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Integral Role of the EGF Receptor in HGF-Mediated Hepatocyte Proliferation

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Hepatocyte growth factor (HGF), insulin, and TGF-α stimulate DNA synthesis in cultured hepatocytes. Each ligand activates a distinct tyrosine kinase receptor, although receptor cross-talk modulates signaling. In rat hepatocytes, HGF can stimulate TGF-α production while TGF-α antibodies or antisense oligonucleotides suppress HGF-stimulated DNA synthesis. We report that the epidermal growth factor receptor (EGFR) kinase inhibitor PKI166 blocked both basal and ligand-induced tyrosine phosphorylation of the EGFR (IC_{50} = 60 nM), but not of the insulin receptor or c-met. Pharmacologic inhibition of the EGFR kinase abolished the proliferative actions of HGF and EGF, but not insulin, whereas PI-3 kinase inhibition blocked both EGF and insulin actions. We conclude that in cultured hepatocytes (i) PI-3 kinase is required for EGF- and insulin-induced proliferation and (ii) EGFR mediates both the basal rate of DNA synthesis and that induced by EGF and HGF, but not insulin. The mitogenic effect of HGF may be secondary to increased synthesis or processing of EGFR ligands such as TGF-α.

Key Words: **liver; hepatocyte; ErbB; hepatocyte growth factor; c-*met;* transforming growth factor-α; liver regeneration; PI-3 kinase; PKI166.**

Transforming growth factor-α (TGF-α) and hepatocyte growth factor (HGF) are among the most potent hepatocyte mitogens both *in vitro* and *in vivo,* and are strongly implicated in the regulation of liver regeneration. Each peptide binds to and activates a distinct tyrosine kinase receptor. TGF-α, a member of the epidermal growth factor (EGF) ligand family, binds to the EGF receptor (EGFR), whereas HGF binds to the protooncogene c-met. When combined, HGF and TGF-α stimulate DNA synthesis in an additive manner, suggesting distinct and largely independent downstream signaling pathways.

However, TGF-α overexpression in chemically transformed rat liver epithelial cells (RLEC) induces tyrosine phosphorylation of c-met in the absence of exogenous HGF, suggesting that receptor cross-talk may integrate the actions of TGF-α and HGF (1). Furthermore, HGF has been shown to induce TGF-α synthesis in hepatocyte cultures, and stimulation of hepatocellular DNA synthesis by HGF was partially blocked *in vitro* by incubating hepatocytes with either an antibody or an antisense probe to TGF-α (2). These results suggest that HGF stimulation of DNA synthesis may involve increased synthesis or release of TGF-α in hepatocytes.

To test this, we evaluated the ability of HGF to stimulate DNA synthesis in the presence of PKI166 (originally denoted as CGP75166), a highly selective and potent inhibitor of the EGFR kinase (3, 4). We found that EGFR kinase inhibition blocked the mitogenic actions of HGF. These results strongly support the hypothesis that EGFR mediates or regulates the growth-promoting actions of HGF, possibly through increased synthesis or processing of TGF-α or other EGF-like ligands. PKI166 also inhibited basal DNA synthesis and decreased cell survival, underscoring the importance of EGFR signaling in the regulation of hepatocyte growth and viability (5).

MATERIALS AND METHODS

Reagents. Mouse EGF was obtained from Sigma, rat TGF-α from Penninsula, and HGF from R & D Systems, Inc. The antibodies against EGFR (sc-03) and c-met (SP260) were from Santa Cruz. The polyclonal anti-phosphotyrosine antibody was from Transduction Laboratories. PKI166 was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). This compound was prepared in a 10 mM stock solution in DMSO and equal amounts of DMSO were added to control cells. The MEK inhibitor (PD98059) and the PI-3

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kinase inhibitor (LY294002) were from BioMol. Culture medium was from Gibco.

Animals. Male Sprague–Dawley rats (150–200 g), from Harlan (Indianapolis, IN) were housed under conditions of regulated lighting (lights on 0600–1800) and *ad libitum* access to water and Purina rodent chow (Ralston–Purina, St. Louis, MO). Cultures were prepared in the early to mid light phase to minimize circadian variation (6). All protocols employed were approved in advance by the Animal Use Subcommittee of the Vanderbilt Animal Care and Use Committee.

Cell culture and DNA synthesis. The basic culture medium was Williams' Medium E (WE) supplemented with 10^{-8} M dexamethasone and 20 mM pyruvate. Hepatocytes were obtained from adult male rats as previously described using a two-step collagenase-based perfusion method Nonparenchymal cells were removed by centrifugation through Percoll prior to plating (8). Cells were plated on Type I collagen with medium containing 6% calf serum. After an attachment period of 60 min, the medium was replaced with serum-free medium containing test substances. At 48 h in culture, the medium was refreshed and 1 μCi/ml [^{3}H]methylthymidine (0.36 Ci/mmol) was added for an additional 24 h. At 72 h, cells were fixed *in situ* and washed free of unincorporated label with 5% TCA. The monolayer was then solubilized in 1.0 N NaOH, precipitated, hydrolyzed in perchloric acid, counted, and the DNA quantified. DNA synthesis is expressed as cpm of [^{3}H]thymidine incorporated per μg DNA in 24 h; mean ± SD.

Immunoprecipitation and Western blotting. Cells were lysed in TGH buffer (20 mM Hepes, 1% Triton X-100, 10% glycerol, 50 mM NaCl) with protease inhibitors (1 mM PMSF, 100 μM sodium orthovanadate, 10 μg/ml aprotinin, and 1 μg/ml leupeptin) as well as phosphatase inhibitors (10 mM sodium molybdate and 10 mM β-glycerol phosphate). Lysates were microfuged at 13,000*g* for 30 min and the protein content of the supernatants determined. Four hundred micrograms of protein was incubated overnight at 4°C with antibodies against EGFR or c-met. The insulin receptor β-subunit (IR-β) was electrophoresed without immunoprecipitation, and identified by its molecular mass. The following day, complexes were precipitated for 2 h at 4°C with protein G–Sepharose (Pierce). Pellets were washed four times with TGH at room temperature, dissolved in 20 μl 1× SDS-gel sample buffer and heated to 95°C for 5 min. The solubilized supernatant protein was resolved on a 6% SDS–polyacrylamide gel, transferred to nitrocellulose and then immunoblotted with antibodies against EGFR or c-met receptors. Immunoreactive species were detected using the ECL method (Pierce) and exposed to X-ray film for autoradiography. Duplicate immunoprecipitations were carried out to determine the phosphotyrosine status of the proteins; all bands disappeared when the antibodies were pre-incubated with the immunizing peptides.

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

RESULTS AND DISCUSSION

PKI166 Is a Highly Effective Inhibitor of Hepatocyte EGFR Tyrosine Phosphorylation

PKI166 is a highly selective, membrane-permeable EGFR kinase inhibitor developed by Novartis for possible cancer therapy (3, 4, 9). It is a protein–tyrosine kinase inhibitor of the 4-(phenylamino)-7H-pyrrolo{2,3-d} pyrimidine class, and interacts with the ATP-binding pocket of the EGFR kinase domain. It has been shown to significantly inhibit primary and metastatic human pancreatic carcinoma in nude mice (4).

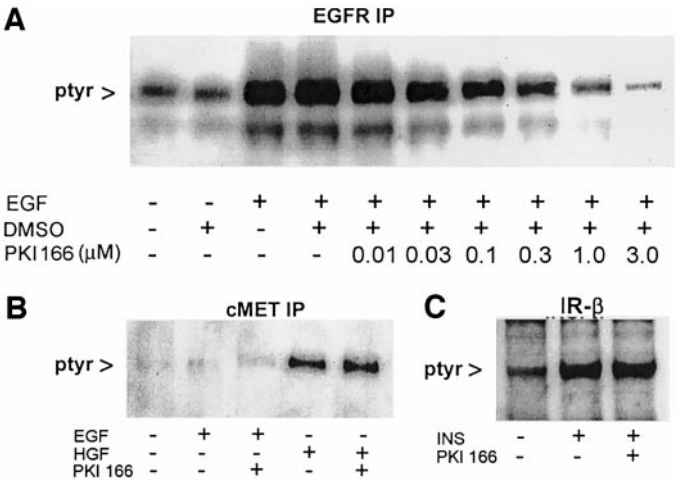

FIG. 1. PKI166 effects on ligand-activated phosphorylation of EGFR, c-met, and IR-β. Primary cultures of hepatocytes were preincubated with DMSO or PKI166 for 90 min and then exposed to ligands for 5 min at 37°C. (A) Phosphorylation of EGFR by 20 ng/ml EGF: dose response to PKI166. (B) Phosphorylation of c-met by 20 ng/ml HGF, but not 20 ng/ml EGF: lack of response to PKI166. (C) Phosphorylation of IR-β by 115 nM insulin: lack of response to PKI166.

Initially, we established the dose range to prevent EGF dependent tyrosine phosphorylation of the EGFR. Hepatocytes were cultured for 20 h in serum-free WE, and then replaced with fresh WE containing either 0.3% DMSO or PKI166. Ninety minutes later, EGF was added to individual cell plates for 5 min at 37°C. The 170-kDa EGFR protein was immunoprecipitated, electrophoresed, and then blotted with an anti-phosphotyrosine antibody.

Figure 1A shows that PKI166 inhibited EGF-induced hepatocyte EGFR phosphorylation at concentrations as low as 30 nM. The IC_{50} was 60 nM, comparable to other cell-based estimates (51 nM) (3). At 1 μM, EGFR phosphorylation was reduced to basal levels whereas at 3 μM, phosphorylation was barely detectable, considerably below basal levels. These results show that PKI166 effectively inhibited EGF-induced EGFR tyrosine phosphorylation. They also demonstrate a significant basal level ofEGFR phosphorylation in the absence of exogenous ligand. Since these cells express several EGF-like ligands such as TGF-α, basal phosphorylation of EGFR may represent an autocrine or juxtacrine response to endogenous ligands or the transphosphorylation of EGFR by other tyrosine kinases (10). However, the inhibitor data suggest that the EGFR kinase is critical to this process.

PKI166 Does Not Inhibit HGF- and Insulin-Stimulated Tyrosine Phosphorylation of c-met and the Insulin Receptor

We evaluated the specificity of PKI166 by determining its actions on the receptor-tyrosine kinase activa-

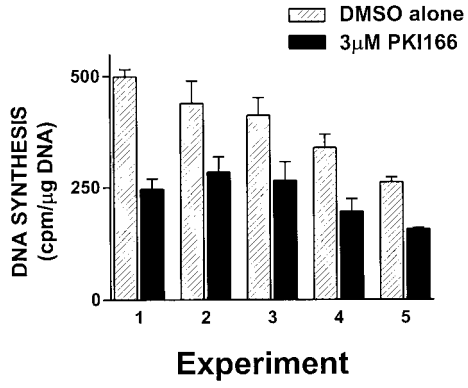

FIG. 2. PKI166 inhibits hepatocyte DNA synthesis in the absence of added ligand. Results from five different hepatocyte preparations. Data are means ± SD of triplicate plates.

tion of two other hepatocyte mitogens, HGF and insulin. We evaluated its ability to inhibit HGF-mediated tyrosine phosphorylation of c-met and insulin-mediated phosphorylation of the IR-β chain. Cultures were pre-exposed to PKI166 for 90 min and then to HGF, EGF, or insulin for 5 min at 37°C. Figure 1B shows that HGF, but not EGF, effectively stimulated c-met phosphorylation, and that 3 μM PKI166 had no effect on HGF-induced c-met receptor autophosphorylation, or c-met levels (data not shown). This is in keeping with *in vitro* data on c-met inhibition (3). Others have shown that TGF-α causes low-level phosphorylation of c-met in rat liver epithelial cells (RLEC) but not until a 30-min exposure of cells to exogenous ligand. The prolonged time course suggests that the EGFR may indirectly phosphorylate c-met (1).

Figure 1C shows that insulin-induced tyrosine phosphorylation of the insulin receptor β chain is not influenced by PKI166, providing additional support that PKI166 is a highly selective inhibitor of the EGFR kinase in primary hepatocytes.

Basal Hepatocellular DNA Synthesis in Vitro Depends on the Tyrosine Kinase Activity of EGFR

Hepatocytes in primary culture have a low basal rate of DNA synthesis in the absence of exogenous growth factors (11). Still, some low-level DNA synthesis can be detected normally between 48 and 72 h of cell culture. In five separate cell preparations, we found that continuous exposure of cells to 3 μM PKI166 reduced basal DNA synthesis by 25 to 50% (Fig. 2). This suggests that basal hepatocellular DNA synthesis requires activation of EGFR and probably arises secondary to the production of EGF-like ligands, principally TGF-α. In preliminary experiments, we have found that cultured hepatocytes actively secreted TGF-α, that a TGF-α neutralizing antibody inhibited basal DNA synthesis by about 20%, and that metalloproteinase inhibitors that inhibit TGF-α processing also reduced basal DNA synthesis by 20 to 30% (data not shown).

PKI166 Inhibits EGF- but Not Insulin-Induced DNA Synthesis

To verify the downstream consequences of PKI166 actions on the EGFR kinase, we examined the inhibitor's ability to influence the individual and combined actions of EGF and insulin on hepatocyte DNA synthesis. Both EGF and insulin independently stimulated DNA synthesis of primary hepatocytes. When combined, they usually stimulate it in a synergistic manner, as shown in Fig. 3A. We found that the MEK

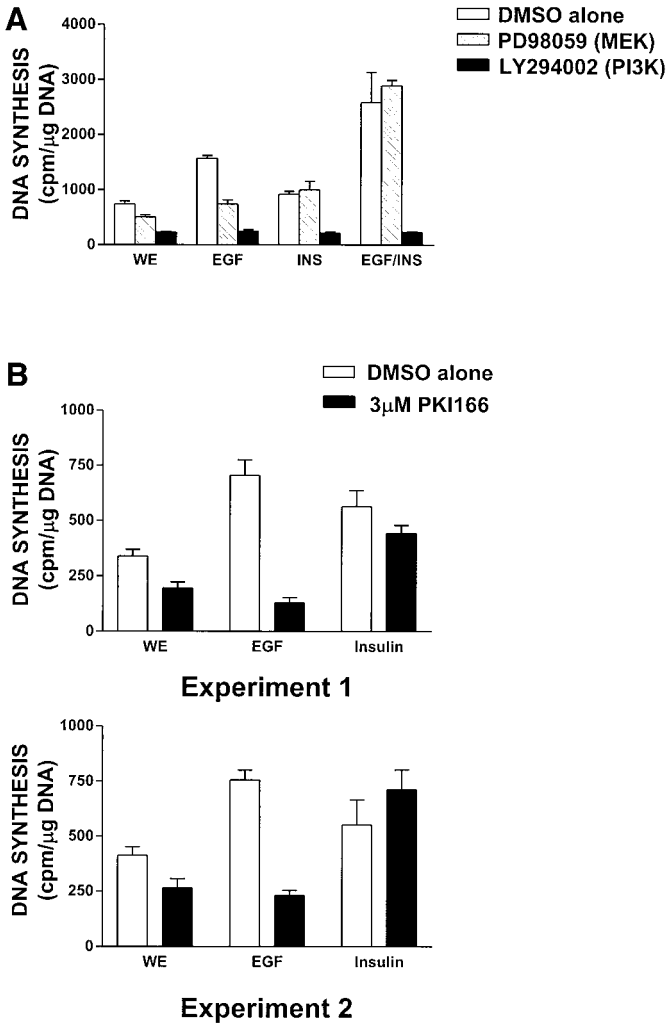

FIG. 3. Insulin-stimulated DNA synthesis is not inhibited by PKI166, but both insulin- and EGF-stimulated DNA syntheses require PI-3 kinase activation. (A) Hepatocytes were cultured for 72 h with 20 ng/ml EGF, 115 nM insulin, or insulin/EGF in the presence or absence of 50 μM PD98059 (MEK inhibitor) or 50 μM LY294002 (PI-3K inhibitor). (B) Hepatocytes were cultured in the presence of 20 ng/ml EGF or 115 nM insulin in the presence or absence of 3 μM PKI166. Data are means ± SD of triplicate plates.

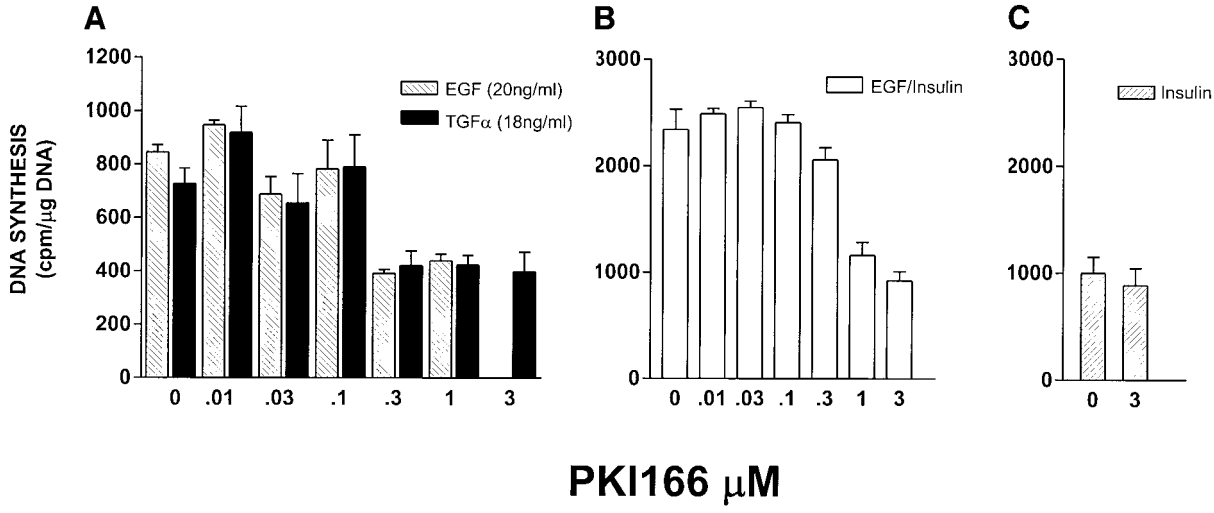

FIG. 4. PKI166 inhibits EGF- (20 ng/ml) and TGF-α- (18 ng/ml) stimulated DNA synthesis with comparable kinetics. Insulin reduces the sensitivity of EGF-stimulated DNA synthesis to PKI166. Data are means ± SD of triplicate plates.

inhibitor PD98059 (14) only partially suppressed EGF stimulation of DNA synthesis, and had no effect on insulin-stimulated DNA synthesis. However, consistent with prior reports (12) both hormones stimulated DNA synthesis in a PI-3 kinase-dependent manner as shown by the ability of the PI-3 kinase inhibitors wortmannin (data not shown) and LY294002 (13) to completely block the EGF and insulin growth stimulating actions (Fig. 3A). Since the EGFR lacks the YXXM motif necessary for the docking and activation of the p85 subunit of PI-3 kinase, another protein must couple the activated EGFR with PI-3 kinase (7, 15). Studies have shown that Gab2 (Grb-associated binder 2) is the principal EGFR-induced phosphoprotein linking the EGFR to PI-3 kinase in hepatocytes, although Shc and the EGFR homolog, ErbB3, also recruit p85 to EGFR (16).

As shown in Fig. 3B, cells cultured in the continuous presence of PKI166 (3 μM) did not respond to EGF with increased DNA synthesis. In contrast, PKI166 had no effect on insulin-stimulated DNA synthesis, consistent with its lack of effect on insulin receptor autophosphorylation (Fig. 1C). These data indicate that PKI166 does not directly inhibit other proximal or distal kinases involved in S-phase cell cycle progression, including PI-3 kinase.

To define the concentration effect of PKI166 on EGFR-mediated DNA synthesis, we carried out a dose–response study. Cells were incubated with DMSO or varying concentrations of PKI166. They were also exposed to one of two EGF ligands (EGF or TGF-α; Fig. 4A), EGF plus insulin (Fig. 4B), or insulin alone (Fig. 4C). PKI166 inhibited DNA synthesis more effectively in the absence of insulin (maximal inhibition at 0.3 μM) than in its presence, but did not discriminate between EGF ligands (Fig. 4A). Again, PKI166 had no effect on insulin-stimulated DNA synthesis regardless of the presence (Fig. 4B) or absence (Fig. 4C) of EGF: the inhibitor reduced insulin/EGFR-stimulated DNA synthesis to the level of insulin alone. The dose dependency curves of PKI166 for EGF-induced tyrosine phosphorylation and DNA synthesis differed somewhat. Inhibition of EGF-induced tyrosine phosphorylation occurred at a lower dose (0.03 μM) than that for DNA synthesis. However, maximal inhibition of tyrosine phosphorylation occurred at a dose 10 times higher (3.0 μM) than that required for maximal DNA synthesis inhibition (0.3 μM).

PKI166 Inhibits HGF-Induced DNA Synthesis

HGF, which acts through the c-met receptor, is recognized as an important hepatocyte growth factor (17–19). Several lines of evidence have recently suggested that it may act in part through EGFR. HGF stimulates the synthesis of a major endogenous EGFR ligand, TGF-α, in a dose-dependent manner. Furthermore, a neutralizing antibody against TGF-α has been shown to block the mitogenic action of HGF by 30 to 40% as does an antisense oligonucleotide against TGF-α (2). Moreover, in contrast to insulin and EGF, the effects of HGF and EGF on hepatocyte DNA synthesis are additive (20), rather than synergistic. This suggests that the latter pair may act through a common mechanism.

To test the hypothesis that EGFR is involved in the actions of HGF, we examined the individual and combined actions of EGF and HGF on hepatocyte DNA synthesis in the presence or absence of PKI166 (Fig. 5). As mentioned above, initial studies demonstrated that PKI166 at a concentration of 3 μM did not interfere with the ability of HGF to activate c-met (Fig. 1B), or of insulin to activate the IR-β (Fig. 1C). In contrast to

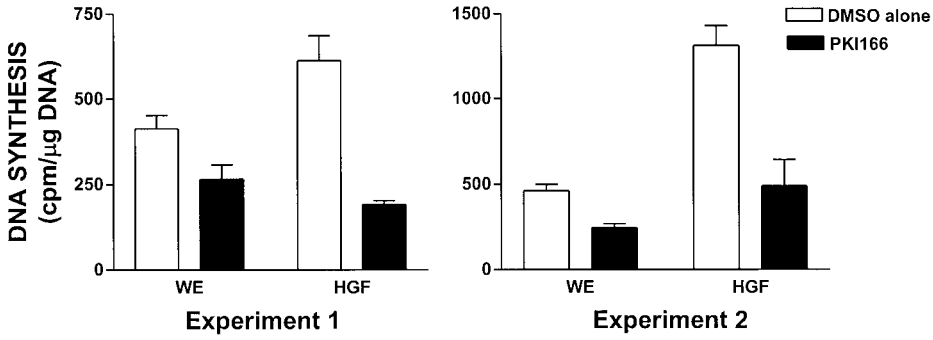

FIG. 5. PKI166 inhibits HGF-stimulated DNA synthesis. Cells were exposed to 10 ng/ml of HGF in the presence or absence of 3 μM PKI166. Data are means ± SD of triplicate plates; two separate cell preparations are shown.

insulin-stimulated DNA synthesis, however, PKI166 consistently reduced the ability of HGF to stimulate hepatocellular DNA synthesis, as shown by the replicate experiments in Fig. 5.

PKI166 had striking effects on the morphology of cultured hepatocytes exposed to EGF or HGF. When insulin-treated cells were examined by phase contrast microscopy at 72 h of culture (Fig. 6A), EGFR kinase inhibition had no discernable effect on the cell number or appearance. Regardless of PKI166, insulin caused the discrete cell islands seen in WE media alone (data not shown) to form a confluent monolayer of large, flattened cells. Although EGF or HGF by themselves caused a similar confluent morphology, the PKI166-treated EGF or HGF treated cells more closely resembled cells exposed to WE media alone. The cell islands in PKI166/HGF plates, however, were slightly larger, more irregular and coalescent than those in the PKI166/EGF plates.

The morphologic differences were reflected to some extent in the total DNA content (cellularity) of the cultures (Fig. 6B). Whereas EGFR kinase inhibition had no effect on the DNA content of the insulin-treated cultures, it reduced the total amount of DNA by about 25% in the HGF-treated group and 50% in the untreated and EGF-treated cells. The reduction in cellular DNA suggests that EGFR kinase inhibition can accelerate cell death, consistent with a recent report that EGFR kinase inhibitors decreased EGF's ability to protect mouse hepatocytes against Fas-induced apoptosis (21). Interestingly, insulin and to a lesser extent HGF rescued cells from the cytocidal effects of PKI166.

Several possibilities account for the ability of EGFR kinase inhibition to extinguish the growth-promoting actions of HGF. One possibility is that HGF normally stimulates hepatocyte growth by causing them to synthesize or release EGF-like ligands, such as TGF-α (10). A second possibility is that basal EGFR activation by endogenous TGF-α may be required to preserve the integrity of c-met signaling in long-term culture, perhaps by supporting a kinase involved in the transactivation of c-met (1). A third possibility is that PKI166 inhibits an unknown kinase downstream from both EGFR and c-met that is structurally similar to the EGFR kinase. This hypothetical kinase would be essential to the mitogenic action of EGF or HGF but extraneous to that insulin.

If TGF-α does play a pivotal role in the action of HGF, it will be important to evaluate the role of other hepatotrophic factors for their dependency on EGFR. Recently, Li *et al.* demonstrated that the growth-promoting actions of hepatopoietin (also known as hepatic stimulator substance in the rat) on a human hepatoma line require the EGFR and could be blocked using a specific EGFR kinase inhibitor (22). However, in this case, hepatopoietin acutely stimulated EGFR phosphorylation within 5 min. The hepatopoietin activation of EGFR persisted when cells were exposed to the monoclonal antibody 528, which blocks EGF ligand binding to human EGFR, preventing endogenous ligand signaling. These findings indicate that the action of hepatopoietin is not due to increased synthesis or processing of an EGF-like ligand or to direct interaction of hepatopoietin with EGFR ligand binding site.

Tumor necrosis factor-α (TNF-α), acting in part through interleukin-6 (IL-6) (23), also plays a major role in the initiation of cell proliferation after partial hepatectomy (24), but the potential dependency of TNF-α or IL-6 on EGFR has not been defined. Gallucci *et al.* showed that TNF-α directly upregulates TGF-α mRNA by as much as sevenfold in mouse hepatocytes (25). As shown for HGF, the mitogenic action of TNF-α is abrogated by the presence of neutralizing antibodies to TGF-α, suggesting that the EGFR may indirectly mediate the hepatotrophic effects of TNF-α. Do TNF-α or IL-6 regulate liver regeneration *in vivo* partly by altering the synthesis and processing of TGF-α or other EGFR ligands? It will be of interest to determine whether the TGF-α increase observed during liver regeneration persists in the TNF-α type I receptor knockout mouse, and whether infusion of TGF-α or EGF after partial hepatectomy can rectify liver regeneration in these mice or in IL-6-deficient ones.

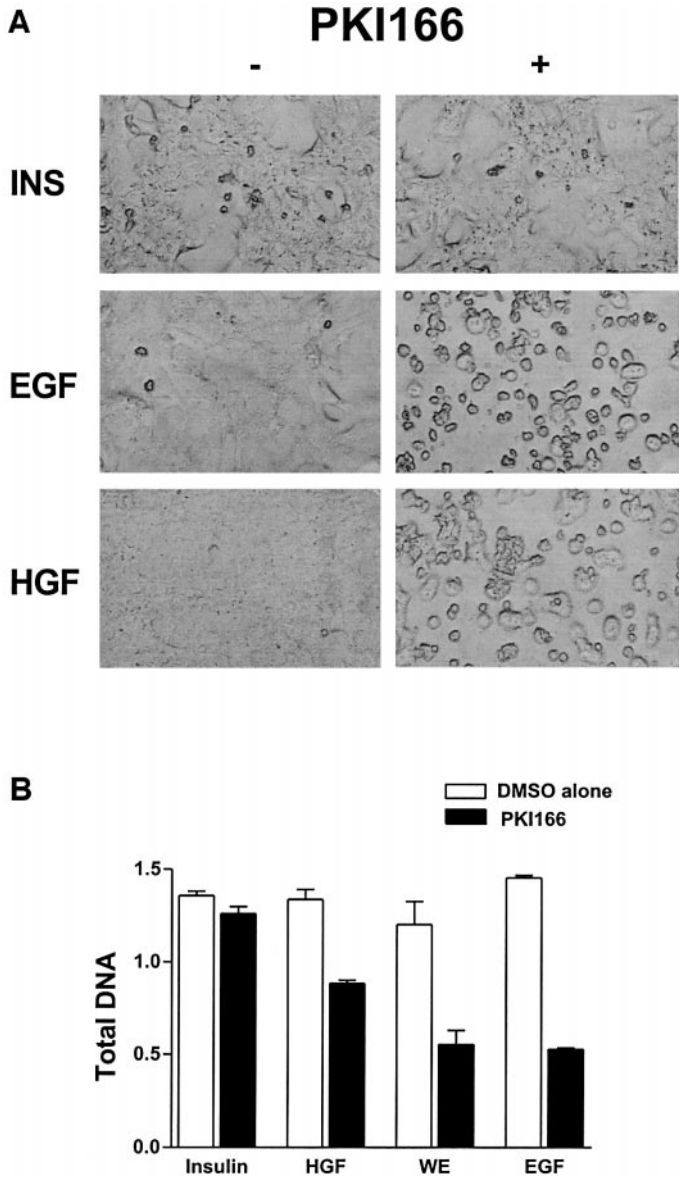

FIG. 6. Insulin preserves hepatocyte morphology and cell mass in the presence of PKI166. (A) Phase-contrast microscopy (magnification 75×) and (B) DNA content of cell monolayers (micrograms of DNA per culture dish, means ± SD of triplicate plates) after 72 h in culture with 115 nM insulin, 20 ng/ml EGF, or 20 ng/ml HGF with or without 3 μM PKI166.

Although the EGFR signaling system has been strongly implicated in liver regeneration, TNF-α, IL-6, and HGF have become focal points in recent regeneration work. The recent findings suggesting that both TNF-α and HGF can stimulate TGF-α synthesis and our current observations that the mitogenic effect of HGF can be abolished by EGFR kinase blockade reiterate the potential centrality of EGFR signaling in normal and regenerative hepatocyte growth. Our laboratory has previously examined liver regeneration in TGF-α knockout and wild-type mice (26). Although we found no significant difference in cell proliferation between the gene deleted and wild-type mice, there are at least six ligands for EGFR. Overexpression of other members of the EGF-ligand family may compensate for TGF-α deletion. Alternatively, only low, ligand-independent, levels of EGFR kinase activation may be required to permit the mitogenic action of HGF. In TGF-α overexpressing RLEC, c-met phosphorylation occurred in the absence of exogenous HGF and the cells became more sensitive to the mitogenic effects of low doses of HGF (1).

Major questions remain to be answered regarding our observations. They include a determination of which HGF effects, in liver or elsewhere, are modulated by the EGFR and a determination of the specific mechanism(s) by which c-met signaling regulates hepatocellular proliferation, both EGFR dependent and independent.

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