The emergence of ErbB2 expression in cultured rat hepatocytes correlates with enhanced and diversified EGF-mediated signaling

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Scheving, Lawrence A., Linda Zhang, Mary C. Stevenson, Eun Soo Kwak, and William E. Russell. The emergence of ErbB2 expression in cultured rat hepatocytes correlates with enhanced and diversified EGF-mediated signaling. Am J Physiol Gastrointest Liver Physiol 291: G16–G25, 2006; doi:10.1152/ajpgi.00328.2005.—The proliferative effects of EGF in liver have been extensively investigated in cultured hepatocytes. We studied the effects of EGF, insulin, and other growth regulators on the expression, interaction, and signaling of ErbB receptors in primary cultures of adult rat hepatocytes. Using immunological methods and ErbB tyrosine kinase inhibitors, we analyzed the expression and signaling patterns of the ErbB kinases over 120 h of culture. Basal and EGF-stimulated protein tyrosine phosphorylation increased as cells adapted in vitro. EGF receptor (EGFr) expression declined in the first 24 h, whereas ErbB3 expression rose. Although ErbB2 was not present in freshly isolated hepatocytes, EGF and insulin independently induced ErbB2 while suppressing ErbB3 expression. Low concentrations of EGF and insulin synergistically stimulated ErbB2 expression and DNA synthesis. The greatest increase in ErbB2, which is normally expressed by fetal and neonatal hepatocytes, occurred shortly before the onset of DNA synthesis (>40 h). EGF promoted EGFr and ErbB2 coassociation, stimulating tyrosine phosphorylation of both proteins. In contrast, heregulin β1 (HRG-β1) did not promote ErbB2 and ErbB3 coassociation. A selective tyrphostin inhibitor of ErbB2 tyrosine phosphorylation increased as cells adapted in vitro. ErbB4 (17). ErbB2 is a potent tyrosine kinase and signal transducer that has no ligand (11). ErbB2 can heterodimerize with ligand-activated ErbB receptors (EGFr, ErbB3, or ErbB4). Alternatively, ErbB2 molecules can homodimerize into ligandless signaling complexes when overexpressed by certain cancer cells. In contrast to other ErbB proteins, ErbB3 has an inactive kinase domain. Although it can bind ligands, it must dimerize with other kinase-active family members such as EGFr or ErbB2 to transduce HRG signals (4). Because each ErbB receptor can activate distinct intracellular signaling cascades, diverse biological response patterns arise from different ErbB associations.

We previously reported that ErbB expression patterns and combinatorial interactions vary during rat liver development (5). Fetal hepatocytes express three of the four ErbB-receptor tyrosine kinases: EGFr, ErbB2, and ErbB3. However, ErbB2 expression ceases at or near weaning. The adult rat hepatocyte, therefore, expresses only EGFr and ErbB3, lacking ErbB2 or ErbB4, even during periods of intense proliferation, such as regeneration following 70% hepatectomy.

Hepatocyte cultures from adult rats are highly useful for the study of factors that regulate cell proliferation, but the hepatocyte in primary culture does not always mirror the hepatocyte from the intact liver. For example, over 80% of cultured hepatocytes can respond to exogenous EGF with increased DNA synthesis. In the intact liver, the DNA synthesis response to EGF is considerably less, unless hepatocytes are primed to respond to EGF in vivo, such as by collagenase pretreatment (14). In this case, the apparent labeling index reaches 50%, which is comparable to that seen after partial hepatectomy. Despite the greater fractional growth response of hepatocytes from adult rats in vitro, their entry into the S phase is slower than in vivo. For example, DNA synthesis begins to rise 44 h after EGF is added to the initial growth medium shortly after cell plating. However, when EGF is injected intraperitoneally, DNA synthesis increases as early as 8–12 h after administration (30, 37). DNA synthesis in the regenerating rat liver also starts to increase 15 h after partial hepatectomy, peaking at 20–24 h. In contrast to adult hepatocytes, cultured fetal or neonatal hepatocytes show a shorter lag phase than adult cells, with peak EGF-stimulated DNA synthesis occurring by 24 h (10).

EGF-stimulated DNA synthesis is strongly amplified by insulin in adult but not fetal hepatocytes (27). In the presence of EGF stimulation and insulin, adult fetal hepatocyte DNA synthesis increases approximately threefold compared with EGF stimulation alone. In contrast, fetal hepatocytes show no induction in DNA synthesis in response to EGF or insulin alone. The mechanisms responsible for this differential effect of insulin on fetal hepatocytes have not been determined.

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of insulin, low concentrations of EGF (high picomolar to low nanomolar) can stimulate DNA synthesis to the same extent as high concentrations of EGF (10 nM) in the absence of insulin. Also, in the presence of insulin, the adult hepatocyte DNA synthesis response to EGF becomes greater as the time in culture increases. For example, the lag phase before DNA synthesis decreases by 20 h for insulin-treated cells exposed to EGF between 48 and 72 h after plating compared with cells exposed to EGF between 0 and 24 h. Hepatocytes exposed to EGF at later times also synthesize DNA in a more synchronized fashion. The level of peak DNA synthesis in the 48- to 72-h treatment group is four times greater than that of the 0- to 24-h treatment group (15). Thus insulin induces changes in cultured adult hepatocytes that increase their sensitivity to EGF as defined by the threshold dose required to stimulate DNA synthesis as well as by the rapidity and synchronicity of response.

Altered patterns of ErbB-receptor expression could explain some of these unique features of the hepatocyte response to EGF in vitro. We now report that insulin and EGF independently induce ErbB2 expression in adult rat hepatocytes after 24 h of culture. The expression of ErbB2 precedes the G1-S transition and correlates with the induction of cyclin D1 expression. We provide evidence that an EGFr-ErbB2 signaling complex emerges in cultured hepatocytes, which mediates EGF-induced DNA synthesis. This complex requires the kinase activity of both the EGFr and ErbB2. Our data implicate ErbB2 induction as a component of the synergy between EGF and insulin in vitro. Our data also suggest that metabolic factors may control ErbB2 expression in the liver.

MATERIALS AND METHODS

Peptides, reagents, and radiochemicals. Human recombinant HRG-1β, (amino acids 177–244) was from R&D Systems (Minneapolis, MN). Insulin (Novolin R) was from Novo Nordisk (Princeton, NJ), and glucagon was from Eli Lilly (Indianapolis, IN). Synthetic rat EGF at later times also synthesize DNA in a more synchronized fashion. The level of peak DNA synthesis in the 48- to 72-h treatment group is four times greater than that of the 0- to 24-h treatment group (15). Thus insulin induces changes in cultured adult hepatocytes that increase their sensitivity to EGF as defined by the threshold dose required to stimulate DNA synthesis as well as by the rapidity and synchronicity of response.

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Culture media and supplies. Williams’ medium E, supplemented with 20 mM pyruvate, 10 nM dexamethasone, and 50 μg/ml gentamicin, was the medium used for all culture studies. Medium and calf serum were from Gibco-BRL (Grand Island, NY). Type I collagenase was from Wako Pure Chemical Industries (Richmond, VA). Falcon six-well dishes were from Fisher. Tyrothrophs AG 1478, AG 825, and AG 879 were from Calbiochem (San Diego, CA) and dissolved in DMSO.

Animals. Adult male Sprague-Dawley rats from Harlan (Indianapolis, IN) were housed under conditions of regulated lighting (lights on 0600–1800). They had access to water and Purina rodent chow ad libitum (Ralston-Purina, St. Louis, MO). The Animal Use Subcommittee of the Vanderbilt Animal Care Committee approved all protocols.

Primary cell cultures. We isolated hepatocytes from the livers of male rats between 10:00 and 11:30 AM to control for circadian variation using a two-stage, collagenase isolation protocol (4). Cells were sedimented through Percoll to reduce nonhepatocyte contamination (4). We plated cells (3.75 × 10⁶ cells/well) in type I collagen-coated, 6-well (35-mm) plates for 60–90 min before adding serum-free medium containing hormones, cytokines, or signaling pathway inhibitors. In some experiments, we exposed the hepatocytes to an EHS mouse tumor extract overlay after 24 h in culture as previously described (20).

Immunoprecipitation and Western blot analysis. Hepatocytes were lysed in TGH buffer (20 mM HEPES, 1% Triton X-100, 10% glycerol, and 50 mM NaCl). This buffer included protease inhibitors (10 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml antiprotein, and 10 μg/ml leupeptin) as well as phosphatase inhibitors (10 mM sodium molybdate and 10 mM β-glycerol phosphate). Lysates were microcentrifuged at 20,800 g for 30 min and then immunoblotted or immunoprecipitated as previously described (5). The affinity-purified antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA), sc-03 for EGFr, sc-284 for ErbB2, sc-285 for ErbB3, and sc-283 for ErbB4. The anti-phosphotyrosine antibody used was RC20H1 (Signal Transduction Laboratories, Lexington, KY) or PY99 (Santa Cruz Biotechnology). The anti-phosphotyrosine (Y1112)-ErbB2 antibody was from Orbigen (San Diego, CA). We normalized all immunoprecipitations or immunoblots by using equal amounts of protein and confirmed equal protein loading and transfer by Ponceau S staining of immunoblots. Immunoreactive signal was detected using the ECL method (Pierce). We performed densitometry using an Epson scanner with Biosoft Quantscan software.

Nucleic acid probes and Northern blot analysis. For ErbB2, we used as a cDNA probe rat neu/t(PS6400 clone (420 bp long) inserted into vector pSP65 (American Type Culture Collection, Rockville, MD). A neu fragment was generated by digestion of the plasmid PS6400 with restriction enzyme BamH1 and then gel purified before labeling. The probe was labeled with [α-32P]dCTP by a random priming method. For EGFr, we used as a cRNA probe the TR-EGFR-human probe containing human EGFr (350 bp, including nucleotides) inserted into a triple script vector (Ambion, Austin, TX). The probe was labeled with [α-32P]UTP by use of the strip-EZ RNA probe synthesis kit (Ambion).

The Northern blot was carried out as previously described (28). RNA from hepatocytes was isolated by TRI Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer’s instructions. The glyoxal-dimethylsulfoxide-denatured RNA was electrophoresed on a 1% agarose gel (20 μg/lane), transferred to a positively charged nylon membrane (Ambion Northern Max kit), and cross-linked to the membrane using ultraviolet light (Stratalinker; Stratagene, La Jolla, CA). Equal loading was confirmed by visualization of the 28S and 18S subunits. Prehybridization and hybridization were carried out at 42°C for the DNA probe or 68°C for the RNA probe using buffers provided by the kit. After hybridization, the membranes were washed with low- and then high-stringency washing buffers as directed by the manufacturer’s instructions. The blots were then exposed to Kodak XAR-5 film at −80°C with double intensifying screens.

DNA synthesis assays. After 48 h in culture, cells were refed with medium containing specified growth factors and 1 μCi/ml of [3H]thymidine. At 72 h, the experiment was terminated, and the incorporation of tracer was determined as detailed previously (4). The results of assays in triplicate are expressed as the specific activity of the DNA (disintegrations min⁻¹ μg DNA⁻¹).

Statistical analysis. Statistical analysis was performed using an unpaired, two-tailed Student’s t-test assuming equal variances between compared groups.

RESULTS

Hepatocytes show increased protein tyrosine phosphorylation in response to EGF with increasing time in culture. Because cultured hepatocytes display an increased DNA synthesis response when the addition of EGF to medium is delayed until several days after plating (15), we studied how the
EGF-stimulated and basal tyrosine phosphorylation patterns varied with time in culture. We cultured cells in the presence or absence of insulin (100 nM) and a submitogenic dose of TGF-α (0.1 nM). At different times after plating, we then exposed them to EGF (100 nM) or HRG-β1 (100 nM) for 1 min and assessed tyrosine phosphorylation of lysate proteins by immunoblot analysis. Figure 1A shows that the overall basal and EGF-stimulated levels of phosphorylation increased with time between 48 and 96 h in culture. Figure 1B shows the basal and EGF-stimulated tyrosine phosphorylation signal obtained from each lane of the insulin-treated cells as a function of time. Also, the response of cells to EGF or HRG-β1 varied. HRG-β1 strongly stimulated ErbB3 and EGFr phosphorylation at 4 or 24 h but not at later times. Insulin, which suppresses expression of the HRG receptor ErbB3 (4), decreased HRG-β1-stimulated phosphorylation at 24 h.

Temporal changes in ErbB expression in vitro. To study ErbB expression in hepatocytes over 5 days in culture, we prepared lysates of cells cultured in medium alone (referred to as “basal”) or in the presence of insulin (100 nM), EGF (6.3 nM), or insulin plus EGF. We quantified ErbB protein expression by densitometric analysis of immunoblots. Figure 2 shows the expression patterns of ErbB1, -2, and -3 over 160 h after cell plating. EGFr declined by over 80% within 24 h of culture in all conditions (Fig. 2, top); however, there was a slight rebound in expression between 48 and 72 h in the cells exposed to insulin or EGF. Others have reported a reduction in EGF binding during the first 24 h of culture, correlating with a loss of high-affinity EGF binding sites (36). Consistent with our previous findings (4), ErbB3 expression increased threefold during the first 24 h of culture, although insulin and EGF suppressed this surge, especially when combined (Fig. 2, middle). Although we detected faint basal ErbB2 expression in hepatocytes cultured in basal media between 72 and 96 h (Fig. 2, bottom), insulin and EGF each induced ErbB2. EGF (6.3 nM) induced ErbB2 to a greater extent than insulin alone or...
insulin and EGF together. Figure 3A shows the immunoblot images of ErbB2 and ErbB3 expression, highlighting their inverse expression patterns. We could not detect ErbB4 during the 166-h harvest period (data not shown).

Figure 3B shows a Northern blot analysis of EGFr and ErbB2 mRNA in cultured hepatocytes. We have previously demonstrated that the increase in ErbB3 protein expression in vitro is associated with increased ErbB3 mRNA (4). Figure 3 shows that the major mRNA transcripts for EGFr and ErbB2 had different expression patterns. Consistent with the in situ hybridization and Northern blot results of Radaeva et al. (22), adult rat hepatocytes did not express ErbB2 mRNA, but the message did appear in cultured cells. For cells exposed to insulin or EGF, the initial decrease in EGFr mRNA within the first 24 h was followed by a second increase at 48 and 72 h. This latter increase may be responsible for the small rebound in EGFr protein expression seen with immunoblot (Fig. 2, top). Also, cells cultured without insulin or EGF showed low levels of ErbB2 protein expression, even though the levels of ErbB2 mRNA compared favorably to insulin- or EGF-treated cells, which expressed much higher levels of ErbB2 protein.

EGF and insulin synergistically stimulate ErbB2, cyclin D1, and DNA synthesis. EGF ligands and insulin synergistically stimulate hepatocyte DNA synthesis (15, 27). EGF acts as a partial agonist of the EGFr-compared with TGF-α (33). To confirm these findings, we examined the effects of different concentrations of EGF or TGF-α in the presence or absence of insulin on DNA synthesis. As shown in Fig. 4A, both EGF and TGF-α synergistically stimulated DNA synthesis between 0.1 and 1 nM, but this synergy disappeared at higher EGF concentrations. We also confirmed in insulin-deficient media that TGF-α stimulates hepatocyte DNA synthesis more effectively than EGF.

We then studied whether EGF and insulin synergistically stimulated ErbB2 or cyclin D1 expression. The de novo expression of cyclin D1 occurs shortly after 44 h and is thought to play a key role in DNA synthesis (15). We prepared lysates and immunoblots from cells that had been treated with different concentrations of EGF with or without insulin for 50 h of culture. Figure 4, B and C, shows that in insulin-containing medium both ErbB2 and cyclin D1 increase with increasing concentrations of EGF. Insulin did not augment the effect of higher concentrations of EGF (10 nM, data not shown; see Fig. 2 also). These results show that low concentrations of EGF (<1 nM) and insulin synergize to stimulate ErbB2, cyclin D1, and DNA synthesis.
EGF, but not HRG-β1, stimulates ErbB2 tyrosine phosphorylation. To evaluate the tyrosine phosphorylation state of ErbB2 and possible intermolecular associations between EGFr and ErbB2, we performed immunoprecipitation experiments. We cultured hepatocytes in the presence of insulin (100 nM) for 72 or 96 h. We then exposed the hepatocytes for 2 min to insulin-free medium containing the EGFr ligand EGF (100 nM) or the ErbB3 ligand HRG-β1 (100 nM). We evaluated the tyrosine phosphorylation patterns of ErbB2 and EGFr immunoprecipitates and determined whether ErbB2 coassociated with EGFr. Figure 5, top, shows the total ErbB2 and EGFr immunoprecipitated from subdivided lysates from cells treated with either no peptide, EGF, or HRG-β1. Note that the total amounts of ErbB2 or EGFr immunoprecipitated from the different lysates are similar. Figure 5, middle, shows the phosphotyrosine signal associated with the immunoprecipitated ErbB3s in Fig. 5, top. Figure 5, bottom, shows the specific phosphotyrosine signal of an antibody raised against a phosphopeptide derived from ErbB2 (Y1112). EGF, but not HRG-β1, strongly phosphorylated ErbB2 as well as EGFr and promoted the coassociation of these two ErbB3s. There is also some basal phosphorylation of EGFr, which is perhaps related to the known ability of these cells to secrete endogenous TGF-α (1). The inability of HRG-β1 to increase ErbB2 tyrosine phosphorylation in either the ErbB2 or EGFr immunoprecipitates argues against the formation of active ErbB2-ErbB3 heterodimers or EGFr-ErbB2-ErbB3 higher ordered oligomers. This is consistent with the absence of HRG-β1-stimulated total tyrosine phosphorylation after 72 and 96 h, as shown in Fig. 1, which we attribute mainly to the greatly diminished ErbB3 expression at these times.

ErbB2 expression is regulated by factors associated with increased cell proliferation. We next evaluated the ability of various “growth activators” to modulate ErbB2 expression in cultured hepatocytes. In the presence of EGF and insulin, nicotinamide (7.5 mM) delays the onset of DNA synthesis by 10–15 h, increases the magnitude and synchrony of peak DNA synthesis, and releases cells from mitotic arrest (19). Figure 6A shows that nicotinamide, which raises the intracellular NAD⁺ levels, had two effects: it stabilized high levels of EGFr expression, and it delayed the down- and upregulation of ErbB3 and ErbB2, respectively (compare with Fig. 2). Figure 6 with a greater sampling density highlights the reciprocal expression patterns of ErbB2 and ErbB3.

TGF-α stimulates hepatocyte DNA synthesis to a greater extent than EGF because a TGF-α-EGFr complex recycles to the surface membrane, whereas an EGF-EGFr complex is routed to lysosomes for degradation (17). Figure 6B shows that EGF and TGF-α differentially regulated EGFr and ErbB2 expression at 72 h of culture. TGF-α stimulated ErbB2 expression at a lower concentration (0.1 nM, as detected in longer autoradiographic exposures). It also induced more ErbB2 at all concentrations tested. EGF, but not TGF-α, caused a marked downregulation of EGFr. Finally, we evaluated if HRG-β1 or PMA stimulated ErbB2 expression. These agents have little endogenous growth stimulatory ability but can potentiate TGF-α-mediated DNA synthesis (32). Figure 6C shows that neither HRG-β1 nor PMA alone induced ErbB2; however, both increased the TGF-α induction of ErbB2, akin to their effects on DNA synthesis.

ErbB2 expression is not a target of inhibitors of hepatocyte DNA synthesis. The ability of growth activators to induce ErbB2 suggested that its expression may be a checkpoint for cell cycle progression, potentially sensitive to cell cycle inhibitors. Thus we examined ErbB2 expression in several models associated with decreased cell proliferation, including increased cell density, DMSO (32), and treatment with TGF-β1. Increasing the plating cell density inhibited DNA synthesis in
response to EGF (Fig. 7B). High plating density increased EGFr levels but did not block ErbB2 expression (Fig. 7A). Figure 7C shows that EGF also induced ErbB2 equally well in the presence or absence of 2% DMSO, even though DMSO markedly suppressed DNA synthesis (data not shown). DMSO, however, did reduce EGFr expression in the same cultures.

TGF-β is a potent inhibitor of hepatocyte proliferation (24, 26). TGF-β1 had a complex dose-response effect on ErbB2 expression (Fig. 7). Low concentrations stimulated ErbB2 expression at 72 h, but the highest doses inhibited it. The stimulatory effect of TGF-β1 on ErbB2 levels at low concentrations may relate to its ability to induce IGF-1 expression (34), which we have shown also induces ErbB2 (data not shown). Cells treated with the highest doses of TGF-β1 were apoptotic and likely unable to synthesize a normally induced protein, such as ErbB2.

ErbB2 expression persists in EHS overlay cultures. Hepatocytes cultured on type I collagen lose their polarity as well as their canalicular network, resulting in cholestasis (20). To determine whether ErbB2 expression was a response to aberrant polarity or to bile salt accumulation, we overlaid the cells with EHS basement membrane after 24 h on type I collagen. Under these conditions, the flattened cells assume a polygonal shape and form an anastomosing network of bile canaliculi (20). Figure 7E shows that ErbB2 expression persisted in the EHS overlay cell cultures. Thus the induction was not secondary to the lack of polarity or to cholestasis. EHS overlay did result in increased expression of EGFr and ErbB3, however.

Effects of ErbB2 blockade. To determine the relative contribution of the EGFr and ErbB2 tyrosine kinases to EGF-induced signal transduction, we tested the effects of two ErbB-selective tyrphostins: AG-1478, an EGFr-selective tyrosine kinase inhibitor, and AG-825, an ErbB2 inhibitor (Table 1). The ErbB2 kinase inhibitor AG-825 has at least 60-fold higher selectivity to ErbB2 (IC50 = 0.35 μM) than to the EGFr (IC50 = 19 μM) in cell-free systems and does not inhibit EGFr tyrosine phosphorylation at the concentration used in cell culture (13). AG-1478 and AG-825 each inhibited DNA synthesis at the dosages used by 50%. When combined, the EGFr and ErbB2 tyrphostins inhibited DNA synthesis by nearly 100%, suggesting that both EGFr and ErbB2 play roles in EGF stimulation of hepatocyte DNA synthesis. This is consistent with our prior finding that the dual kinase EGFr-ErbB2 inhibitor PKI-166 was a surprisingly potent inhibitor of DNA synthesis in primary hepatocytes. Because we did not know that ErbB2 was induced in primary hepatocytes at the time of this original study, we originally assumed that this inhibitory effect was entirely related to EGFr blockade. We have obtained similar findings in experiments using AG-1478 and AG-879, an even more selective ErbB2 selective tyrphostin, which has over a 500-fold selectivity to ErbB2 (IC50 = 1 μM) than to the EGFr (IC50 = 500 μM) in cell-free systems (13). The combination of these two tyrphostins at concentrations as low as 0.3 μM completely blocked TGF-α-stimulated DNA synthesis (data not shown). These data strengthen the implications shown in Fig. 4 demonstrating that EGFr-ErbB2 heterodimers have a role in regulating hepatocyte proliferation.
DISCUSSION

EGF ligands have marked effects on liver cell proliferation, differentiation, apoptosis, cell signaling, metabolism, and carcinogenesis. Hepatocytes express more than 200,000 EGF binding sites per cell. These were originally assumed to be EGF monomers or homodimers. Recently, we showed that hepatocytes at different developmental stages can also express ErbB2 and ErbB3, which can interact with EGF monomers to form kinase-active heterodimers (3–5).

In this study, we showed that hepatocyte adaptation to cell culture results in rapid and substantial changes in the abundance, availability, and use of ErbB proteins for signal transduction. Many laboratories rely on primary cultures of hepatocytes in serum-free medium to study the influence of EGF-like ligands on hepatocyte growth and function. However, there is often variation in experimental design, particularly with respect to the culture medium and the time cells spend in culture before experimentation. Some investigators study freshly isolated cells, either attached or in suspension. Others use cells at variable periods after cell attachment. Investigators who transfsect cells to express new or dominant negative genes frequently culture cells for as long as 72 h before performing experiments to ensure sufficient foreign protein expression. Our work documents dramatic changes in ErbB receptor expression as cells adapt to culture. Given these changes, differences in experimental design will generate divergent findings when EGF-mediated signaling events are studied.

Several lines of evidence suggest that cultured hepatocytes respond to EGF differently than their counterparts in vivo, and our results implicate the expression of ErbB2 in vitro for many of these differences. The magnitude of the EGF response in cultured hepatocytes rivals that of regenerating liver following partial hepatectomy (as judged by nuclear bromodeoxyuridine or thymidine labeling). Yet despite immediate-early gene activation during cell isolation (18), DNA synthesis does not begin in cultured hepatocytes until 40 h, peaking between 48 and 72 h (15, 24, 26, 35). In the intact adult animal, however, a 70% hepatectomy or an EGF injection given after a priming stimulus will elicit hepatic DNA synthesis within 12 h, with peak DNA synthesis occurring before 24 h (30, 35, 37).

Studies by Sand and Christoffersen (27) and by Loyer et al. (15) have suggested the existence of a late G1 event that sensitizes cultured adult hepatocytes to EGF and facilitates entry into the S phase. This restriction point occurs at about 44 h of culture. One consequence of this restriction point is that cells cultured in insulin become more responsive to EGF with time in culture. When ligand addition is delayed until after this late G1 event, the DNA synthesis response to exogenous EGF becomes synchronized and amplified. For example, when added between 72 and 96 h of culture as opposed to 0 and 24 h,
EGF stimulation of peak DNA synthesis occurs in about half the time and is fourfold greater.

We propose that the EGF-sensitizing event that occurs in late G1 is the induction of ErbB2 by insulin and EGF in vitro. Our work thus far has not suggested that ErbB2 induction is a general mechanism controlling cell proliferation in the adult liver. It is not required for DNA synthesis after partial hepatectomy (5) or in a model of hepatic growth following protein deprivation and amino acid gavage (data not shown). However, microarray experiments have shown that adult hepatocytes revert to an embryonic or neonatal phenotype as they adapt to culture (2). Adult rat hepatocytes can express some fetal/embryonic genes, such as γ-glutamyl transpeptidase, as early as 72 h of culture (31). The reemergence of ErbB2 as a signaling molecule in these cells may be representative of this process. In contrast to adult hepatocytes, fetal hepatocytes, which already express ErbB2, exhibit no synergy between insulin and EGF, and their G1/S transition occurs rapidly, within the first 24 h of exposure to EGF (7, 10) (I. Fabregat, personal communication). Thus we speculated that the growth response of cultured adult hepatocytes to EGF required a fetal/neonatal pattern of ErbB expression, including ErbB2.

We demonstrated that hepatocytes expressed ErbB2 shortly before entering the S phase (Figs. 2–3) and that blocking ErbB2 tyrosine kinase reduced DNA synthesis (Table 1). Even though many stimulators of DNA synthesis strongly induced ErbB2 (Fig. 6), its expression was insufficient for DNA synthesis. In fact, ErbB2 expression persisted in some models associated with a decreased proliferative response to EGF (Fig. 7). Collectively, our data suggest that ErbB2 plays a permissive role in the regulation of cell progression into the S phase. However, its expression neither requires nor mandates S phase entry.

The active receptor unit of the EGF signaling complex in the liver can be either an EGFr homodimer or an EGFr heterodimer with ErbB2 or ErbB3. Therefore, changes in the ErbB expression profile, especially the upregulation of ErbB2, may alter EGF cell signaling (8, 9). In various cell systems, the mitogenic signaling capacity of an EGFr-ErbB2 heterodimer is more potent than that of an EGFr homodimer. In contrast to the

| Table 1. Effects of ErbB-selective tyrosine kinase inhibition on hepatocyte EGF-stimulated DNA synthesis |
|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------------------------|
| Control                                           | AG-825                                           | AG-1478                                           | AG-825 + AG-1478                                    |
| Basal                                             | 20,685 (1,929)                                    | 13,170 (1,538)†                                   | 4,896 (625)†                                        |
| EGF                                               | 189,611 (20,104)                                  | 106,828 (13,785)†                                 | 100,928 (9,105)†                                   |
| Insulin                                           | 34,723 (4,842)                                    | 26,134 (1,599)*                                   | 24,241 (2,047)*                                    |

Values are means with SDs in parentheses. The results of triplicate assays are expressed as the specific activity of the DNA (disintegrations·min⁻¹·µg DNA⁻¹). DNA synthesis was measured in hepatocytes cultured for 72 h in the absence of growth factors (basal) or in the presence of EGF (6.3 nM) or insulin (100 nM) and in the absence (control) or presence of ErbB-selective inhibitors AG-1478 (EGFr, 4 µM), AG-825 (ErbB2, 4 µM), or the combination of both.

*P < 0.05 vs. control; †P < 0.01 vs. control; ‡P < 0.01 vs. AG-825 and vs. AG-1478 alone.

AJP-Gastrointest Liver Physiol • VOL 291 • JULY 2006 • www.ajpgi.org
latter, the EGFr-ErbB2 heterodimer has a prolonged retention at the plasma membrane (presumably from a decreased ability of the heterodimer to engage c-cbl), resulting in increased mitogenic signaling capacity (21). Indeed, we noted that basal and EGFr-stimulated protein tyrosine phosphorylation increased with time in culture (Fig. 1). Interestingly, others have reported that the overall tyrosine kinase activity in ErbB2-expressing hepatocytes of fetal/newborn animals exceeds that in the ErbB2-negative hepatocytes of adult animals (12, 16).

ErbB2 may associate with either EGFr or ErbB3, but we found evidence for the association and phosphorylation of ErbB2 only with EGFr (Fig. 5). Immunoprecipitation of ErbB2 from lysates of EGFr-treated cells at 44 h of culture showed increased ErbB2 tyrosine phosphorylation and EGFr coassociation, suggestive of heterodimerization. Cells treated with HRG-β1, an ErbB3 ligand, showed no ErbB2 phosphorylation, even though the ErbB2-ErbB3 heterodimer is the most potent mitogenic signaling pair among the ErbB receptors. We speculate that the relative excess of EGFr favors the formation of EGFr-ErbB2 dimers, possibly having a dominant negative effect on the assembly of ErbB2-ErbB3 dimers. The complementary actions of ErbB2- and EGFr-selective tyrophostins suggest that full EGF signaling depends on the heteromeric association of both tyrosine kinases. These findings are consistent with our prior work showing that a dual EGFr-ErbB2 kinase inhibitor, PKI-166, fully blocked the mitogenic effects of EGF (29).

We have extended our previous observations to define the nature of the “EGF” signaling system in cultured adult rat hepatocytes. We previously showed that ErbB-receptor expression and signaling options vary with the developmental state of the liver in vivo. We now show that a dramatic and rapid change in ErbB expression patterns and signal transduction options occurs during hepatocyte culture. Our findings suggest caution when making inferences about EGF signaling in the liver based solely on signaling studies in cultured hepatocytes. EGF signaling will change as hepatocytes revert to a more fetal pattern of ErbB expression. This includes the expression of ErbB2, which may be the event that allows insulin to amplify the mitogenic effects of EGF. Finally, the action of insulin to induce ErbB2 expression also suggests that it may be under metabolic control, like its homologs ErbB3 (3) and EGFr (6).

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