Growth hormone (GH) promotes signaling by causing activation of the non-receptor tyrosine kinase, JAK2, which associates with the GH receptor. GH causes phosphorylation of epidermal growth factor receptor (EGFR; ErbB-1) and its family member, ErbB-2. For EGFR, JAK2-mediated GH-induced tyrosine phosphorylation may allow EGFR to serve as a scaffold for GH signaling. For ErbB-2, GH induces serine/threonine phosphorylation that dampens basal and EGF-induced ErbB-2 kinase activation. We now further explore GH-induced EGFR phosphorylation in 3T3-F442A, a preadipocytic fibroblast cell line that expresses endogenous GH receptor, EGFR, and ErbB-2. Using a monoclonal antibody that recognizes ERK consensus site phosphorylation (PTP101), we found that GH caused PTP101-reactive phosphorylation of EGFR. This GH-induced EGFR phosphorylation was prevented by MEK1 inhibitors but not by a protein kinase C inhibitor. Although GH did not discernibly affect EGF-induced EGFR tyrosine phosphorylation, we observed by immunoblotting a substantial decrease of EGF-induced EGFR degradation in the presence of GH. Fluorescence microscopy studies indicated that EGF-induced intracellular redistribution of an EGFR-cyan fluorescent protein chimera was markedly reduced by GH cotreatment, in support of the immunoblotting results. Notably, protection from EGF-induced degradation and inhibition of EGF-induced intracellular redistribution afforded by GH were both prevented by a MEK1 inhibitor, suggesting a role for GH-induced ERK activation in regulating the trafficking itinerary of the EGF-stimulated EGFR. Finally, we observed augmentation of early aspects of EGF signaling (EGF-induced ERK2 activation and EGF-induced Cbl tyrosine phosphorylation) by GH cotreatment; the GH effect on EGF-induced Cbl tyrosine phosphorylation was also prevented by MEK1 inhibition. These data indicate that GH, by activating ERKs, can modulate EGF-induced trafficking and signaling and expand our understanding of mechanisms of cross-talk between the GH and EGF signaling systems.

Growth hormone (GH) is a four-helix bundle protein that shares structural similarity with a large class of hormones and cytokines, including prolactin and various interleukins and colony stimulating factors. GH exerts its profound somatogenic and metabolic regulatory effects by interacting with the GH receptor (GHR), a cell surface glycoprotein member of the cytokine receptor superfamily (1–3). Like other cytokine receptors, the GHR initiates its signal transduction by physical and functional association with JAK2, a cytoplasmic tyrosine kinase member of the JAK family (4, 5). GH activates several intracellular signaling pathways. GH-induced STAT5b activation has been particularly intensively studied and is strongly implicated in the regulation of certain GH-responsive hepatic genes (6). Other signaling pathways induced by GH include the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways, both also targets of a variety of other growth factors and cytokines.

GH induces activation of ERK1 and ERK2 in several model systems, including the murine 3T3-F442A preadipocytic fibroblast (7–10). In contrast to STAT5 activation, which requires the presence of the receptor cytoplasmic domain in addition to activation of JAK2, the ability of GH to activate ERKs appears to require none of the distal GHR tail and instead corresponds to whether it can promote JAK2 activation (9, 11–13). However, ERK is not activated in all cells in which GH activates JAK2 (14). Though it remains uncertain what factors determine whether a GHR-expressing cell or tissue will respond to GH with ERK activation, evidence exists among responsive cells for the potential involvement of a number of molecules. These include the Shc-Grb2-Sos-Raf pathway (15, 16), the phosphatidylinositol 3-kinase 3-kinase (or a related enzyme) pathway (17–19), IRS-1 (19) and the IRS-like Gab-1 adapter molecule (20), and c-src (21).

Recent studies of the mechanisms and consequences of GH-induced ERK activation have revealed interesting relationships between the GH and epidermal growth factor (EGF). These studies suggest previously unrecognized cross-talk between the GHR and members of the EGFR family, examples of seemingly disparate types of signaling receptors (a cytokine receptor and a family of tyrosine kinase receptors, respective-
ly). The EGFR family of structurally related transmembrane glycoproteins includes the EGFR itself (ErbB-1), ErbB-2 (c-neu), ErbB-3, and ErbB-4 (22, 23). Except for ErbB-3, each has intrinsic tyrosine kinase activity in its cytoplasmic domain. Ligands such as EGF, transforming growth factor-α, and neu-regulins induce signaling through these receptors by binding to specific EGFR family members in such a way as to promote specific homo- or heterodimers among the family members. ErbB-2 has no known ligand, but it is the preferred heterodimer partner for other family members when they engage their ligands. EGF, for example, promotes formation of EGFR homodimers or EGFR/ErbB-2 heterodimers and thus causes activation of both of these tyrosine kinases in cells that express them. EGF signaling through EGFR and ErbB-2 has a number of biologically relevant signaling outcomes in both normal and neoplastic cells.

Yamauchi et al. (24) first demonstrated that GH caused tyrosine phosphorylation of the EGFR, both in vivo in the livers of mice and in cell culture. This GH-induced EGFR tyrosine phosphorylation was shown to require JAK2, but not EGFR, kinase activity. Partial mapping by mutagenesis suggested that EGFR Tyr-1068, which when phosphorylated resides in a consensus Grb-2 association motif, was a site of GH-induced phosphorylation and that GH caused enhanced EGFR-Grb-2 association. Further, GH-induced EGFR tyrosine phosphorylation was shown in cell culture to likely contribute to GH-induced ERK activation. Thus, this study suggested that EGFR may be a docking molecule involved in GH-induced, JAK2-dependent ERK activation. Our previous study (25) in 3T3-F442A cells confirmed that GH caused EGFR kinase-independent EGFR tyrosine phosphorylation but also suggested GH effects on ErbB-2. GH caused a decrease in basal and EGF-induced ErbB-2 tyrosine kinase activation and tyrosine phosphorylation that was associated with retardation of the electrophoretic migration of ErbB-2. This retarded migration was shown to be because of serine or threonine, rather than tyrosine, phosphorylation of ErbB-2, and both the GH-induced change in migration and inhibited tyrosine kinase activation were prevented by blocking GH-induced ERK activation. These findings suggested that GH caused an ERK pathway-dependent phosphorylation of ErbB2 that rendered it desensitized to activation in response to EGF.

We now explore further the mechanisms and consequences of cross-talk between GH and EGFR signaling. In particular, we examine GH-induced phosphorylation of EGFR and ErbB-2. Our findings suggest that GH causes an ERK pathway-dependent threonine phosphorylation of both ErbB-2 and EGFR that is recognized by a state-specific antibody reactive with ERK consensus phosphorylation sites. In contrast to our findings for ErbB-2, we observe that this GH-induced EGFR phosphorylation does not significantly alter the intrinsic tyrosine kinase activation of EGFR, but instead slows the rate of EGF-induced EGFR intracellular redistribution and degradation, thereby potentiating EGF-induced EGFR signaling. These results suggest that GH may affect EGF signaling by multiple mechanisms, including modulation of EGF-induced EGFR trafficking.

### EXPERIMENTAL PROCEDURES

**Materials**—Recombinant human GH was kindly provided by Lilly. Recombinant human EGF was purchased from Upstate Biotechnology (Lake Placid, NY), and recombinant human PDGF-BB was from Intergen (Purchase, NY). Murine IGF-1 (150–200 μCi/μg) was purchased from PerkinElmer Life Sciences. PMA was obtained from Sigma. The PKC inhibitor, GF109203X (Calbiochem), and the MEK1 inhibitors, PD98059 (New England Biolabs, Beverly, MA) and U0126 (Promega, Madison, WI), were purchased commercially.

**Antibodies**—Polyclonal anti-ErbB-2, anti-EGFR, and anti-Cbl-b antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal anti-phospho-threonine-proline antibody (PTP101, polyclonal anti-phospho-seryl-proline antibody (Tyr-845, Tyr-992, Tyr-1045, and Tyr-1068 (Cell Signaling Technology, Beverly, MA), anti-active mitogen-activated protein kinase affinity-purified rabbit antibody (recognizing both ERK1 and ERK2), polyclonal anti-PDGFR antibody (recognizing both PDGFR type A and B receptors), and monoclonal anti-phospho-EGFR antibody (Tyr-1173 (Upstate Biotechnology, Lake Placid, NY) were all purchased commercially.

**Cell Culture and Transfection**—3T3-F442A cells (26), kindly provided by Drs. H. Green and M. Baltz, were maintained in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose (Cellgro, Inc.), supplemented with 10% calf serum, 50 μg/ml gentamicin sulfate, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Biofluids, Rockville, MD). 3T3-L1 cells from American Type Culture Collection (Manassas, VA) were grown in the above medium, supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD) instead of 10% calf serum.

To generate stable 3T3-L1 transfectants expressing the CFP-tagged EGFR (EGFR-CFP), cells were seeded in 60-mm dishes and used at 50–80% confluency. The plasmid pCFP/EGFR, kindly provided by Dr. L. Samuelson, Laboratory of Cellular and Molecular Biology, National Institutes of Health, Bethesda, MD, encodes the human EGFR with the CFP fused to its C terminus (27). pCFP/EGFR was transfected into 3T3-L1 cells using GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego, CA) as described previously (28). 3T3-L1 cells expressing CFP-CRF were selected in 1 mg/ml of G418 (Invitrogen) and cloned. Transfectants were maintained in culture medium containing 200 μg/ml of G418.

**Cell Starvation, Inhibitor Pretreatment, Cell Stimulation, and Protein Extraction**—Serum starvation of 3T3-F442A or 3T3-L1 cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V: Roche Molecular Biologicals) for serum or fetal bovine serum for the culture medium 24–26 hours prior to experiments. Treatments and stimulations were carried out at 37°C in binding buffer (consisting of 25 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 0.1% (w/v) bovine serum albumin, and 1 mM dextrose). Serum-starved cells were pretreated with PD98059 (100 μM), U0126 (10 μM), GF109203X (1 μM), or vehicle (as a control) for 30 or 60 min prior to treatment with GH (500 ng/ml), EGF (1 μM), PDGF (40 ng/ml), or vehicle, as specified in each experiment. Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline supplemented with 0.4 mM sodium orthovanadate (PBS-vanadate) and then harvested by scraping in PBS-vanadate. Cells were collected by brief centrifugation, and pelleted cells were incubated for 15 minutes at 37°C in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 5 μg/ml aprotinin, and 5 μg/ml leupeptin). A 15,000 × g for 15 min at 4°C, the detergent extracts (supernatant) were subjected to immunoprecipitation or were directly electrophoresed and immunoblotted, as indicated below. For examining the abundance of EGFR and ErbB-2, total cell lysates were extracted in the presence of 1% SDS.

**Immunoprecipitation and Immunoblotting**—For immunoprecipitation, cell extracts (500–1000 μg) were mixed with 5 μl of polyclonal anti-ErbB-2, -EGFR, -PDGFR, or -Cbl-b antibody (1 μg) and incubated at 4°C for 2 hours with continuous agitation. Protein A-Sepharose (20 μl) (Amersham Biosciences) was added and incubated at 4°C for an additional hour. The beads were washed four times with lysis buffer. Laemmli sample buffer eluates were resolved by SDS-PAGE and immunoblotted as indicated below.

**Densitometric Analysis**—Densitometry of immunoblots was performed according to the manufacturer’s suggestions (Amersham Biosciences).
formed using a solid state video camera (Sony-77; Sony Corp.) and a 28-mm MicroNikkor lens over a light box of variable intensity (Northern Light Precision 890; Imaging Research, Inc., Toronto, Canada). Quantification was performed using a Macintosh II-based image analysis program (Image 1.49, developed by W. S. Rashband; Research Services Branch, NIMH, Bethesda, MD). Pooled data from several experiments are displayed as mean ± S.E. The significance (p value) of differences of pooled results was estimated using unpaired t tests.

To verify the fidelity of this densitometric method, we performed a control experiment in which total protein concentration of 3T3-F442A cells was determined using BCA protein assay reagents (Pierce), and serially diluted total protein aliquots (2.5–100 µg) were resolved by SDS-PAGE and immunoblotted with anti-EGFR. Densitometry of the EGF intensities plotted against the known loaded protein amounts yielded a straight line with a correlation coefficient (R) of 0.97 (not shown). This suggested a high degree of reliability for this densitometric analysis.

Fluorescence Microscopy—3T3-L1 transfectants expressing EGFR-CFP were grown on Corning glass coverslips precoated with gelatin (Sigma) in 6-well plates for 48 h in culture medium until they reached ~50% confluence. The cells were starved, pretreated with PD98059 (100 µM) for 1 h, and stimulated with GH (500 ng/ml), EGF (1 nM), or vehicle as described above and specified in the experiment. The cells were rinsed with PBS and fixed with 4% formaldehyde solution in PBS for 15 min at room temperature. After rinsing with PBS, the coverslips were mounted on microscope slides (Fisher) in Vectashield mounting medium for fluorescence (Vector Laboratories Inc., Burlington, CA). Fluorescence patterns were visualized with an Olympus fluorescence microscope at the University of Alabama Cell Biology Imaging Core Facility. Images were collected and analyzed using IPLab Spectrum software (Scanalytics Inc., Fairfax, VA).

FIG. 1. GH and EGF acutely induce appearance of PTP101-reactive ErbB-2 and EGF-R in 3T3-F442A cells. A, serum-starved 3T3-F442A cells were stimulated with GH (500 ng/ml), EGF (1 nM), or vehicle for 10 min. Detergent extracts (50 µg) were immunoprecipitated with anti-ErbB-2 (lanes 1–3) and anti-EGFR (lanes 4–6) antibodies, respectively. Eluted proteins were analyzed by immunoblotting with anti-ErbB-2 (upper panel, lanes 1–3), anti-EGFR (upper panel, lanes 4–6), pTyr (middle panel), and PTP101 (lower panel), respectively. B, detergent extracts (30 µg) as in A were resolved without immunoprecipitation by SDS-PAGE and immunoblotted with an anti-active ERK1/2 antibody. The experiments shown are representative of five such experiments.

Results

GH Acutely Promotes ERK-dependent Phosphorylation of EGFR at Sites Recognized by an ERK Phosphorylation Site Antibody—The murine 3T3-F442A fibroblast expresses EGFR and ErbB-2, as well as the receptor for GH (25). We and others (4, 10, 18, 24, 25, 29, 30) have detected biochemical responses to both GH and EGF in this cell line, making it an appealing target for evaluation of potential cross-talk between the GH and EGF signaling pathways. Our previous studies (25) indicated that in 3T3-F442A cells GH treatment causes ErbB-2 to undergo serine/threonine phosphorylation that results in a decrease in its basal activation state and its desensitization to EGF-induced activation. These effects of GH were inhibited by drugs that block activation of the MEK1/ERK pathway.

To further characterize such serine/threonine phosphorylation of EGFR family members, we employed a state-specific monoclonal antibody (PPTP101) that specifically detects proteins phosphorylated at the consensus site(s) for proline-directed protein kinases, such as the ERKs (31–33). In the experiment shown in Fig. 1A, serum-starved cells were exposed to GH, EGF, or vehicle (--) for 10 min prior to detergent extraction and immunoprecipitation with anti-ErbB-2 (lanes 1–3) or anti-EGFR (lanes 4–6) antibodies. Eluates were resolved by SDS-PAGE and immunoblotted sequentially with anti-ErbB-2 (upper panel, lanes 1–3), anti-EGFR (upper panel, lanes 4–6), antiphosphotyrosine antibodies (anti-pTyr; middle panel, lanes 1–6), and PTP101 (lower panel, lanes 1–6). Consistent with our previous findings (25), neither GH nor EGF acutely changed the abundance of ErbB2 or EGFR (upper panel, lanes 1–6), but both stimuli caused retardation in the SDS-PAGE migration of ErbB-2 (upper panel, lanes 1 and 3 versus 2). For EGF, this retarded migration was accompanied by an increase in ErbB-2 tyrosine phosphorylation (middle panel, lane 3 versus 2), whereas GH caused a decrease in ErbB-2 tyrosine phosphorylation (middle panel, lane 1 versus 2). As expected (24, 25), EGFR tyrosine phosphorylation was promoted by both GH and EGF, although EGF was more potent than GH (middle panel, lanes 4 and 6 versus 5). Notably, the immunoblot in the lower panel (lanes 1–6) revealed that GH and EGF promoted the appearance of forms of ErbB-2 and EGFR that were recognized by PPTP101, suggesting phosphorylation at ERK consensus sites in those molecules. Indeed, immunoblotting of unfractionated cell extracts from the same cells with anti-active ERK antibodies confirmed that both GH and EGF promoted robust ERK activation in these cells (Fig. 1B, lanes 1 and 3 versus 2). Interestingly, whereas GH induced less EGFR tyrosine phosphorylation than did EGF, comparison of the GH- and EGF-induced PPTP101 signals in Fig. 1A suggests that GH promoted substantially more EGFR ERK consensus site phosphorylation than did EGF.

The data in Fig. 1 indicated that both GH and EGF caused phosphorylation of ErbB-2 and EGFR at potential ERK consensus sites, suggesting that this is mediated by activation of the ERK pathway by these stimuli. To further test this propo-
EGFR (Fig. 2, lower panel) shown in are representative of two such experiments. Eluted proteins were resolved and immunoblotted with anti-EGFR (B, lower panel), anti-ErbB-2 (A, lower panel), or anti-ERK1/2 (upper panel) and anti-ERK1/2 (lower panel). The experiments shown in are representative of two such experiments.

We employed inhibitors of MEKI, the upstream activator of ERK1 and ERK2 (Fig. 2). Two separate inhibitors, PD98059 and the more potent U0126 (34–36), were utilized. As already observed, GH and EGF induced the PTP101 reactivity of both ErbB-2 (Fig. 2A, upper panel, lanes 4 and 7 versus 1) and EGFR (Fig. 2B, upper panel, lanes 4 and 7 versus 1). Pretreatment of the cells with either PD98059 or U0126 dramatically inhibited both GH- and EGF-induced PTP101 reactivity of ErbB-2 (Fig. 2A, upper panel, lanes 5 and 6 versus 4 and lanes 8 and 9 versus 7) and EGFR (Fig. 2B, upper panel, lanes 5 and 6 versus 4 and lanes 8 and 9 versus 7). As seen in Fig. 2C, immunoblotting of detergent extracts with anti-active ERK (upper panel) verified the inhibitory effects of PD98059 and U0126 on GH- and EGF-induced ERK activation (lanes 5 and 6 versus 4 and lanes 8 and 9 versus 7). Consistent with our previous findings (25), the GH-induced retardation of migration of ErbB-2 was blocked by the MEKI inhibitors (Fig. 2A, lower panel, lanes 4–6 versus 1–3). In contrast, the EGFR did not exhibit detectable change in its SDS-PAGE migration in response to the phosphorylations induced by GH (Fig. 2B, lower panel).

Previous studies suggested that serine/threonine phosphorylation of EGFR family members induced by the phorbol ester PMA and PDGF are attributable to the activation of PKC (37–41). We tested whether the GH-induced ErbB-2 and EGFR phosphorylations detected by PTP101 were related to activation of PKC by using the PKC inhibitor, GF109203X (42) (Fig. 3). PMA, like GH, induced the PTP101 reactivity of ErbB-2 (Fig. 3A, upper panel, lanes 7 and 4 versus 1) and EGFR (Fig. 3B, upper panel, lanes 7 and 4 versus 1) and ERK activation (Fig. 3C, upper panel, lanes 7 and 4 versus 1). As expected, the PMA-induced ERK activation and modifications of ErbB-2 and EGFR were prevented by preincubation of the cells with GF109203X or with the MEKI inhibitor U0126 (Fig. 3, A–C, upper panels, lanes 8 and 9 versus 7). In contrast, neither GH-induced ERK activation nor ErbB-2 and EGFR PTP101 reactivity were affected by the PKC inhibitor, though U0126 was inhibitory, as expected (Fig. 3, A–C, upper panels, lanes 5 and 6 versus 4). These data strongly suggest that PTP101 is detecting ERK-dependent, rather than PKC-dependent, phosphorylation of EGFR family members that occurs in response to GH and that the PMA-induced ErbB-2 and EGFR PTP101-reactive phosphorylations are also mediated by ERK activation; however, the mechanism of PMA-induced (in distinction to the GH-induced) ERK activation in these cells appears to involve the activation of PKC forms that are sensitive to GF109203X. Further, PMA caused retardation of SDS-PAGE migration of both ErbB-2 and EGFR, and this PMA-induced retarded migration was prevented by both the PKC and MEKI inhibitors (Fig. 3, A and B, lower panels, lanes 7–9 versus 1–3),...
Tyr-1068 was a preferred site for GH-induced EGFR tyrosine phosphorylation of the EGFR family members, but not of the PDGFR.

GH-induced activation of ERKs results in ERK kinase condensation on the ErbB-2 activation mechanism. As detailed above, GH-promoted PDGFR tyrosine phosphorylation, as assessed by anti-PDGFR immunoprecipitation followed by anti-pTyr blotting (Fig. 4A, upper panel, lane 2 versus 3), verifying the responsiveness of these cells to PDGF. In contrast, GH failed to promote either tyrosine phosphorylation (Fig. 4A, upper panel, lane 1) or PTP101-reactive phosphorylation of PDGFR (Fig. 4A, middle panel, lanes 1 and 2 versus 3), despite the rough equivalence of PDGFR immunoprecipitation in GH, PDGF, and control stimulations (Fig. 4A, lower panel, lanes 1–3). As a positive control, the GH-induced PTP101-reactive phosphorylation of ErbB2 in the same experiment was monitored (Fig. 4B, upper panel, lane 1 versus 2), confirming the ability of these reagents and methods to detect such a modification. We conclude that GH-induced activation of ERKs results in ERK kinase consensus sequence phosphorylation of the EGFR family members, ErbB-2 and EGFR, but not of the PDGFR.

GH Affects EGF-induced EGFR Trafficking in 3T3-F442A Cells—We demonstrated previously (25) that acute treatment of 3T3-F442A cells with the combination of GH and EGF resulted in substantially less activation of ErbB-2 than did exposure to EGF alone, suggesting a dampening effect of GH signaling on the ErbB-2 activation mechanism. As detailed above, GH promoted both tyrosine- and PTP101-reactive phosphorylation of the EGFR. Thus, we examined whether cotreatment with GH appreciably affected EGF-induced EGFR tyrosine phosphorylation. For these experiments, we employed a panel of state-specific antibodies, each specific for phosphorylation of particular EGFR tyrosine residues, Tyr-845, Tyr-992, Tyr-1045, Tyr-1068, or Tyr-1173, in addition to anti-pTyr antibody (Fig. 5). Consistent with our prior treatment protocol (25), cells were treated with vehicle (−), GH, or EGF alone for 15 min or EGF for 15 min in the presence of GH (added 10 min prior to EGF). As expected, GH and EGF each alone caused EGFR tyrosine phosphorylation (Fig. 5, row F (anti-pTyr blot), lane 2 and 3 versus 1), with the effect of EGF being more potent. Yamauchi et al. (24) used EGFR mutagenesis to suggest that Tyr-1068 was a preferred site for GH-induced EGFR tyrosine phosphorylation. Blotting of EGFR precipitates with various phosphotyrosine state-specific antibodies (Fig. 5, rows A–E) confirmed that this site (row D, lane 2 versus 1) is phosphorylated in response to GH. However, we also detected substantial GH-induced phosphorylation of Tyr-845 (row A, lane 2 versus 1), a site not known previously to be targeted. Additionally, we observed less substantial phosphorylation of Tyr-992 and Tyr-1045 (C, Tyr-1068 (D), Tyr-1173 (E), anti-pTyr (F), or anti-EGFR (G). The experiments shown are representative of three such experiments.
difference in the level of EGFR tyrosine phosphorylation in EGF-treated versus EGF plus GH-treated samples in which the GH cotreatment was simultaneous to the EGF treatment, rather than being added 10 min prior. Thus, unlike our findings for ErbB-2, GH-induced activation had no appreciable effect on the level of acute EGF-induced EGFR tyrosine phosphorylation, a reflection of the earliest steps in EGF-induced EGFR triggering.

We next investigated the effects of GH on the EGF-induced fate of the EGFR and ErbB-2. It is well documented that EGF induces down-regulation of EGFR, but not ErbB-2, mass (reviewed in Ref. 45). We used immunoblotting of total cell lysates (extracted in the presence of 1% SDS) to estimate the abundance of the EGFR and ErbB-2 in 3T3-F442A cells treated with EGF and GH over a 4-h period (Fig. 6). As expected, EGF treatment caused a time-dependent loss of EGFR, detectable after 30 min and progressive over the remainder of the period (Fig. 6A, upper panel, lanes 2–6 versus 1). In contrast, no loss of EGFR mass was observed in response to treatment with control vehicle alone (not shown) or with GH alone (Fig. 6A, upper panel, lanes 12–16 versus 1), likely consistent with the lack of EGFR tyrosine kinase activation by GH (24, 25). Interestingly, however, treatment with EGF in the presence of GH (added 10 min prior to EGF) resulted in attenuation of the loss of EGFR mass in comparison with exposure to EGF alone (Fig. 6A, upper panel, lanes 7–11 versus 2–6). These parameters were estimated quantitatively by densitometric analysis of the results of five such experiments (Fig. 6B). Though GH itself did not affect EGFR mass, GH cotreatment significantly inhibited the EGFR loss induced by EGF treatment of 60, 120, or 240 min duration (p values less than 0.05, 0.01, and 0.001, respectively). This analysis indicated that the EGFR loss promoted by 240 min of EGF treatment was reduced by roughly 50% in samples also exposed to GH. Thus, GH partially, but substantially, protects EGFRs in 3T3-L1 cells from the ligand-activated down-regulation of the EGFR in this system. Notably, ErbB-2 abundance was not affected by treatment with EGF alone, GH alone, or EGF plus GH (Fig. 6A, lower panel, lanes 2–6, 12–16, and 7–11, respectively, versus 1), consistent with previous observations of the lack of ErbB-2 down-regulation in response to EGF.

Because GH did not affect acute EGF-induced EGFR tyrosine phosphorylation (Fig. 5) but did cause EGFR PTP101-reactive phosphorylation (Figs. 1–4) and attenuated EGF-induced EGFR loss (Fig. 6), we considered whether the GH-induced ERK-mediated phosphorylation might relate to the effects of GH on EGFR trafficking. To address this issue, cells were treated with GH, EGF, or EGF plus GH for 2 h in the absence or presence of the MEK1 inhibitor, PD98059. As expected, in the absence of PD98059, EGF caused substantial loss of EGFR (Fig. 7A, lane 3 versus 1), GH alone did not affect EGFR abundance (lane 2 versus 1), and the presence of GH blunted the EGF-induced EGFR loss (lane 4 versus 3). In the presence of PD98059, EGF-induced EGFR loss was similar to that seen in the absence of the drug (compare lane 6 versus 8 to lane 3 versus 1), whereas GH caused no loss of EGFR (lane 7 versus 8). Notably, however, the EGF-induced loss of EGFR was not prevented by GH cotreatment in the presence of PD98059 (lane 5 versus 6), in contrast to the findings in the absence of the MEK1 inhibitor (lane 3 versus 4). For ease of visualization, the immunoblot in Fig. 7A was scanned densitometrically, and the relative intensity of the EGFR band (relative EGFR mass) is plotted for each lane in Fig. 7B. Pooled data from four independent experiments were densitometrically evaluated and are shown in Fig. 7C. In this display, the loss of EGFR induced by a 2-h incubation with EGF is considered as 100%. Cotreatment with GH in the absence of PD98059 blunted the EGF-induced loss, resulting in a 64 ± 8% loss of EGFR (p < 0.05 versus EGF alone). In contrast, in the presence of PD98059, GH plus EGF resulted in a 102.9 ± 1.7% loss of EGFR, not different from the 100% loss induced by EGF alone. Thus, blockade of the ERK activation pathway reversed the effect of GH on EGF-induced EGFR loss. This suggests that the GH-induced activation of ERKs and possibly ERK-dependent EGFR PTP101-reactive phosphorylation substantially affect the ligand-activated trafficking of the EGFR, slowing its degradation and down-regulation.

These biochemical studies suggested that GH may impinge on early aspects of EGF-induced EGFR trafficking, because the protective effect of GH on EGFR loss could be discerned as early as 30 min into EGF treatment, albeit not statistically significantly so until 1 h (Fig. 6B). We thus sought to examine the effects of GH on acute EGF-induced EGFR trafficking. As we have shown previously (25, 44), 3T3-L1 fibroblasts, like 3T3-F442A cells, endogenously express GHRs and biochemically respond similarly to GH. Notably, the abundance of EGFRs in 3T3-L1 cells was found to be substantially less than

Modulation of EGF-induced Trafficking and Signaling
thermore, GH treatment resulted in ERK activation in the L1/EGFR-CFP, as expected (data not shown).

We next performed fluorescence microscopy of 3T3-L1/EGFR-CFP cells to track the subcellular distribution of the EGFR chimera (Fig. 8D, upper panel). As expected, untreated serum-starved cells (control) exhibited prominent steady-state cell surface EGFR-CFP expression, as has been noted previously (27) for this chimera and for endogenous EGFRs (reviewed in Ref. 45). Treatment with GH for 25 min had no discernable effect on the distribution of EGFR-CFP. In contrast, EGF treatment for 15 min caused a substantial redistribution of EGFR-CFP away from the cell surface to perinuclear and peripheral intracellular aggregates, similar to those observed by others (27, 41) in the course of EGFR endocytosis. Notably, treatment with EGF in the presence of GH (GH/EGF, in which GH was added 10 min prior to EGF) markedly reduced this translocation, resulting in substantial remaining EGFR-CFP on the cell surface and diminished accumulation of intracellular chimera. We further tested the impact of inhibition of the ERK pathway on these EGF- and GH-induced effects on EGFR trafficking by performing the same experiments in the presence of the MEK1 inhibitor, PD98059 (Fig. 8D, lower panel). ERK inhibition had no discernable effect on the distribution of EGFR-CFP in control, GH-treated, or EGF-treated cells, consistent with the biochemical data on EGFR abundance in Fig. 7A. Remarkably, however, PD98059 treatment precluded the inhibitory effect of GH on EGF-induced EGFR-CFP intracellular redistribution (Fig. 8D, GH/EGF treatments, upper versus lower panel). Together with the data in Fig. 7, these fluorescence microscopy experiments suggest that GH lessens EGF-induced EGFR loss in an ERK pathway-dependent fashion, likely by inhibiting an early step(s) in EGFR trafficking.

To further probe the effect of GH, we assessed radiolabeled EGF internalization in these cells in the presence or absence of GH (Fig. 9). 3T3-F442A cells were treated at 37 °C for 10 min with GH prior to the addition of murine 125I-EGF (1 ng/ml). At intervals over 10 min after radiolabeled EGF addition, internalized 125I-EGF was measured, as described under “Experimental Procedures.” Radiolabeled EGF internalization, expressed as a fraction of total specific EGF binding, is plotted as a function of the duration of incubation in Fig. 9. Notably, GH pretreatment did not appreciably change the rate of 125I-EGF internalization. We thus conclude that the changes in EGF-induced EGFR fate promoted by GH are not likely exerted at the earliest steps corresponding to EGF internalization.

**GH Affects Acute EGF-induced EGFR Signaling**—The findings above suggested that GH affects EGF-induced EGFR trafficking in such a way as to delay its degradation and down-regulation. We explored whether this effect of GH might have impact upon early EGF signaling events. We first examined the effects of GH on EGF-induced ERK activation (Fig. 10). 3T3-F442A cells were exposed to GH for 0–60 min, and detergent extracts were evaluated with anti-active ERK immunoblotting (Fig. 10A, upper panel). Consistent with previous reports (7, 8, 10), treatment with GH (500 ng/ml) caused ERK activation as early as 5 min with peak activation occurring between 5 and 15 min. Thereafter, ERK activation markedly declined. We next compared the level of ERK activation induced by EGF alone (15 or 30 min) with that induced by EGF in the presence of GH (Fig. 10B). As in the experiments above, in the cotreated samples, GH was added 10 min prior to EGF; thus, comparison treatments with GH alone were for 25 or 40 min. The level of ERK activation achieved by the combination of EGF plus GH was greater than EGF alone at both 15 and 30 min of EGF stimulation (Fig. 10B, upper panel, lane 4 versus 3 and lane 7 versus 6, respectively).

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*Y. Huang and S. J. Frank, unpublished observations.*
To determine whether this augmentation reflected simply the summation of GH-induced plus EGF-induced ERK activation or instead reflected synergistic ERK activation (more than the summation) by EGF in the presence of GH, we performed densitometric evaluation of three such experiments. The results for ERK2 activation are displayed in Fig. 10C. In this figure, the open bars indicate the sum of the relative activation of ERK2 elicited by 15 min (left side) or 30 min (right side) of EGF alone (corresponding to lanes 3 and 6, respectively of Fig. 10B, upper panel) plus the ERK2 activation caused by GH alone (25 min, left side; 40 min, right side, corresponding to lanes 2 and 5, respectively, in Fig. 10B, upper panel). That summation is compared with the closed bars, which reflect the activation elicited by the cotreatment with EGF plus GH (15/25 min, left side; 30/40 min, right side, corresponding to lanes 4 and 7, respectively, in Fig. 10B, upper panel). Notably, no synergy was detected in comparing EGF only for 15 min with EGF for 15 min in the presence of GH (no difference in the height of the open and closed bars on the left of Fig. 10C), but significant synergistic augmentation was observed in comparing EGF treatment for 30 min in the presence of GH with EGF treatment alone for 30 min (p < 0.05 for the roughly 60% increase in the height of the closed bar versus the open bar on the right of Fig. 10C). Thus, GH cotreatment appeared to sensitize the EGF-induced ERK2 activation signals that continue after 30 min (but not 15 min) of EGF exposure. Because GH-induced ERK2 activation is minimal at this time (40 min of GH exposure), this suggests that changes in either the EGFR activation state or the itinerary of the EGF-activated EGFR brought about acutely by GH might affect the duration or strength of the EGF-induced ERK2 activation signal.

EGF-induced tyrosine phosphorylation of Cbl is a well-documented early event in EGFR signaling in a range of cell types (46–50). To further investigate the effects of GH on EGFR-mediated signaling, we examined the tyrosine phosphorylation of Cbl (Fig. 11). We first tested whether EGF also induced tyrosine phosphorylation of Cbl in 3T3-F442A cells (Fig. 11A). Serum-starved cells were treated with EGF for 15, 30, or 90
inhibition of the ERK pathway (Fig. 11C, upper panel, lanes 3, 6, and 9 versus 1). In other experiments (not shown), we also detected the tyrosine-phosphorylated EGFR and SHC in anti-Cbl-b precipitates in response to EGF, as has been observed in other cell types. In contrast to EGF treatment, GH stimulation for 15, 30, or 90 min did not result in detectable tyrosine phosphorylation of Cbl (Fig. 11A, upper panel, lanes 2, 5, and 8 versus 1). Notably, however, cotreatment with GH plus EGF at both 15 and 30 min of EGF exposure resulted in substantially increased Cbl tyrosine phosphorylation when compared with treatment with EGF alone (Fig. 11A, upper panel, lanes 4 versus 3 and lanes 7 versus 6). These findings strongly suggested that GH enhanced the ability of EGF to cause Cbl tyrosine phosphorylation.

To examine the basis for this augmentation of EGF-induced Cbl tyrosine phosphorylation by GH, we tested the effect of inhibition of the ERK pathway (Fig. 11B). Pretreatment with the MEK1 inhibitor, PD98059, had no effect on EGF-induced Cbl tyrosine phosphorylation (Fig. 11B, upper panel, lane 6 versus 3), nor did the drug reverse the inability of GH to cause Cbl tyrosine phosphorylation (Fig. 11B, upper panel, lane 5 versus 2). PD98059 did, however, prevent the augmentation of EGF-induced Cbl tyrosine phosphorylation by GH (Fig. 11B, lane 7 versus 6 compared with lane 4 versus 3). Densitometric evaluation of three such experiments (Fig. 11C) indicated that GH cotreatment in the absence of PD98059 augmented EGF-induced Cbl tyrosine phosphorylation (15 min of treatment with EGF) by 70 ± 13% (p < 0.05) but that cotreatment in the presence of PD98059 eliminated the GH-induced augmentation.

DISCUSSION

Emerging data suggest that the EGFR family signaling system has interrelationships with various other signaling systems. Interactions between EGFR itself or EGFR family members and the GHR have been recognized in the past several years. Yamachi et al. (24) demonstrated that GH promotes EGFR tyrosine phosphorylation that is dependent on JAK2 activation but independent of an intact EGFR kinase domain. The implication of that study was that the EGFR serves as a docking molecule for the GHR-JAK2 system, allowing GH activation to trigger loading of the tyrosine phosphorylated EGFR with Grb-2, a key activator of the ERK pathway. Our studies in 3T3-F442A cells have confirmed that GH-induced EGFR tyrosine phosphorylation is not dependent on EGFR kinase activation (25). In the current study, we extend these findings to indicate that several EGFR tyrosine residues become phosphorylated in response to GH, most notably Tyr-845 and the previously implicated Tyr-1068. Our inability to discern more abundant overall EGFR tyrosine phosphorylation in the presence both GH and EGF is likely related to the overall stoichiometry for tyrosine phosphorylation induced by each peptide, with that induced by EGF far outweighing that induced by GH alone.

FIG. 9. Effect of GH on internalization of 125I-EGF. Serum-starved 3T3-F442A cells were pretreated with GH (500 ng/ml) or vehicle at 37 °C for 10 min. Cells were then incubated with 125I-EGF (1 ng/ml) at 37 °C for various time intervals (0–10 min). The internalized 125I-EGF was expressed as a fraction of total cell-associated radioactivity, as described under “Experimental Procedures.” The mean and range (error bars) of duplicate determinations are shown. The same results were obtained in another experiment in which 125I-EGF binding occurred at 4 °C for 1 h prior to allowing internalization to occur by warming to 37 °C (not shown).

FIG. 10. GH augmentation of EGF-induced ERK1/2 activation in 3T3-F442A cells. A, kinetics of GH-induced ERK1/2 activation. Serum-starved 3T3-F442A cells were treated with 500 ng/ml GH for indicated durations. Total cell lysates (extracted in the presence of 1% SDS) (15 μg) were resolved by SDS-PAGE and immunoblotted with anti-active ERK1/2 (upper panel) and anti-ERK1/2 (lower panel). B, activation of ERK1/2. Cells were treated with 1 nM EGF alone (lanes 3 and 6) or 500 ng/ml GH plus 1 nM EGF (GH was added 10 min prior to EGF) (lanes 4 and 7) for 15 and 30 min, respectively. Note that the incubation durations for GH in lanes 4 and 7 are 25 and 40 min, respectively. Therefore, cells treated with 500 ng/ml GH alone for 25 and 40 min were used as controls (lanes 2 and 5) in addition to vehicle treatment (lanes 1 and 8). Total cell lysates (15 μg) were resolved by SDS-PAGE and immunoblotted with anti-active ERK1/2 (upper panel) and anti-ERK1/2 (lower panel). C, augmentation of EGF-induced ERK2 activation by cotreatment with GH. Data shown in B, along with those obtained from two other experiments, were subjected to densitometric analysis. Data are normalized to the level of GH-induced ERK2 activation (the GH-induced ERK2 activation is considered as 100%) and are mean ± S.E. of three experiments. Comparison of the level of ERK2 activation by cotreatment of GH plus EGF where GH was added 10 min prior to EGF (referred to as GH alone + EGF; closed bars) with the sum of those induced by EGF alone plus GH alone (referred to as GH alone + EGF alone; open bars) at 15 and 30 min, respectively, is shown. For ERK2 activation by GH/EGF versus GH alone + EGF alone at 30 min, p < 0.05, as indicated.
cells were preincubated without (lanes 1–4) B lanes 4 (GH was added 10 min prior to EGF) (cell-surface precursors (51–59). The ligands then can engage protease-catalyzed release of EGF family ligands from their among others, and in those instances is mediated by metallo-lysophosphatidic acid, endothelin, and glucagon-like peptide-1, of receptors for angiotensin II, thrombin, carbachol, bombesin, with anti-pTyr (500 ng/ml GH (lanes 2 and 5), or GH plus EGF (GH was added 10 min prior to EGF) (lanes 4 and 7, and 10) for 15, 30, or 90 min, respectively. B, cells were preincubated without (lanes 1–4) or with 100 µM PD98059 (lanes 5–8) for 60 min prior to stimulation with vehicle (lanes 1 and 8), 500 ng/ml GH (lanes 2 and 5), 1 nx EGF (lanes 3 and 6), or GH plus EGF (GH was added 10 min prior to EGF) (lanes 4 and 7) for 15 min. In A and B, detergent extracts (500 µg) were immunoprecipitated with anti-Cbl-b. Eluted proteins were resolved by SDS-PAGE and immunoblotted with anti-pTyr (A and B, upper panels) and anti-Cbl-b (A and B, lower panels), respectively. C, data shown in B, along with those obtained from two other experiments, were subjected to densitometric analysis. In each experiment, the EGF-induced Cbl-b tyrosine phosphorylation is considered as 100%. Data are mean ± S.E. of three experiments. For Cbl-b tyrosine phosphorylation by GH/EGF versus EGF alone in the absence of PD98059, p < 0.05, as indicated.

unaccompanied by generation of EGF or EGF-family ligands but was instead envisioned to occur by oligomerization of ErbB-2 molecules associated with the gp130 subunit of the IL-6R complex. Because GH-induced activation of the EGFR kinase does not underlie GH-induced EGFR tyrosine phosphorylation, we view the cross-talk of GH with EGFR as mechanistically and probably functionally distinct from these examples of EGFR family member transactivation by G protein-coupled receptor activators and IL-6. Our findings that GH alone does not promote EGFR internalization and degradation are consistent with the lack of induction of EGFR kinase activation by GH.

Although we cannot completely rule out the possibility that GH can cause phosphorylation of particular ErbB-2 tyrosine residues, our data in 3T3-F442A and 3T3-L1 cells strongly suggest that, unlike its effects on EGFR, GH induces a lessening of both basal and EGF-induced overall tyrosine phosphorylation of ErbB-2 and also decreases ErbB-2 tyrosine kinase activity (25). This finding is also in contrast to the effects of IL-6 on ErbB-2 in cancer cells noted above (60). We note that both GH and IL-6 couple to signaling pathways that involve JAK kinases. The dissimilarities of the effects of the two ligands on ErbB-2 kinase activity in these two different systems remain as yet unexplained, but strongly suggest that the milieu in which the JAKs are activated and/or the downstream signaling pathways activated likely dictate the net effect of non-EGF family ligands on EGFR family responses.

The current study in 3T3-F442A, a preadipocytic fibroblast that endogenously expresses GHR, ErbB-2, and EGFR, highlights the finding that both ErbB-2 and EGFR are also targets for GH-induced phosphorylation at sites other than tyrosine residues. In particular, use of the PTP101 monoclonal antibody for immunoblotting allowed us to detect prominent GH-induced phosphorylation of both molecules at what are most likely ERK consensus phosphorylation sites. The GH-induced PTP101-reactive phosphorylations of ErbB-2 and EGFR were strongly inhibited by two different MEK1 inhibitors and not inhibited by a PKC inhibitor, furthering the conclusion that GH-induced ERK activation is involved. The EGFR family members are at least relatively specifically targeted in that, by contrast, GH-induced ERK activation does not lead to PTP101-reactive phosphorylation of the PDGFR in the same cells. Notably, although GH induces ERK consensus phosphorylation of both ErbB-2 and EGFR, the consequences of that phosphorylation may differ substantially. As noted previously, ErbB-2 becomes relatively desensitized for basal and EGF-induced kinase activation in a manner correlated to the GH-induced phosphorylation. In contrast, EGFR kinase activation and trafficking appear unaffected by GH treatment alone. Rather, EGF-induced EGFR trafficking is substantially influenced by GH in a pattern corresponding to the ability of GH to activate the ERK signaling pathway. Our data indicate that both EGF-induced EGFR degradation and early EGF-induced EGFR redistribution from the cell surface to internal pools are substantially lessened by GH and that these GH effects are blocked by MEK1 inhibition.

Previous studies have implicated serine/threonine kinases as important in regulation of EGFR function. In particular, two major threonine phosphorylation sites in the juxta-membrane cytoplasmic domain have been identified, Thr-654 and Thr-669 (37, 61–63). PMA can lead to the phosphorylation of both, but protein kinase C is believed to directly mediate Thr-654 phosphorylation, whereas Thr-669 phosphorylation is thought to be phosphorylated by ERKs (64, 65). Indeed, Thr-669 lies in the only ERK phosphorylation consensus in the EGFR cytoplasmic domain and is a known target for EGF-induced EGFR phos-
phosphorylation. The exact role of these phosphorylations, if any, in EGFR signaling is as yet unclear. For example, although PKC activation leads to decreased EGFR-induced EGFR kinase activation, direct phosphorylation of EGFR by either PKC or ERKs is not believed to be sufficient to mediate this effect (66).

In contrast to the MEK1-dependent dampening effects of PKC activation on EGFR kinase activity reported previously (66), GH treatment of 3T3-F442A cells in our experiments does not cause detectable decrease in EGFR-induced EGFR tyrosine phosphorylation. Instead, the effect we observe is a GH-induced, MEK1-dependent alteration of the EGF-induced fate of phosphorylation. Notably, Bao et al. (41) reported a FMA-induced change in EGFR-induced EGFR trafficking and down-regulation that was PKC-dependent and attributable to Thr-654 phosphorylation. Our findings that GH protects the EGFR from EGFR-induced intracellular redistribution, degradation, and down-regulation, in each case in a fashion reversible by PD98059, suggest, to our knowledge, a heretofore unappreciated role of regulation, in each case in a fashion reversible by PD98059, in the post-ligand engagement trafficking of the activated EGFR.

In this regard, we suggest that GH, in addition to its known IGF-1-generating and direct effects, may also exert its actions by regulatory cross-talk with an important tyrosine kinase growth factor receptor system.

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