Cultured rat hepatocytes upregulate Akt and ERK in an ErbB-2-dependent manner

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Departments of 1Pediatrics, Division of Endocrinology and 2Cell and Developmental Biology, the 3Digestive Disease Research Center, the 4Vanderbilt Diabetes Center, and the 5Vanderbilt Ingram Cancer Center, Vanderbilt University Medical Center, Nashville, Tennessee

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Scheving LA, Stevenson MC, Zhang X, Russell WE. Cultured rat hepatocytes upregulate Akt and ERK in an ErbB-2-dependent manner. Am J Physiol Gastrointest Liver Physiol 295: 322–331, 2008. First published June 5, 2008; doi:10.1152/ajpgi.00597.2007.—ErbB-2-dependent signaling of EGF and homologous ligands (40) is known to require interactions among the four related ErbB tyrosine kinase receptors. Whereas the EGF receptor (EGFr), also known as ErbB1, is the only member of the family that can bind EGF, it forms heterodimers with other members of the ErbB family. We have previously defined a developmental profile of ErbB expression in liver. Fetal hepatocytes express EGFr, ErbB2, and ErbB3, but adult hepatocytes express only EGFr and ErbB3. ErbB4 cannot be detected (8, 32).

Our recent studies identified the induction of ErbB2 as a critical event in the responsiveness of cultured hepatocytes to EGF and showed that the restriction point in the hepatocyte cell cycle that occurs at 40–44 h in culture corresponds to the induction of ErbB2 and the dedifferentiation of the cells to a more fetal phenotype. Shortly after plating, EGFr expression rapidly declines, whereas ErbB3 expression increases over the first 24 h of culture but then plummets in cells cultured in EGF and insulin. As ErbB3 levels decline, de novo ErbB2 expression begins and steadily increases thereafter. The most potent receptor signaling complex for EGF in vitro is an EGFr/ErbB2 heterodimer.

Two important downstream effectors of EGF action in cells are the Akt and ERK 1/2 families of serine/threonine protein kinases (3, 22). The Akt kinases are anchored to the plasma membrane by lipid products of the phosphatidylinositol kinase-3 (PI3K) pathway and are activated by PDK1 and mTORC2 kinases. Akt kinases regulate multiple factors that influence cell survival and metabolism, such as NF-κB, Bcl-2 family proteins, and glycogen synthase kinase 3 (GSK-3). The ERK (mitogen activated protein kinases, MAPK) kinases are activated by cell surface receptors such as the EGFr through the intermediacy of G proteins in the Ras/Raf/ERK pathway. Activated ERK kinases regulate a variety of transcription factors such as CREB and c-Myc, which regulate transcription of important cell cycle genes. ERK phosphorylation of the 40S ribosomal protein S6 kinase also regulates the translation of mRNA into protein.

To define the downstream pathways of ErbB signaling in hepatocytes we examined the temporal changes in the Akt and ERK 1/2 proteins in cultured hepatocytes. We found that hepatocytes upregulate Akt and ERK prior to DNA synthesis. This upregulation is partly dependent on the presence of insulin or EGF in the medium. Suppression of de novo ErbB2 expression blocks the induction of these proteins without influencing EGFr expression. We propose that newly synthesized ErbB2 is

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required for Akt and, to a lesser extent, ERK 1/2 expression in cultured hepatocytes and that this upregulation of Akt and ERK 1/2 contributes to the enhanced responsiveness of hepatocytes to EGF as they are maintained in culture.

EXPERIMENTAL PROCEDURES

Peptides, reagents, and radiochemicals. Synthetic rat TGF-α was from Peninsula Laboratories (Belmont, CA). Insulin (Novolin R) was from Novo Nordisk Pharmaceuticals (Princeton, NJ). Dexamethasone, pyruvate, bovine serum albumin (fatty acid-free), Percoll, and all buffer reagents were from Sigma (St. Louis, MO). ECL reagents were from Pierce (Rockford, IL). Nitrocellulose membranes were from Osmonics (Minnetonka, MN).

Animals. Adult male Sprague-Dawley rats (200–250 g) from Harlan (Indianapolis, IN) were raised under conditions of regulated lighting (lights on 0600-1800). They had ad libitum access to water and Purina rodent chow (Ralston-Purina, St. Louis, MO). The Animal Use Subcommittee of the Vanderbilt Animal Care Committee approved all protocols.

Culture media and supplies. Williams’ Medium E, supplemented with 20 mM pyruvate and 50 μg/ml gentamicin, was the medium used for all culture studies. The media typically contained insulin (100 nM), needed to preserve EGF or TGF-α responsiveness. The concentration of dexamethasone was generally 10−8 M. Medium and calf serum were from GIBCO, Invitrogen (Grand Island, NY). Type I collagenase was from Wako Pure Chemical Industries (Richmond, VA). Falcon six-well dishes were from Fisher Scientific.

Primary cell cultures. Hepatocytes were isolated 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 (8). To reduce nonhepatocyte contamination, cells were sedimented through Percoll (8). We plated cells (3.75 × 10^5/well) in type-I collagen-coated six-well 35-mm plates for 60–90 min before addition of serum-free medium, growth factors, or dexamethasone. In some experiments, growth factor was added at different times after the change from plating to growth medium.

siRNA transfection experiments. The small interfering (si)RNA were obtained from Xeragon (Huntsville, AL). Sequences, with two 3’ deoxythymidine overhangs, were siErbB2 (GAGAGGGA-CCAGCTCTTTGA) and a nonsilencing control siRNA (AAT-TCTCCGAACGTTCAGGTCACTGA). The ErbB2 sequence and the control sequence did not match that of any other rat gene according to the NCBI nucleotide-nucleotide blast program. Transfection of siRNAs was performed 90 min after the initial plating by use of oligofectamine reagent (Invitrogen; Carlsbad, CA). Hepatocytes in six-well collagen-coated plates were incubated with the transfection mix to give a final siRNA concentration of 120 nM siRNA (9 μl). The siRNA was originally complexed with 3 μl oligofectamine in OptiMEM (Invitrogen; final volume 70 μl) and was added to 1.5 ml of the growth medium. The cells had been in the growth medium for 2 h before transfection.

Immunoblotting. Hepatocytes were lysed in TGH buffer (20 mM HEPES, 1% Triton X-100, 10% glycerol, 100 mM NaCl). This buffer included protease inhibitors (1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM EDTA, and 1 mM EGTA) as well as phosphatase inhibitors (10 mM sodium molybdate, 10 mM β-glycerol phosphate, 10 mM sodium pyrophosphate, and 10 mM NaF). Lysates were microfuged at 20,800 g for 30 min and then immunoblotted or immunoprecipitated as previously described. The phospho, total, and isoform-specific Akt (AKT Isoform Sampler kit) and ERK 1/2 antibodies were from Cell Signaling Technology (Beverly, MA). Antibodies against CDK-2 and cyclin D1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against cytchrome P-450 2E1 was from Abcam (Cambridge, MA). Antibody against rat albumin was from ICL (Newberg, OR). We normalized immunoblots by using equal amounts of protein, as determined by either the Bio-Rad DC Protein Assay (Bio-Rad Laboratories; Hercules, CA) or the Micro BCA protein assay (Pierce Biotechnology, Rockford, IL). After each transfer, we confirmed equal protein loading and transfer by Ponceau S staining of immunoblots, scanning the image for future reference. Immunoreactive signal was detected using the ECL method (Pierce Biotechnology). We performed densitometry using an Epson scanner with Biosoft Quantiscan Software or the Image J program.

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

RESULTS

ERK and Akt increase in primary hepatocytes with time in culture. Given their importance in EGF signaling, we examined the time course of Akt and ERK 1/2 expression in primary cultures of rat hepatocytes. We prepared lysates of cells cultured for up to 5 days in Williams’ E medium alone (designated “basal” medium) or in the presence of insulin (100 nM), EGF (6.3 nM), or insulin in combination with EGF. We measured Akt and ErbB1/2 expression by densitometry of immunoblots (Fig. 1B). Figure 1A shows the immunoblot results of Akt (left) and ERK 1/2 (right). In the absence of insulin or EGF there was a steady increase in the amount of Akt and ERK 1/2 with time in culture. This increase began earlier with ERK 1/2 than Akt. The presence of insulin and/or EGF accelerated this increase for both signaling proteins, particularly for Akt. Scanning densitometry revealed that this increase was over 15-fold (graphs of expression are shown under each blot). ERK 1 and 2 both increased in culture; however, the ratio of ERK 1 to ERK 2 increased from 0.5 to over 1.5, regardless of the hormonal treatment (Fig. 1B, bottom right). This may be important because distinct functions have been reported for ERK 1 and 2 (20). Moreover, Ponceau S staining of these blots revealed no change in the levels or pattern of expression of most of the major hepatocellular proteins (not shown).

Akt expression relative to the expression of differentiation or G1/S transition markers. To define the expression of Akt and ERK 1/2 relative to other hepatocyte markers, we probed blots of lysates with antibodies against protein markers of hepatocyte differentiation or proliferation. For differentiation, we immunoblotted with antibodies against albumin or cytchrome P-450 2E1 (CYP2E1). For the G1/S transition, we immunoblotted with antibodies against CK2 or cyclin D1. EGF-stimulated S-phase entry in a hepatocyte culture system identical to ours has been shown to be preceded by and coupled to the de novo synthesis of cyclin D1, with peak DNA synthesis occurring between 48 and 72 h for cells exposed to EGF at the time of plating (21).

As shown in Fig. 2, the two markers of differentiation showed distinct expression patterns. CYP2E1 began to fall off as early as 10 h and then disappeared after 34 h particularly in the presence of EGF without insulin (Fig. 2, left). This pattern also occurred in cells cultured in media without insulin or EGF (data not shown). In contrast, albumin expression persisted through 4 days of culture, particularly in the insulin-EGF-treated hepatocytes. In contrast, the markers of the G1/S transition appeared with a time course similar to that seen for Akt. However, in contrast to Akt, cyclin D1 and CDK2 did not appear in the absence of EGF, even when insulin was present (data not shown).
The phosphorylated forms of Akt and ERK increase with time in culture. The phosphorylated forms of Akt and ERK 1/2 have been shown to be indirect indicators of PI3K and MEK activity, respectively. To determine whether an increase in the total Akt or ERK correlated with an increase in the basal levels of phosphorylated Akt (Ser-473) or the dually phosphorylated ERK 1/2, we probed parallel immunoblots with phospho-specific antibodies that recognize these “active” forms. Figure 3 shows the densitometric results of these blots. We found a low level of phosphorylated Akt that appeared most rapidly with cells treated with insulin alone (Fig. 3B). The greatest increase in pAkt in insulin-treated cells occurred after 48 h. Although EGF by itself (Fig. 3C) increased pAkt relative to the basal medium (Fig. 3A), it also blunted the sustained increase seen in the insulin-treated cells (Fig. 3D).

A similar analysis was carried out for the dually phosphorylated ERK 1/2 (ppERK 1/2). This form increased with time in culture, paralleling the increase in total ERK 1/2. The extent of ERK1 phosphorylation paralleled that of ERK 2, enabling us to sum the ERK 1 and ERK 2 data for this figure. The greatest increases in phosphorylation of Akt and ERK 1/2 in EGF treated cells occurred before the increases in CDK-2 and cyclin D1, whose synthesis is required for DNA synthesis to proceed. In addition, the phosphorylated isoforms also appeared earlier in cells exposed to insulin and/or EGF. In contrast to phospho-Akt, which appeared to be elevated by insulin to a greater extent than EGF, ppERK 1/2 appeared earlier in cells exposed to EGF (Fig. 3G) than insulin (Fig. 3F). The combination of EGF and insulin caused the ppERK 1/2 levels to peak even earlier (Fig. 3H), but the elevation was not sustained as in cells treated with either insulin or EGF.

EGF stimulation of acute ERK and Akt phosphorylation increases with time in culture. To explore the acute activation of Akt at different times of culture, we exposed parallel plates of cells to EGF for 0, 1.5, or 3 min and then immunoblotted lysates prepared from these cultures as above. We also examined the effect of dexamethasone on the expression or EGF responsiveness of Akt or ERK 1/2. We initially cultured parallel plates of cells in either 10^{-8} M dexamethasone, our standard concentration, or 10^{-6} M, a growth-inhibitory concentration (31). Figure 4 shows that both total Akt (Fig. 4A) and total ERK 1/2 (Fig. 4B) increased from 24 to 48 h of culture as expected. The high dexamethasone concentration inhibited the increase of total ERK 1/2 to a greater extent than that of total Akt. The ability of EGF to increase acutely the phosphorylation of Akt (Fig. 4A) or ERK 1/2 (Fig. 4B) also depended on the time of culture. In contrast to total
Akt, high-dose dexamethasone inhibited the ability of EGF to trigger phosphorylation of both Akt and ERK 1/2.

The increase in Akt and ERK 1/2 is regulated by PI3K and ErbB2. To determine whether signaling components upstream of Akt or ERK 1/2 mediated the increased expression, we used a pharmacological approach. We focused on kinases that are responsible for the phosphorylation of these molecules and instrumental in EGF stimulation of DNA synthesis, namely PI3K and MEK. Cells were treated for 48 h with EGF and insulin in the presence or absence of LY94002, an inhibitor of PI-3 kinase or U0126, an inhibitor of MEK. The de novo synthesis of cyclin D1 after 40 h of culture has been shown to be an obligatory step in the initiation of DNA synthesis by EGF. Consistent with prior reports, Fig. 5A shows that inhibitors of PI3K and to a lesser extent MEK each inhibited cyclin D1 expression (35). These inhibitors also prevented the increase in total Akt that occurs during culture. There was less effect on ERK in this experiment, although the highest dose of LY294002 modestly depressed ERK 1/2 in other experiments (data not shown).

ErbB2, not normally expressed in adult rat hepatocytes, upregulates as primary hepatocytes adapt to culture. This ErbB forms a potent heterodimeric signaling complex with ligand-bound EGFr (9). ErbB2 kinase blockade suppresses EGF-stimulated DNA synthesis in primary hepatocytes. This raises the possibility that the increase in Akt or ERK 1/2 is related to the acquisition of ErbB2 signaling. To test this, we treated cells with a selective inhibitor of ErbB2, the tyrphostin AG879. AG879 blocks the activity of ErbB2 with a 500-fold selectivity (IC_{50} = 1 μmol/l) than to EGFr (IC_{50} > 500 μmol/l) (18). To
determine whether an inhibitor of the ErbB2 tyrosine kinase blocks the increased synthesis of either cyclin D1, total Akt, or total ERK 1/2. We cultured cells as above in the presence of varying concentrations of AG879. As illustrated in Fig. 5B, AG879 diminished the cyclin D1 synthesis, consistent with its ability to inhibit DNA synthesis in primary hepatocytes. Moreover, it also suppressed the increased synthesis of total Akt, total ERK 2, and to a lesser extent total ERK 1.

**Decreased expression of ErbB2 by siRNA blunts the increased expression of Akt and ERK 1/2.** To determine whether the increase in Akt and ErbB2 could be blocked by downregulating the expression of ErbB2, we used an siRNA approach. In preliminary studies, we found that specific siRNA’s decreased the expression of hepatocellular proteins when the transfection reagent (oligofectamine) was introduced shortly after the time of plating, but not at 24 h (data not shown). Figure 6A shows that an ErbB2-specific siRNA depressed ErbB2 expression by as much as 50–70%. The right panel shows the immunoblot results for ErbB2 at 48 h (n = 3). Figure 6B shows the immunoblot results for ErbB3 (left) and EGFr (right). Note that whereas the downregulation of ErbB2 had no affect on the expression of EGFr, it resulted in increased expression of ErbB3. This is of interest because EGF itself inhibits ErbB3 expression and we frequently see an inverse expression pattern of ErbB2 and ErbB3 in cultured hepatocytes (32).

Akt 1, 2, and 3 show divergent patterns of expression in primary hepatocytes. The total and phospho-specific Akt antibodies used in the initial studies recognize three homologous isoforms that show divergent patterns of expression in different organs and physiological states. Although all three isoforms...
have been detected in the liver, little is known about the expression of these isoforms in cultured hepatocytes. In most published studies, these isoforms are not distinguished from one another. We therefore probed immunoblots from lysates obtained at different times of culture with antibodies that recognized all three isoforms (total Akt) or the specific isoforms Akt 1, Akt 2, and Akt 3. Figure 7 shows the results of this experiment. Generally, all three isoforms were detected but the expression level and temporal expression pattern differed for the three molecules. The main isoform at the time of plating is Akt 1. This isoform increases in hepatocytes cultured at the later times (48 and 70 h). Both EGF and, to a lesser extent, insulin enhance this increase of Akt 1 by 48 h. In contrast to Akt1, Akt2 shows low-level expression that essentially remains low in cells cultured in basal medium, even with insulin. Like Akt1, Akt2 shows enhanced expression in the presence of EGF, with or without insulin at later times of culture. Finally, Akt3 shows little or no expression at the beginning of culture but EGF upregulates it, particularly at the later time (70 h). Thus the increase detected in total Akt at later culture times is partly due to increases in its Akt 2 and Akt 3 isoforms. Indeed, the ratio of Akt 1 to Akt 2 and 3 decreases as cells synthesize DNA in response to EGF stimulation.

DISCUSSION

Primary cultures of hepatocytes are widely used to study hepatocellular function and proliferation because they can be prepared in large numbers and isolated from the nonparenchymal cells that complicate in vivo analysis. The generation of hepatocytes that proliferate in vitro while maintaining their function has clinical relevance because of their potential use in cell therapies to treat liver diseases (13, 34). Unfortunately, hepatocytes from adult livers respond sluggishly to growth factors in vitro. Although they eventually synthesize DNA, they usually fail to divide. Understanding the mechanisms of cell cycle progression may yield clues that will allow researchers to expand hepatocyte populations in vitro.

Primary hepatocytes respond to EGF in serum-free medium by synthesizing DNA, but they must first traverse a prolonged lag phase (~44 h) before entering the S phase (21). However, provided that insulin remains in the medium, they become increasingly responsive to EGF with increasing time in culture (21). By the third day of culture, the overall cell population responds to exogenous EGF in an increasingly synchronized manner, as evidenced by a shorter lag phase (~20 h) and an increased DNA synthesis peak. The reasons for this enhanced responsiveness to EGF have been a focus of our attention.

The molecular events that heighten responsiveness to EGF must impinge on the synthesis of cyclin D1 immediately before the DNA synthesis restriction point. Cyclin D1 appears to be a key regulator of hepatocyte growth in culture and during liver regeneration following hepatectomy (2). Indeed, when cyclin D1 is transfected into hepatocytes, they synthesize DNA even in the absence of exogenous growth factors (1). Other signaling molecules acting between ErbB activation and cyclin D1 synthesis have been shown to play key roles in EGF-stimulated...
DNA synthesis as well. Based on the use of inhibitors and dominant negative enzyme strategies, the action of EGF requires both PI3K and MEK (3), which phosphorylate Akt and ERK 1/2, respectively. Constitutively activated Akt (22) and MEK1 (35) by themselves will increase cyclin D1 and DNA synthesis. Moreover, a newly developed chemical inhibitor of Akt (A-433654) inhibited the hepatocellular DNA synthesis in response to EGF and insulin (22). In light of our previous observations of dramatic changes in the expression of ErbB 1, 2, and 3 as hepatocytes adapt to culture, we examined the levels and phosphorylation status of Akt and ERK 1/2 at different times of culture.

We found that primary hepatocyte cultures show a surprising temporal increase in the total expression of both Akt and ERK (Figs. 1 and 2), independent of EGF stimulation, resulting in a greater level of basal (Fig. 3) and EGF-stimulable (Fig. 4) Akt and ERK activity. The increase in Akt and ERK precedes the synthesis of cyclin D1. Whereas others have noted an increase in total ERK1 between 3 and 48 h of culture (29), the increases in Akt and ERK 2 have not been documented before. Increased ERK 2 expression during culture is important because this isoform is singularly responsible for ERK-mediated cell cycle progression of hepatocytes in vitro or during liver regeneration (12). We hypothesize that elevated expression of Akt and ERK 2 during culture may prime hepatocytes to respond to exogenous EGF in a brisk and synchronized fashion.

The increase in Akt and ERK 1/2 likely occurs through a transcriptional mechanism. Indeed, the work of Boess et al. (5) complements our data and supports this idea. They characterized the mRNA expression profiles of several hepatic in vitro systems, comparing them to gene expression in liver tissue. They studied primary hepatocytes in conventional monolayer or in sandwich culture, liver slices, and rat liver cell lines...
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(BRL3A and NRL clone 9). Although not a focus of their study, their supplemental data revealed that during the initial 54-h culture period the relative expression of Akt1 and ERK 1 mRNA doubles relative to basal liver expression. This level still is only ~25% the level seen in the BRL3A and NRL clone 9 cell lines.

Little is known about the regulation of Akt or ERK 1/2 gene expression (28). Typically, increases in Akt and ERK 1/2 signaling activities are related to the activating or destabilizing influences of kinases and phosphatases, respectively, independent of gene expression. This is partly because homogeneous cell lines, like BRL3A and NRL clone 9, express Akt and ERK 1/2 at a high and consistent level, perhaps a reflection of their high rate of cell proliferation. Increased activities in the absence of changes in expression also manifest during liver regeneration following partial hepatectomy (4). Despite this, in vivo work reveals many tissue-specific or developmentally related differences in the expression of Akt (7) and ERK 1/2 mRNA (6). Indeed, hepatocytes show regulated expression of these molecules in the intact animal (36). For example, although the liver of the adult rat expresses little or no Akt 3, the fetal liver abundantly expresses this isoform compared with other tissues (7). In addition, a comparative microarray analysis of genes upregulated during liver development vs. posthepatectomy regeneration revealed increased expression of Akt1 and 3 during development but not regeneration (25). Similarly, early work showed that the mRNA transcripts for ERK 1 and ERK 2, but not ERK 3, were markedly upregulated in fetal compared with adult rat liver (6).

An analysis of the promoter for the human Akt1 reveals multiple potential binding sites for transcription factors, such as CREB, AP-1, GC, NF-kB, and STAT3 (27). STAT3 has the greatest number of binding sites in the human Akt1 promoter (27). It has 12 putative STAT3 binding sites, 4 of which have been proven to bind to STAT3 in vivo and in vitro. Indeed, blocking STAT3 by antisense or genetic knockdown decreased Akt1 expression (27, 39). In this paper, we have shown that during culture hepatocytes upregulate Akt expression in an ErbB2- and PI3K-dependent manner (Figs. 5 and 6). During culture, hepatocytes also express de novo ErbB2, acquiring the ability to form ErbB2-EGFr heterodimers that complement EGFr-EGFr homodimers. Although both dimers are equally potent in activating the Ras/mitogen-activated protein kinase pathway, ErbB2-EGFr heterodimers are superior to EGFr-EGFr homodimers in PI3K activation, which is required for Akt hyperexpression (41). ErbB2 activation also frequently correlates with constitutively active STAT3. In transformed human epithelial cells, TGF-α constitutively activates STAT3 via an ErbB-1/-2 heterodimer complex that requires the ErbB2 kinase activity (11). Importantly, EGFr homodimers by themselves did not activate STAT3. Since the Akt 1 promoter has numerous STAT3 binding sites (27), the emergence of ErbB2-EGFr heterodimers in primary hepatocytes likely increases STAT3 activation (Fig. 8A), driving Akt 1 expression.

Akt and ERK 1/2 hyperexpression is also seen in many cancers, including hepatocellular cancer (26, 24), colon cancer (10), breast cancer (23), human acute leukemias (17), and follicular and papillary thyroid cancer (16, 30, 33). When Sprague-Dawley rats were administered diethylnitrosamine for up to 3 mo, they developed hepatomas (26). These hepatomas showed elevations in Akt and ERK 1/2 levels and activities that were associated with cyclin D1 upregulation. Others have reported similar findings in patients who had poorly differentiated-type hepatocellular carcinomas or intrahepatic metastases (19). In these studies, increased Akt and ERK 1/2 protein expression and activity frequently correlated with increased gene expression. Although Akt1 is elevated in some human tumors, Akt2 appears to be the major Akt isoform at work in human tumorigenesis. An analysis of Akt 1 and 2 expression in 56 patients with hepatocellular carcinoma revealed an upregulation of Akt 2, but not Akt 1, in 21 patients (37, 38). Our work suggests that cultured adult hepatocytes, fetal hepatocytes in vivo, and hepatoma cells each upregulate the expression of Akt and ERK 1/2. This upregulation presumably enables them to resist apoptosis, to enhance their proliferative ability, and to alter cell-cell polarity and integrity.

In normal nontransformed cells, Akt1 and Akt 2 may have opposite roles (Fig. 8B). During progression through the various phases of the cell cycle, nontransformed cells synthesize p21, the CDK and cyclin inhibitory protein, twice. They synthesize it once in G1 before entry into the S phase and then again in G2 before entry into the M phase. p21 must be degraded for cells to progress into the S and M phases to proceed. Its presence provides cells with a “pause” period to shuffle in and out the various enzymes required to complete the S and M phases without error. The degradation of p21 is mediated in part by Akt 1 and Akt2. Akt 1 phosphorylates p21 on threonine 145 (14). This causes p21 to exit the nucleus and ultimately to be degraded by ubiquitination. In contrast, Akt 2 cannot phosphorylate p21. However, Akt 2 can bind to p21 and

Fig. 8. Proposed models for Akt1 and p21 modulation in cultured rat hepatocytes. A: induction of ErbB2 leads to the formation of ErbB2-ErbB1 heterodimers. This leads to STAT3 activation, dimerization, and translocation to the nucleus, where it drives Akt1 expression (A role for Stat3 in Akt 2 transcription is not clear). B: synthesis of p21 before and after S phase implements a G1 and a G2 pause before the S and M phases of the cell cycle, respectively. Akt-1 activation by EGFr leads to p21 phosphorylation, nuclear export, and ultimate degradation. Akt-2 cannot phosphorylate p21, but it can bind it, blocking p21 phosphorylation and degradation. Because the induction of Akt2 is delayed relative to Akt1, the S phase can progress. However, because EGFr induces Akt-2, p21 synthesized during G2 is stable, favoring the formation of polyploid cells that do not divide.

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block Akt 1 phosphorylation of p21. p21 has been shown to accumulate at higher and higher levels in EGF-stimulated hepatocytes after they synthesize DNA (15). Since Akt 2 expression manifests exclusively in EGF-treated hepatocytes, we hypothesize that the induction of Akt 2 is responsible not only for the increase in p21, but also for the failure of hepatocytes to progress through the M phase (14).

In summary, we have shown that as hepatocytes adapt to culture they spontaneously upregulate Akt and ERK 1/2. In this sense, they begin to resemble less differentiated transformed cell lines. This coincides with an upregulation of ErbB2, which is also expressed in fetal hepatocytes and in a sizable subtraction of hepatomas, but not in normal adult hepatocytes. These adaptations may explain why primary cultures and transformed hepatocytes become more responsive to exogenous growth factors during culture. Because primary hepatocytes are easy to isolate and culture, they provide a model to examine the transcriptional mechanisms that alter Akt and ERK 1/2 expression.

This should be useful in defining the mechanisms that regulate Akt and ERK 1/2 gene expression in the liver and understanding the adaptational response that allows primary cultures of hepatocytes to synthesize DNA and survive in culture but fail to divide and proliferate.

**GRANTS**

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