractionation scheme (40) to biochemically evaluate the subcellular distribution of the GHR and the relevant GH signaling molecules in cells that endogenously express the receptor and are known to respond to GH. In this scheme, cells are extracted in the absence of detergents, and the plasma membranes are isolated first. This PM fraction is then further fractionated, on the basis of differential buoyancy, into caveolar (or lipid raft) and non-caveolar (or non-lipid raft) components. Our aim was to rigorously define the extent to which the receptor segregates into lipid-rich (buoyant) microdomains within the PM and to learn to what degrees its various signaling molecules co-segregate. Despite its relative complexity, we chose this fractionation method, as opposed to more simple detergent extraction-based protocols, because of its reliability in achieving separation of caveolae/rafts from other membrane elements and preserving intact membrane structure.

We found that, in 3T3-F442A cells, GHR was highly concentrated (on a per milligram total protein basis) in caveolae. We note that only ~5% of total plasma membrane protein was recovered in the caveolae membrane fraction. Therefore, when the yields of protein in the various fractions are considered, we calculate that, in non-GH-treated 3T3-F442A cells, roughly 35% of the total PM-associated GHR is in the CM fraction. Thus, in terms of both their abundance and their concentration, receptors in this fraction are poised to contribute substantially to GH signal transduction. In 3T3-F442A cells, we found that, in response to GH, ERKs associated with the CM fraction were markedly and preferentially activated, whereas (non-nuclear) STAT5 activation was only minimally detected in that fraction. Furthermore, treatment with a cholesterol-depleting reagent, methyl-β-cyclodextrin, significantly decreased GH-induced ERK activation, which was restored by repletion of cholesterol; however, cholesterol depletion did not affect STAT5 activation. Likewise, the degree of GH-induced JAK2 activation detected in total cell extracts was not influenced by methy-[H]-cyclodextrin (data not shown). This analysis leads us to conclude that, to a first approximation, the activation of two major GH-induced signaling pathways in these cells may emanate from receptors that reside in different subregions of the plasma membrane. GHRs in the CM fraction of the plasma membrane appear to couple preferentially to ERK activation, whereas those not extracted with the PM, or those that reside in the PM but are excluded from the CM fraction, couple to STAT5 activation.

GHRs in IM-9 cells were also found to be highly concentrated in lipid rafts. Although GH treatment of these cells activates JAK2 and STAT5, no ERK activation is detected (24, 39, 62). We think it is likely relevant that ERKs themselves and the important ERK activation pathway adaptor protein, Grb2, are significantly more concentrated in the CM fraction of 3T3-F442A cells than they are in the LR fraction of IM-9 cells. The reason for this difference between the two cell types is as yet unknown. Although this differential distribution of ERK pathway proteins may not completely explain the difference in the capacity of the two cells to allow GH-induced activation of this pathway, we see merit in further studies of the mechanistic impact of this correlation. We note that GH induced further accumulation of GHR (roughly 1.4-fold) and Grb2 (roughly 1.7-fold) in the CM fraction in 3T3-F442A cells. Results in IM-9 cells have been more variable on this point but have shown no such consistent acute GH-induced accumulation of receptors or Grb2 in the LR fraction (data not shown). As yet, we do not know the degree to which induced translocation of GHRs confers the ability to activate ERKs. This is another topic worthy of study that may be approachable when the structural determinants in the receptor that are required for its association with cholesterol-rich microdomains are better understood. ERK activation by GH has been linked in some systems to GH-induced c-fos gene activation and proliferation and to cross-talk between the GH and EGFR-family signaling systems (8–12). Thus, differential degrees of ERK activation in response to GH could underlie elements of the heterogeneity of GH responses.

Previous studies of the importance of caveolar/raft localization in receptor signaling have been focused on systems such as the intrinsic tyrosine kinase growth factor receptors (e.g., EGFR, insulin receptor, and IGF-1 receptor), serpentine receptors, and immunoreceptors. Relatively little information is available regarding the influence of cholesterol-rich microdomains on the signaling properties of cytokine receptor superfamily members and the JAKs. Some investigators report concentration of JAKs in rafts (74, 75), whereas others report exclusion from these microdomains (76). Interleukin-2 and interferon-γ receptor components have been localized to rafts, although no unified view has emerged to indicate how this localization affects signaling (43, 75–78). The only report to date in which the influence of caveolae/rafts on GH was studied was that of Lobie et al. (35), who used Chinese hamster ovary cells stably transfected with the rat GHR to demonstrate by electron microscopy that GHR colocalized with caveolin-1 and that this colocalization was increased after GH treatment. In that system, 125I-GH internalization was reduced by treatment with cholesterol-depleting reagents. Furthermore, transient expression of the GHR and overexpression of caveolin-1 in Chinese hamster ovary cells resulted in enhanced GH-induced GHR internalization and augmented GH-induced transactivation of reporter genes driven by STAT-binding elements.

Our results share some features in common with Lobie et al. (35) in that we observed substantial caveolar/raft association of the GHR. As mentioned above, our signaling studies implicate the caveolae/rafts as a potential platform for GH signaling. However, our data suggest that ERK signaling (perhaps because of interactions of the GHR and JAK2 with ERK pathway activators) may be particularly reliant on the GHRs in the lipid microdomains, whereas coupling to STAT5 activation may not utilize receptors in this fraction. More investigation of GHRs in different cellular contexts is warranted to determine the validity of these interpretations. However, we note that the cells used in the current study endogenously express the GHR and its signaling components. We view this as important in understanding the role of subcellular compartmentalization in that it may avoid the potential difficulties of aberrant protein trafficking and localization that are sometimes inherent when overexpressing receptors and signaling molecules.

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Caveolar and Lipid Raft Localization of the Growth Hormone Receptor and Its Signaling Elements: IMPACT ON GROWTH HORMONE SIGNALING
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