Growth Factor Signal Transduction Immediately after Two-Thirds Partial Hepatectomy in the Rat

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ABSTRACT

Liver regeneration after partial hepatectomy (PHx) of the liver serves as a model for studying normal growth factor signals that become aberrant in cancer. Growth factor signals that may play a role in initiating the proliferation of hepatocytes after 70% PHx in the rat were investigated immediately after surgical resection of the liver. Presumptive activity was evaluated by determining the tyrosine phosphorylation state of receptors for epidermal growth factor (EGF) and hepatocyte growth factor (HGF) in the liver after PHx and after sham operation as a control. Under these conditions, it was determined that the EGF receptor was constitutively phosphorylated. EGF receptor tyrosine phosphorylation, however, was increased over basal levels by 60 min after resection. The HGF receptor, c-Met, was minimally phosphorylated in control livers, but a biphasic increase in phosphorylation was observed at 1-5 min after PHx and 60 min postsurgery. A slight increase in c-Met phosphorylation was observed in the sham-operated livers, but the signal was significantly less when compared with that in resected livers. Furthermore, 1 min after PHx, but not sham operation, urokinase-type plasminogen activator (u-PA) and u-PA receptor were observed in the immunoprecipitates of c-Met. Signaling downstream of growth factor receptor activation was also examined. There were no discernible phosphorylation changes in focal adhesion kinase during the early events after surgery in PHx; however, a rapid and sustained increase in the tyrosine phosphorylation of paxillin beginning 1 min after PHx, and a gradual increase in the phosphorylation beginning 5 min postsurgery, were observed. Changes in the activated state of the small GTP-binding protein Rho A and its associated proteins were seen but only after 3 h after PHx. The results indicate that HGF-related signal transduction cascades, which contribute to hepatocyte proliferation, are initiated within one min after PHx.

INTRODUCTION

Liver regeneration is a system well-suited to investigating normal, regulated growth. After surgical removal of 70% of the mass of a healthy liver (two-thirds PHx), the remaining tissue undergoes rapid regeneration of its lost mass in entirety, usually within a week after surgery (1-3). The hepatocytes, which comprise approximately 80% of the liver by weight and roughly 60% by cell number, are the first to enter into the cell cycle in response to the regenerative stimulus. In the rat, the major peak of proliferation for hepatocytes occurs at 24 h after surgical resection, followed by the ductular epithelia at 48 h, Kupffer and Ito (stellate or fat-storing) cells at 72 h, and sinusoidal endothelial cells at 96 h (4-6).

Given the rapidity and extent of the parenchymal response to surgical resection, our laboratory has focused on the immediate signaling events after PHx to identify factors that can stimulate normal hepatocyte proliferation. Previous studies (7) have shown that an immediate increase in u-PA activity occurs within 1 min after PHx. This finding is of critical significance in liver regeneration for three reasons:

1. u-PA has been shown to be an activator of HGF from single chain to its two-chain form in vitro (8);
2. u-PA can also act as a proteolytic enzyme to activate plasminogen to plasmin and thus initiate breakdown of ECM constituents such as fibrinogen, fibronectin, and by plasmin-catalyzed activation of latent metalloproteinases (9, 10). This may constitute a “priming” response that predisposes hepatocytes to the mitogenic action of growth factors (11, 12). This has been suggested by the work of Liu et al. (13) that showed that the infusion of rat liver with TGF-α or HGF could efficiently result in a mitogenic response only when the liver was pretreated with collagenase. Likewise, Kim et al. has shown that very rapid ECM remodeling occurs within 15 min after PHx (14); and
3. many growth factors and inhibitors are known to be sequestered within the ECM of many tissues (15) including liver (16, 17). Proteolysis of the ECM components could spatially and temporally release significant amounts of either hepatotrophic growth factors or negative regulators and could result in signal transduction within the liver lobule.

A large variety of growth factors have been investigated as to their roles in the regeneration process; however, of the growth factors associated with signaling via tyrosine kinase receptors, most of the studies have focused on HGF as well as the EGF ligands EGF and TGF-α because these growth factors exhibit potent mitogenicity on hepatocytes in vitro (1, 3). Additionally, HGF (18, 19), EGF (20, 21), and TGF-α (22-24) protein levels and/or mRNA levels have been shown to increase in the liver during the prereplicative phase of hepatocytes. Although both HGF and EGF/TGF-α seem to elicit motility, morphogenic and mitogenic effects on hepatocytes, it has been shown that HGF and EGF ligands do not always stimulate redundant signaling pathways on hepatocytes in vitro (25-28), which suggests that each growth factor is capable of eliciting a unique response resulting in specific downstream events.

The increase in mRNA and protein levels that have been observed at intermediate early time points after PHx are likely to reflect the replacement of vital proteins used during the earliest regenerative response. Therefore, we examined whether EGF/TGF-α and/or HGF can signal hepatocyte proliferation immediately after PHx. To do so, we examined PY of the EGF and HGF receptors at very early times after PHx, then evaluated activation of potential known downstream effectors to determine the timing of signaling events leading to the first wave of hepatocyte proliferation. Our results suggest that EGF and HGF signaling pathways play very distinct roles in initiating the regenerative process in rats, with HGF receptor-related signaling...
increasing rapidly after PHx, whereas the EGFR is maintained in a constitutively phosphorylated state.

**MATERIALS AND METHODS**

All of the chemicals were obtained from Sigma Chemical Company (St. Louis, MO) unless indicated otherwise.

**Animals and Surgery.** All of the rats were humanely treated using approved procedures in accordance with the guidelines of the Institutional Animal Use and Care Committee at the University of Pittsburgh School of Medicine and the NIH. Male Fisher 344 rats (National Cancer Institute, Frederick, MD), averaging 200–225 g, were 70% partially hepatectomized as described previously (29). Before surgery, animals were allowed standard rat chow and water ad libitum and maintained on a 12-h light-dark schedule. For the immediate time points of 1–15 min postsurgery, the incision was covered with sterile gauze, saturated with sterile saline, before harvesting liver remnant. For longer time points, incisions were sutured closed until the liver was harvested. Shams were prepared exactly as described for the resected animals, the xiphoid process was removed, but the livers were not manipulated because this was found to cause minor proliferation of hepatocytes at 24 h postsham, presumably the result of cell damage. At specific time points after surgery, animals were anesthetized with 100 μg/200-g Nembutal i.p., and livers were harvested and immediately snap-frozen in liquid nitrogen. Control livers were obtained from Metophone- or Nembutal-anesthetized nonmanipulated animals. Frozen tissue was stored at −80°C until use.

**Protein Lysates.** All of the protein lysates were obtained from flash-frozen liver tissue. Frozen tissue was cut and weighed on frozen surfaces to assure that proteinolysis was maintained at a minimum. Then 0.35 g of liver tissue was homogenized in 2 ml of ice-cold lysis buffer containing a panel of broad spectrum protease and phosphatase inhibitors [10 mM Tris·HCl (pH 7.4); 10 μg/ml each leupeptin, pepstatin A, 1, 10 phenanthrolin, and E-64; 1 mM EDTA, 1 mM sodium ortho-vanadate, and 4 mM di-isopropylfluorophosphate]. Aliquots were removed from this solution and frozen at −80°C for additional use. An aliquot was retained and SDS was added to a final concentration of 1%. The solution was briefly sonicated on ice to resuspend the proteins and fragment the DNA. This resulted in a solution with a protein concentration that was routinely 25 mg/ml using the bicinchoninic-acid protein determination with BSA as a standard.

**IPP.** Liver lysate (100 μl) containing 1% SDS consisting of 2.5 mg of total protein was used in all of the IPP procedures. To this volume of lysate in a 1.5-ml microfuge tube was added 400 μl of H2O and 500 μl of 2× IPP buffer containing 300 mM NaCl, 20 mM Tris·Cl (pH 7.4), 2% Triton X-100, 1% NP40, 2 mM EDTA, 2 mM EGTA, 10 μg/ml each leupeptin, pepstatin A, 1, 10 phenanthrolin, and E-64, 1 mM EDTA, 1 mM sodium ortho-vanadate, and 4 mM di-isopropylfluorophosphate (DFP). The resulting solution, which had now a final concentration of 0.1% SDS, was precleared with 20 μl of packed, washed Protein A or Protein G Sepharose for 30 min while shaking at 4°C. This solution was centrifuged at 4°C at 14,000 × g for 10 min. The supernatant was added to a new microfuge tube containing the antibody as well as the absorbant of choice as indicated in the figure legends. IPP proceeded for 2 h at 4°C while shaking. The IPP suspension was centrifuged at 14,000 × g for 5 min at 4°C; the supernatant was discarded, and IPP complexes were washed three times with 1× IPP buffer containing protease inhibitors. After the final wash, proteins were solubilized in reducing gel electrophoresis buffer and then frozen until use. The following primary antibodies were used for IPP and Western blotting: (a) sheep antihuman EGFR (UBI, Lake Placid NY); (b) rabbit antimonuse c-Met, rabbit antihuman Rho A, Rho-GDI, and monoclonal antihuman PCNA (Santa Cruz Biotechnology, Santa Cruz, CA); (c) monoclonal anti-PY (PY-20), monoclonal antiphospho, p190 RhoGAP, and FAK (Transduction Laboratories, Lexington KY); and (d) rabbit antimonus u-PA and rabbit antirat u-PAR (ADI, Greenwich, CT). All of the HRP-conjugated secondary antibodies were purchased from Sigma, except anti-PY blots were performed using HRP-conjugated anti-PY (RC20) from Transduction Laboratories.

**ADP-Ribosylation of Rho A by C3 Exoenzyme in Liver Membranes.** Crude liver membrane fractions were obtained by centrifugation of total lysates processed without SDS for 1 h at 14,000 × g at 4°C. Supernatants were removed, and the pellet was resuspended in an equal volume of ribosylation buffer [20 mM Tris (pH 7.4), 10 mM NaCl, 1 mM EDTA, 1 mM MgCl2, 2 mM DTT, 10 mM thymidine, and 0.5 μCi [32P]NAD] by brief sonication on ice (30). Twenty-five μg of membrane protein, as determined by Bradford method, were retained on ice while 0.1 μg of C3 Exoenzyme (List Biochemicals, Campbell, CA) was added to each tube. Reaction was initiated and preceded by incubation at 37°C for 30 min with occasional agitation. Reaction was stopped by the addition of an equal volume of reducing electrophoresis buffer, followed by boiling for 5 min.

**Gel Electrophoresis and Western Blotting.** To reduce the background that we find inherent to anti-PY blots, all of the electrophoreses buffers and blotting buffers (before the addition of blocking proteins) were filtered through 0.22-μm nitrocellulose filters (Corning-Costar, Cambridge, MA). Protein-containing lysates or immunoprecipitated samples were resolved on reducing discontinuous SDS polyacrylamide gels and then electropherotically transferred at 250 mAmps in 25 mM Tris, 192 mM glycine, and 0.01% SDS overnight at room temperature to Immobilon-PVDF membrane (Millipore, Bedford, MA). The even transfer of proteins was determined by reversible staining with Ponceau Red S solution before blocking. ADP-ribosylation of Rho A was examined by autoradiography overnight at −80°C.

Blots were blocked with either 5% fish gelatin (5% Fish Blotto) in TBST for anti-PY blots or 5% nonfat dry instant milk (5% Milk Blotto) in TBST for other antigens for at least 1 h at room temperature or 4°C overnight. Blotting proceeded with primary antibodies for 2 h at room temperature or overnight at 4°C in 5% milk or fish gelatin. Blots were washed three times in 1% gelatin or milk in TBST (1% Blotto). Secondary antibodies were applied in 1% Blotto for 1 h at room temperature. Blots were washed three times in 1% Blotto and then once in TBST before development on X-ray film using enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL). PVDF membranes were stripped by 2 × 1-h incubation in 0.2 μg/mL glycine, 0.05% Tween 20 (pH 2.5) at 80°C. Densitometric analysis of Western blots were performed using the BioImage Analytical Scanning Densitometer equipped with the Whole Band Analysis software package (Millipore/BioImage, Bedford, MA). Statistical data were generated using the InStat 2.01 statistical software.

**RESULTS**

The time frame in which hepatocytes enter into DNA synthesis after PHx has been shown to occur between 12 and 24 h after surgery (4). To correlate our results with the timing of DNA synthesis in the animals used for subsequent phosphorylation studies, we examined proliferating PCNA levels in whole liver lysates at various times from PHx, sham-treated, and untreated animals. Figure 1 indicates that in liver lysates prepared from rats subjected to 70% PHx, a substantial increase (>5-fold over controls) in the expression of PCNA—a G1-to-S-phase marker—is seen 24 h after resection, whereas no changes are noted in the sham-treated animals.

To determine whether EGF and HGF have a role in the initial wave of hepatocyte proliferation before PCNA up-regulation, we examined the signaling of their receptors after PHx. Increases in the proteins and mRNAs for these growth factors occur within the liver at immediate and immediate-early times after PHx. Growth factor protein concentrations have been shown to increase at very early time points after liver injury, whereas their mRNAs increase somewhat later, which suggests replenishment of growth factors that have been depleted. Nevertheless, the increases of a specific growth-factor protein or mRNA do not necessarily indicate that signaling is occurring, as much as the protein may not be in an active conformation or may be suppressed by an inhibitor, or else the intended receptor may not be available for binding. To verify that signal transduction occurs, we used an indirect approach by determining the PY state of the receptors for EGF/TGF-α and c-Met, the receptor for HGF after PHx or sham.

To examine the signaling of these two receptors with relation to one

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another, a coimmunoprecipitation experiment was performed. EGFR and c-Met were immunoprecipitated together from total lysates prepared from livers subjected to PHx or sham operation at various time points, resolved on reducing gels, blotted to PVDF membrane, and then probed with anti-PY antibodies. Fig. 2A indicates that the EGFR is constitutively tyrosine-phosphorylated, because phosphorylation is present in control livers, but slight fluctuations in this basal level are observed in both manipulated and sham-treated livers because a decrease in EGFR phosphorylation is seen at 5 and 15 min in both PHx and sham-operated livers (Fig. 2B). After 30 min, EGFR phosphorylation returns to basal levels in sham-treated rats. However, EGFR phosphorylation increases to 2-fold over basal levels at 60 min post-PHx, returning to basal levels 180 min after surgery. c-Met is not visibly tyrosine-phosphorylated in control livers but shows a biphasic increase in phosphorylation beginning at 1 min after PHx, decreasing at 15 min, then increasing until 60 min post-PHx, and subsequently

Fig. 2. A, top, PY of EGFR (EGF-R) and c-Met in normal livers (C) and at specific time points (in min) after PHx and sham operations. Total liver lysates (2.5 mg) were coimmunoprecipitated with antibodies directed toward human EGFR (10 μl) and mouse c-Met (10 μl) and Protein G Sepharose. Immunoprecipitated proteins were resolved on 8% discontinuous SDS-PAGE gels, blotted to PVDF, and probed with anti-PY antibodies conjugated to HRP. Two different control, nonoperated livers were used (C). Middle, top row, gel was stripped and reprobed with antibodies to EGFR to show that equal amounts of EGFR protein were immunoprecipitated during the procedure. Bottom, top row, gel was stripped and reprobed with antibodies to c-Met, to indicate that equal amounts of c-Met protein were immunoprecipitated during the procedure. B, densitometric and statistical analysis representing EGFR and c-Met phosphorylation in PHx and sham-treated animals. Data shown are performed as described in A. The SE of three separate experiments performed using three separate sets of animals is shown. PY signal was divided by the signal from total receptor IPP to normalize the PY signal; then this value was normalized to the untreated control livers to obtain -fold increase or decrease in phosphorylation for each condition. Results for c-Met phosphorylation at 5, 60, and 180 min are statistically significant when compared with the same time points in the sham-treated animals (left, *, P < 0.05). Likewise, EGFR phosphorylation significant over sham-operated animals at 60 min post-surgery (right, *, P < 0.05). C, immunoprecipitation of tyrosine-phosphorylated proteins from control; PHx- and sham-treated livers and complexes were blotted with EGFR or c-Met antibodies. Results shown are representative of two separate sets of PHx, sham, and control livers.
dropping to lower levels between 60 and 180 min. Only a slight increase in the phosphorylation of c-Met is observed in sham-treated rats. When these blots were stripped and reprobed for EGFR and c-Met, an even amount of each protein was immunoprecipitated per condition, normalizing the phosphorylation signals observed in the upper panels. This experiment was repeated three separate times, using three different sets of rats, with similar results. Fig. 2B shows the densitometric analysis of these Western blots scanned, quantified, and normalized to the amount of receptor as well as nonresected controls in each experiment. The left panel compares the fold increase over controls in the phosphorylation of c-Met in resected and sham-operated rats. A significant and reproducible 3.5-fold increase in c-Met PY is evident at 5 min after PHx. A biphasic response is observed when, after a decrease from the initial phosphorylation, an 8-fold increase in c-Met is observed at 60 min post-PHx, followed by another drop in signal at 180 min. A small, but steady increase in c-Met phosphorylation as a result of sham-operation is observed and is statistically significant compared with nonoperated controls. EGFR PY does not fluctuate so dramatically, but a 2-fold increase in phosphorylation is seen at 60 min post-PHx.

The converse experiment described in Fig. 2A was performed on additional aliquots of liver lysates. Fig. 2C indicates that, when all of the tyrosine-phosphorylated proteins are immunoprecipitated and then blotted for EGFR, an increase is observed at 60 min after PHx, whereas sham levels seem essentially stable. As shown in Fig. 2C, a biphasic increase in c-Met phosphorylation is observed after PHx, whereas in sham-treated animals, a slight increase in c-Met is seen.

Previous results from our laboratory have shown that u-PAR was increased in liver lysates within 1 min after PHx (7). Others have also shown that activation of HGF is stoichiometrically regulated by u-PA (31), suggesting the potential formation of a stable complex between u-PA, u-PAR, HGF, and, possibly, c-Met. To examine potential interaction at this early signaling event, we immunoprecipitated c-Met from nonmanipulated, sham- and PHx-treated livers and then probed the resolved immune complexes using antibodies to u-PA or u-PAR. Fig. 3 shows that, indeed, both u-PA and u-PAR coprecipitated with c-Met at 1 min after PHx but not in sham-treated animals nor in nonmanipulated livers (of which livers from four different control animals were used). Interestingly, significant amounts of u-PA were not seen associated with c-Met again until 3 h after PHx, and u-PAR was not observed in the immunoprecipitates for up to 12 h after PHx. A minor association of u-PA, but not u-PAR, was seen with c-Met in some of the later time points after sham operation. As a control, blots were also probed with c-Met antibody to indicate that even amounts of c-Met protein were immunoprecipitated and loaded per lane. As an additional control, u-PA and u-PAR were not seen associated with immunoprecipitated EGFR for the parallel time points described here (data not shown).

The timing of downstream signaling events relevant to HGF, TGF-α, and EGFR binding were analyzed in whole liver lysates. Liver lysates were immunoprecipitated with anti-PY antibodies, and these resulting immune complexes were probed for FAK, Paxillin, and p190 RhoGAP (small GTP-binding protein RhoGAP). These proteins have been shown to be phosphorylated and, therefore, activated by c-Met and/or EGFR on a variety of cell types, including hepatocytes in vitro, which results in mitogenic and motogenic responses (32–34). Because HGF, EGFR, and TGF-α are both mitogenic and motogenic to hepatocytes in vitro, signaling should be indicative of very early events triggered by the growth factors for either of these pathways. As shown in Fig. 4, an immediate increase in Paxillin phosphorylation is evident at 1 min after PHx and stays elevated until 12 h after surgery. A sustained, but much reduced, phosphorylation of Paxillin is seen in the sham-treated rat liver. Interestingly, despite the FAK/paxillin connection normally observed in migrating cells, there are no visible changes in the phosphorylation of FAK observed in either the PHx- or sham-treated liver. An increase in RhoGAP is observed at 3 and 6 h after surgery. No phosphorylation changes are seen in RhoGAP in the sham-operated livers.

Other than the association of u-PAR with c-Met, the increase in RhoGAP phosphorylation was the only phenomenon that was specific to PHx and not seen in shams. Therefore, we decided to investigate this phenomenon in greater detail. Because p190 RhoGAP activation via mitogens is thought to regulate Rho activity, we next examined the amount and activity of Rho A and its negative effector, Rho-GDI (GDP/GTP exchange inhibitor) in liver membranes. Rho A is known to regulate stress fiber formation and motility in mitogen-treated cells (35), and its activity is related to its translocation to the membrane in stimulated cells (36). It has also been implicated in inducing cells to traverse through G1 to the S phase of the cell cycle, independent of MAP kinase activation, after serum activation (37). Active Rho A (not complexed with Rho-GDI) can be assessed indirectly by its ability to be ADP-ribosylated by Clostridium botulinum toxin exoenzyme C3. When Rho is in the inactive state by complexation with its negative effector Rho-GDI, ADP-ribosylation is inefficient (38). When liver membranes were treated with exoenzyme C3 in the presence of 32P-NAD, small fluctuations were seen in the ADP-ribosylation of Rho A until 18 h after PHx, when a large increase in 32P-labeled Rho A was observed (Fig. 5). These changes were not observed in sham-treated livers relative to the amount of Rho present. In addition, no major increases in the amount of membrane-associated Rho A protein was seen at any of the time points tested, which indicates that, although the amount of Rho A at the membrane does not change after PHx, its activity does change significantly at 6, 18, and 72 h post-resection. Additionally, an increase of Rho-GDI is seen in liver membranes between 18 and 72 h after PHx but is not seen in sham livers.

DISCUSSION

In this report we have examined a cascade of immediate growth factor signaling events in the liver after 70% PHx that may contribute to the profound regenerative response in hepatocytes within 24 h after surgery. Before undertaking this study, we expected that HGF activation, changes in EGF and/or HGF receptor phosphorylation, and related downstream signaling events must occur rapidly, probably within 15 min, of the surgical event. DNA synthesis has been shown to begin at 12–18 h in hepatocytes after PHx (4). Therefore, we reasoned that the initial event leading to the hepatocyte proliferative response must be very early after surgery. What was not clear, however, was whether HGF, EGF/TGF-α, and/or some other agent, were cues to initiate the proliferative signaling cascade.

Important clues to this effect were elucidated recently by us and others. The first clue indicated that within 1 min after 70% PHx, active u-PA was present in the liver (7). This finding had relevance to liver regeneration because u-PA is known to proteolytically cleave inactive single-chain HGFs to the biologically active two-chain form (8). Furthermore, both u-PA activity and active HGF increase in liver remnants shortly after PHx (7). Because HGF is a potent, complete mitogen for hepatocytes, we hypothesized that HGF signaling via c-Met activation may also occur rapidly. Indeed, we observed that phosphorylation of c-Met was seen as early as 1–15 min after PHx. The largest increase in phosphorylation—at 60 min—exactly parallels previous results from our laboratory that demonstrated that active, two-chain HGF increases steadily from 15 min and peaks at approximately 60 min post-PHx (7). c-Met signaling in liver regeneration has been shown to occur within 1 h after surgical or chemical manipulation (39–40). This suggests that HGF signaling is necessary for liver regeneration as neutralizing monoclonal antibodies to HGF severely
Fig. 3. Interaction of u-PA and u-PAR with c-Met immediately after PHx. Whole liver lysates in 1% SDS were diluted to 0.1% SDS and then immunoprecipitated with rabbit antimouse c-Met exactly as described in Fig. 2. After gel electrophoresis and electroblotting to PVDF membrane, blots were probed with rabbit antimouse u-PA (top row) or rabbit antirat u-PAR (middle row). To show that immunoprecipitations were even, the bottom row shows the c-Met immunoprecipitates probed with anti-c-Met antibody. Results are representative of two separate sets of PHx- and sham-operated livers and livers removed from four control animals.

Fig. 4. Signal transduction events downstream of growth factor-receptor binding. Several nonreceptor tyrosine kinases were examined in control and in sham- and PHx-treated livers. Proteins were subjected to IPP with anti-PY antibodies, resolved on 8% gels, blotted, and then probed with the primary antibodies to FAK, paxillin, and p190 RhoGAP. Results shown are representative of two separate sets of PHx, sham, and control livers.

The phosphorylation that we observe at the earliest time points are most likely the result of stimulation by growth factors that are distinct from those seen at later time points when paracrine secretion of mitogen occurs within the liver. One interpretation of our findings is that this immediate signaling is the result of proteolytic activation and release of HGF sequestered within the liver ECM (16, 17), possibly by u-PA. Alternatively, growth factors may infuse from an extrahepatic source such as peripheral blood. In addition to the finding of active u-PA at 1 min post-PHx, recent work in our laboratory describes quantifiable breakdown of liver ECM, including plasminogen, fibrinogen, fibronectin, entactin, and laminin by 15 min after PHx (14). By immunohistochemical localization, the ECM changes seem to occur in the perportal area of the liver, which is the area of greatest hepatocyte proliferation at 24 h postresection. Thus, matrix breakdown and HGF activation correspond well with the phosphorylation of c-Met described here; however, additional studies are needed to discriminate between these two possibilities. Additional support for the involvement of ECM breakdown was shown in that u-PA knockout mice exhibit a transiently impaired regenerative response after PHx (42). This delay could also be related to the lack of HGF activation in the absence of u-PA.

Concurrent with our studies of liver regeneration, recent work has shown that HGF activation and subsequent signaling is contingent on a stoichiometric reaction between HGF, u-PA, and c-Met (31). We examined this cooperative effect on initial signaling in the HGF signaling pathway and observed a strong association of u-PA and u-PAR with c-Met but only within the earliest time points after PHx. These results are not likely to be artifactual because the IPPs were performed under high stringency by inclusion of 0.01% SDS in the IPP buffer. Furthermore, we have also observed the c-Met, u-PA, and u-PAR association in vitro using serum-free human hepatocyte cultures (43). This rapid but transient association in vitro may ensure that immediate signaling occurs spatially and temporally within the liver lobule to initiate only specific cells into the proliferative pathway.

Because HGF is the most potent mitogenic factor for hepatocytes, displaying at least a 15-fold increase in mitogenicity over EGF or TGF-α on a molar basis (28), only small quantities should be required for maximal effect in the liver after PHx. It is important to note that these results do not rule out a possible cooperative, additive, or synergistic effect between HGF and the EGFR-based signaling because we observed that the EGFR is constitutively phosphorylated in nonmanipulated as well as in manipulated livers (Fig. 2). Although c-Met- and EGFR-based cross-talk has been shown in TGF-α-transformed rat liver epithelial cells (44), it is unknown whether such cross-talk occurs in nontransformed hepatocytes in situ. Still, it is worth noting that a significant increase in EGFR phosphorylation is observed at 60 min post-PHx, exactly paralleling the peak activation of c-Met. The parallel increase in both EGFR and c-Met phosphorylation may be due to coincident increases in activated EGF/TGF-α and HGF, respectively, or the result of cross-talk between the two receptors activated by only one growth factor (44). The potential for cross-talk requires further study to determine its possible role in normal liver development, regeneration as well as cancer.

Small amounts of c-Met phosphorylation were observed in sham-operated animals. Therefore, an examination of the timing of downstream signaling cascades that modulate hepatocyte proliferation was necessary because receptor phosphorylation is inconsequential unless the proliferative signal can transverse the membrane and initiate DNA synthesis. We looked at several intracellular signaling that are known to be tyrosine-phosphorylated after growth-factor-receptor stimulation in vitro by either HGF or EGF/TGFα. Differential signaling was apparent among specific downstream effectors after PHx. Notably, a rapid rise in tyrosine-phosphorylated paxillin was seen within 1 min after PHx and remained highly phosphorylated until at least 12 h after surgery. Increases in tyrosine-phosphorylated paxillin were also apparent in the sham-operated livers; however, the increases were gradual and of a much lesser extent than seen in resected livers. Inhibitors that prohibit sham-treated livers from traversing the cell cycle, even in the presence of growth factor signal transduction, are a topic worthy of exploration. These same inhibitors may also play a role in delaying compromised liver regeneration after carbontetrachloride damage (41).
the proliferative response of some of the other cell types in the liver, like endothelial and bile ductule epithelial cells, also known to be responsive to HGF and/or EGF/TGFα in vitro (45–48). For example, “priming” via ECM breakdown may be one of the checkpoints that allows resected livers to proceed into a proliferative pathway because sham-operated rats do not exhibit ECM breakdown (14). Thus, under these circumstances, intact matrix is an inhibitor of proliferation.

In view of the increase in phosphorylated paxillin, it is curious that FAK PY does not change from control levels up to 12 h after PHx, because FAK and paxillin are generally linked in processes involving growth-factor signaling and integrin activation (49). FAK phosphorylation has been shown to precede that of paxillin during signaling (50) because paxillin can be phosphorylated by FAK in vitro (51). Furthermore, HGF has been shown to induce PY of FAK in vitro (32). Nevertheless, FAK knockout mice do not exhibit reduced paxillin phosphorylation (52), which indicates that activation and turnover of these two proteins may not be interdependent.

The FAK/paxillin phosphorylation pathway, as well as the activation of mitogen receptor tyrosine kinases, has also been tied to the activation of the small GTP-binding proteins such as Rac, Rho, and Cdc42 (53). As such, the activity of small GTP-binding proteins can initiate various modes of F-actin rearrangement in cells via growth factors or integrin engagement (35) leading to motility. Mitogens such as HGF and EGF have also been shown to activate the Rho-mediated signal transduction cascades (33, 54–56) and, thus, would be relevant to liver regeneration in the context described here. To determine whether the Rho-mediated signal pathway is activated in the early times immediately after resection, we initially examined the phosphorylation status of p190 RhoGAP, which is a negative modulator of Rho. GAPs function to enhance the GTPase activity of small GTP-binding proteins, leading to inactivation of these proteins (57). When RhoGAP becomes tyrosine phosphorylated, it preferentially turns off Rho and, to a lesser extent, Rac and Cdc42, by altering the GTP-bound status to GDP-binding. After PHx, minimal PY of p190 RhoGAP can be detected within 5 min with peaks at 3 and 6 h after surgery. The simplest explanation is that the process of turning off Rho A very early in the regeneration process may serve to restrain cytoskeletal activity relating to the motility of hepatocytes or other liver cells caused by HGF (27, 28, 58), leading to the mitogenicity rather than the motogenicity pathway because these two events cannot proceed simultaneously in hepatocytes (58).

To further characterize the activity of Rho and Rho-related proteins in the early signaling events after PHx, we examined the ability of Rho A to be ADP-ribosylated by C3 exoenzyme. When Rho A is coupled to Rho-GDI—a protein that maintains Rho A in the inactive GDP-bound state—ADP-ribosylation of Rho A is ineffectual (40). When membranes were examined for ADP-ribosylated Rho A, no obvious changes were observed in either the PHx or sham-operated animals from 1 min to 3 h after surgery. Noticeable increases in Rho A ADP-ribosylation were observed in the PHx-treated livers at 6, 18, and 72 h after resection, indicating potential increases in Rho A activity. This was not due to increases in Rho A protein at the membrane because probing the same blot with anti-Rho antibodies did not indicate more Rho A associated with the liver membrane at these times. The increases in the availability of Rho A to be ribosylated by C3 exoenzyme is likely due to a temporal release of Rho-GDI from Rho A at the membrane, allowing for ribosylation and, therefore, potential increase in activity. The increase of Rho A activity at 18 h after resection correlates well with the role of Rho A as a necessary element in the G-to-S-phase transition of hepatocytes as suggested by the micro-injection experiments of Olson et al. (37). Interestingly, despite the increased activity of Rho A at 6, 18, and 72 h, Rho-GDI increases in the membrane fractions at 12–72 after PHx. Removal of inactive Rho A-GDP from the membrane is thought to be mediated by Rho-GDI, although the nature of this translocation is not well understood (36, 54, 56). Although seemingly contradictory, this increase at the liver membrane at times with which Rho A is potentially more active may indicate a higher turnover of Rho A at the membrane (36) because removal of inactive Rho A- from the membrane is mediated by Rho-GDI. Furthermore, the timing of the movement of Rho-GDI to liver membranes after resection may represent temporal and spatial fine tuning of Rho A activity within the liver inasmuch as only specific sections of the lobule, as well as specific cells within the lobule, undergo proliferative and/or motility responses.

In summary, we describe early signaling events after PHx that fall into several categories with respect to growth factor activation and downstream signal transduction cascades before the initial proliferative wave of hepatocytes. Signaling cascades involving uPA, uPAR, and c-Met were speculated by our laboratory to occur very early after PHx (1). Here we show that they are, indeed, interacting and signaling in intact liver immediately after PHx, and the data implicate the EGFR pathway in the early regenerative response also. Additionally, many of these growth factor signaling cascades have been shown to be up-regulated in cancer cells:

(a) constitutive signaling events: for example, EGFR and FAK phosphorylation are found in nonproliferating livers. Because we observe that EGFR is constitutively phosphorylated, EGFR and HGF signaling pathways may be integrated for regeneration to proceed efficiently;

(b) general immediate-early signaling events: within 1 min to 1 h after liver resection, c-Met and paxillin phosphorylation is observed. These are observed to a much lesser extent in shams;

(c) specific early signaling events: c-Met, but not EGFR, forms a PHx-specific interaction with u-PA and u-PAR at 1 min after surgery. Rho-GAP is increased at 3 and 6 h after PHx but not in sham; and

(d) specific delayed-early signaling responses: active Rho A increases at 6 and 18 h after PHx followed by PCNA expression. The results indicate that immediate interaction between components of the HGF, c-Met, and u-PA signaling systems are activated within 1 min after PHx, contributing to the initiation of hepatocyte proliferation.
SIGNAL TRANSDUCTION DURING LIVER REGENERATION

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Growth Factor Signal Transduction Immediately after Two-Thirds Partial Hepatectomy in the Rat

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