Ribosomal Protein S6 Phosphorylation and Function during Late Gestation Liver Development in the Rat

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The phosphorylation of ribosomal protein S6 is thought to be required for biosynthesis of the cell's translational apparatus, a critical component of cell growth and proliferation. We have studied the signal transduction pathways involved in hepatic S6 phosphorylation during late gestation in the rat. This is a period during which hepatocytes show a high rate of proliferation that is, at least in part, independent of mitogenic signaling pathways that are operative in mature hepatocytes. Our initial studies demonstrated that there was low basal activity of two S6 kinases in liver, S6K1 and S6K2, on embryonic day 19 (2 days preterm). In addition, insulin- and growth factor-mediated S6K1 and S6K2 activation was markedly attenuated compared with that in adult liver. Nonetheless, two-dimensional gel electrophoresis demonstrated that fetal liver S6 itself was highly phosphorylated. To characterize the fetal hepatocyte pathway for S6 phosphorylation, we went on to study the sensitivity of hepatocyte proliferation to the S6 kinase inhibitor rapamycin. Unexpectedly, administration of rapamycin to embryonic day 19 fetuses in situ did not affect hepatocyte DNA synthesis. This resistance to the growth inhibitory effect of rapamycin occurred even though S6K1 and S6K2 were inhibited. Furthermore, fetal hepatocyte proliferation was sustained even though rapamycin administration resulted in the dephosphorylation of ribosomal protein S6. In contrast, rapamycin blocked hepatic DNA synthesis in adult rats following partial hepatectomy coincident with S6 dephosphorylation. We conclude that hepatocyte proliferation in the late gestation fetus is supported by a rapamycin-resistant mechanism that can function independently of ribosomal protein S6 phosphorylation.

During the last 3 days of gestation in the rat, fetal weight more than triples, with liver weight increasing proportionately (1). This rate of hepatic growth slows markedly at term; and although there is a restoration of vigorous growth in the neonatal period, the extraordinary rate of growth seen in late gestation is never again attained. We have shown previously that late fetal and neonatal development is associated with a distinctive pattern of hepatocyte proliferation during the perinatal period (2, 3). Our work has focused on the developmentally regulated signal transduction mechanisms that control hepatocyte proliferation during late gestation and beyond. Activation of hepatocyte proliferation following partial hepatectomy in the adult rat has been shown to correlate with the intracellular activation of several interacting protein kinase cascades (Reviewed in Ref. 4). One of the signaling cascades that is activated after partial hepatectomy leads to the phosphorylation of ribosomal protein S6 (5) and is characterized by its sensitivity to the immunosuppressant rapamycin (6). Studies have established that the target of rapamycin is mTOR (mammalian target of rapamycin; also named FRAP/RAFT1) (7). Rapamycin forms a complex with the immunophilin peptidylprolyl isomerase FKBP12, which binds to mTOR and inhibits its ability to phosphorylate substrates such as S6 kinase and 4E-BP1. Phosphorylation of ribosomal protein S6, located in the 40 S subunit, is thought to be required for the translation of a subset of mRNAs that contain a 5′-oligopyrimidine tract at their transcriptional start sites (5′-terminal oligopyrimidine (5′-TOP) mRNAs) (8). 5′-TOP mRNAs may number as few as 100–200, but they can account for 20–30% of total cellular mRNA. They encode many of the components of the translational apparatus, including ribosomal proteins and elongation factors that are necessary for cell cycle progression. Recently, Volarevic et al. (9) developed a system for the conditional deletion of protein S6 in mouse liver. They made the unexpected observation that loss of S6 had no effect on hepatocyte growth in response to refeeding after a fast, but that hepatocyte proliferation in response to partial hepatectomy was completely abolished. This led these authors to conclude that abrogation of 40 S ribosome biogenesis may induce checkpoint control that prevents cell cycle progression.

Thomas and co-workers (10) first purified and characterized the kinase activity responsible for S6 phosphorylation in mitogen-stimulated Swiss mouse 3T3 cells more than 10 years ago. Subsequent purification and cloning showed that two isoforms of this original kinase are produced from the same transcript (reviewed by Dufner and Thomas (8)). Null mutation in mice for the p70/p85 S6 kinases, which we will refer to as S6K1, is associated with a proportional 20% decrease in somatic growth (11). Mouse embryo fibroblasts derived from these animals showed diminished (but not absent) S6 phosphorylation, leading to the discovery of another physiological S6 kinase. This enzyme, a mitogen-responsive S6K1 homolog designated S6K2, has since been identified by other laboratories (12, 13). It has recently been determined that S6K1 and S6K2 are regulated similarly by effectors of the phosphatidylinositol 3-kinase pathway, including Cdc42, Rac, protein kinase Cζ, and phospholipid-dependent kinase-1 (14).

* This work was supported by National Institutes of Health Grants HD24455, HD11343, and HD35831 and by the Rhode Island Hospital Department of Pediatrics Research Endowment. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: 5′-TOP, 5′-terminal oligopyrimidine; S6K, S6 kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; EGF, epidermal growth factor; BrdUrd, 5-bromo-2′-deoxyuridine; E19, embryonic day 19.
Our prior studies on mitogenic signaling during liver development in the rat have suggested that the well-characterized pathways that mediate growth factor-induced mitogenesis in adult rat hepatocytes are not operative in the late gestation fetus. Our results have indicated an alternative means for the up-regulation of c-myc via RNA stabilization (15), uncoupling of the prototypical MAPK pathway that terminates in ERK1/2 (16, 17), and post-transcriptional induction of cyclin D1 (18). Given the established role of S6 phosphorylation in hepatoctye proliferation, we undertook a study of the hepatic signal transduction pathways terminating in ribosomal protein S6 phosphorylation in fetal and adult rats.

**EXPERIMENTAL PROCEDURES**

**Materials**—Prior to the experiment, a study was made to determine the effect of the inhibition of 56 kinase activity in 30 fetal rat liver homogenates. The results were expressed as a percentage of the control value and were determined in triplicate. The values are shown in Table I. The statistical significance of the differences was determined using a paired t-test (19).

**Preparation of Liver Homogenates and Cell Extracts**—Liver homogenates were prepared from fetal rat livers by a two-step collagenase digestion (19). Hepatocyte suspensions were isolated as described previously (20). Fetal rat hepatocytes were isolated by collagenase digestion as described previously (21). The samples were analyzed in duplicate and the results were averaged. The statistical significance of the differences was determined using a paired t-test (19).

**RESULTS**

**Hepatic S6K1 Activity in E19 versus Adult Rats**—Our initial experiments were aimed at determining the levels of basal and insulin-stimulated S6K1 activities in E19 and adult rat livers.
The finding that basal S6K1 activity was low in our hands, we analyzed preparations from a fasted adult rat—without any period of treatment. We interpreted this result as indicating that rapamycin had no stimulatory effect of either agent 

Comparison of Rapamycin Sensitivity in Fetal versus Adult Hepatocytes in Primary Culture—Having seen that rapamycin administered in vivo to E19 rats did not effect DNA synthesis, we performed a parallel in vitro experiment. We compared the rapamycin sensitivity of fetal versus adult hepatocyte DNA synthesis using primary cultures (Fig. 5). Fetal hepatocytes were studied under our usual defined, growth factor-free conditions. Adult hepatocytes were treated with EGF plus insulin to stimulate DNA synthesis. In contrast to our in vivo findings, rapamycin inhibited DNA synthesis in fetal and adult hepatocytes with a similar dose dependence. We reasoned that the discrepancy between the in vivo and in vitro results might be accounted for by a change in the fetal cells that was a result of isolation and culture. To address this, we examined the regulation of S6K1 in fetal hepatocyte primary cultures. The results showed inactivation of S6K1 activity at 5 and 24 h after rapamycin injection in a separate experiment, we confirmed S6K1 inactivation in rapamycin-injected fetuses at 24 h while also confirming that vehicle injection did not produce a decrease in activity. Despite the potent inhibitory effect of rapamycin on S6K1, there was no discernible effect on in vivo DNA synthesis after 24 h, assessed as immunohistochemical staining for BrdUrd incorporation. We interpreted this result as indicating that rapamycin had no cell cycle inhibitory effect on late gestation fetal rat hepatocytes. This was based on the reasoning that any period of inhibition during the 24-h experimental duration would have resulted in a decreased BrdUrd labeling index.

S6K2 Activity in Fetal and Adult Rat Livers—Several groups have recently reported the cloning of a second S6 kinase (S6K2) that has a 70% overall amino acid identity to S6K1 (12, 13, 26, 27). We considered the possibility that S6K2 could represent an alternative pathway mediating rapamycin-resistant S6 phosphorylation in fetal liver. To examine this, we first looked at S6K2 activity in E19 and adult liver homogenates. The results
showed S6K2 activity in E19 livers that was approximately double that seen in adult livers (Fig. 6). In vivo injection of insulin or EGF into E19 fetuses did not increase hepatic S6K2 activity (Fig. 6), a result similar to our findings for S6K1. Differences between S6K2 content in fetal versus adult livers (Fig. 6, inset) were minimal and could not account for differences in basal S6K2 activity.

Consideration of S6K2 as a candidate rapamycin-resistant S6 kinase led us to compare the relative sensitivities of S6K1 and S6K2 in cultured fetal hepatocytes. The hepatocytes were first treated with insulin and EGF to stimulate S6 kinase activity and then exposed to varying doses of rapamycin (0–6 nM). The results showed that S6K2 was somewhat more resistant to rapamycin inhibition than S6K1 (Fig. 7). We also measured attenuation of basal (unstimulated) S6 kinase activity in fetal hepatocytes and found that S6K2 activity was again less sensitive to rapamycin than basal S6K1 activity (45% inhibition of S6K2 versus 80% inhibition of S6K1 activities at a dose of 2 nM rapamycin) (data not shown). A parallel experiment on cultured adult rat hepatocytes gave similar rapamycin sensitivities for S6K1 and S6K2 (data not shown). In addition, we compared the in vivo sensitivity of S6K1 and S6K2 to rapamycin by injecting E19 rats with rapamycin (2.5 μg/g of body weight) and measuring S6K1 and S6K2 activities in total liver homogenates. A maximal inhibitory dose of rapamycin produced similar and marked degrees of inhibition of S6K1 and S6K2.

**Effect of Rapamycin on Ribosomal Protein S6 Phosphorylation in Vivo**—Our findings to this point led us to hypothesize that the phosphorylation of ribosomal protein S6, which is thought to be essential for the translation of 5’-TOP mRNAs and for hepatocyte proliferation, might be maintained by a rapamycin-resistant mechanism in fetal liver. To confirm this, we analyzed liver homogenates for S6 protein content and

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**FIG. 2.** S6 phosphorylation in fetal and adult rat livers. Two-dimensional gel electrophoresis was performed using 40 S ribosomal proteins prepared from livers taken from an uninjected adult rat (Adult Control), an adult rat sacrificed 15 min after injection of 2.5 μg of insulin/g of body weight (Adult, Insulin), and a pooled litter of uninjected E19 rats (Fetal, E19). The gels were stained with Coomassie Blue and photographed. The arrows identify the position of non-phosphorylated S6.

**FIG. 3.** S6K1 activity and rapamycin sensitivity of DNA synthesis in adult rat liver after partial hepatectomy. Adult male rats were subjected to either a sham operation (white bars; n = 1 per time point) or two-thirds partial hepatectomy (black bars; mean for n = 2 per time point). Livers were collected at 6, 24, 48, and 72 h after surgery. The graph illustrates S6K1 activity in the sham-operated and partial hepatectomy animals at all four time points. The photomicrographs show representative immunohistochemical staining for BrdUrd incorporation in control and rapamycin-injected animals 24 h after partial hepatectomy.

**FIG. 4.** Effect of rapamycin on S6K1 activity and DNA synthesis in fetal rat liver. Rapamycin (5 μg/fetus) or vehicle (Control) was administered to E19 fetuses in situ by intraperitoneal injection. Either fetal livers were harvested at 15 min after injection; or the fetuses were replaced, the laparotomy incision was closed, and gestation was allowed to continue for 5 or 24 h. A shows S6K1 activity as determined by the immunoprecipitation kinase assay. B shows representative photomicrographs of BrdUrd immunohistochemical staining of liver sections from fetuses injected with rapamycin along with BrdUrd (50 μg/fetus) 24 h before collecting the livers. C shows nuclear labeling indices for BrdUrd (BrdU) incorporation over 24 h. For A and C, data are shown as means ± S.D. for each group (n = 4 per group).
concentrations ranging from 0 to 200 nM. The fetal hepatocytes were exposed to rapamycin at concentrations ranging from 0 to 200 nM. The fetal hepatocytes were maintained under usual basal conditions. The adult hepatocytes were exposed to EGF plus insulin (100 ng/ml each) starting at time 0. BrdUrd was included in all cultures from 16 to 40 h, at which point the cells were fixed and stained for BrdUrd (BrdU) incorporation. Between 150 and 250 cells were counted to derive a nuclear labeling index. Results (one analysis per condition) are expressed as percent of control (no rapamycin). B, E19 hepatocytes were exposed to 100 ng/ml EGF, insulin (Ins), or a combination of the two for 5 min. Cells were lysed and assayed for S6K1 activity. Results are shown as means ± S.D. for triplicate wells per condition.

Our initial studies led to the unexpected finding that basal hepatic S6K1 activity in the late gestation fetal rat is lower than in adult animals. Given our prior observations demonstrating that late gestation fetal hepatocyte proliferation in vivo lies downstream from mechanisms that control S6 phosphorylation.

DISCUSSION

For a cell to proliferate, it must up-regulate the biosynthetic apparatus needed to support cell growth. Studies have shown that the mRNA transcripts for all ribosomal proteins and protein synthesis elongation factors contain an unusual oligopyrimidine tract at their transcriptional start sites, commonly referred to as a 5'-TOP. The translation of these mRNAs has been shown to be dependent on S6K1 activation, presumably mediated by an increase in S6 phosphorylation (28). Studies have demonstrated that 5'-TOP mRNA translation is selectively inhibited by the immunosuppressant rapamycin, a potent inhibitor of S6 kinase activity (29).

Although equal amounts of protein were loaded onto each lane (confirmed by Coomassie Blue staining), we consistently observed variation in the amount of total S6 ribosomal protein in the fetal samples. In addition to the results shown in Fig. 8, we found similar variability in two other experiments (data not shown). Although this variability in S6 content might be accounted for by position in the uterus or timing of fetal sampling during cesarean section, we were not able to confirm a simple explanation for this finding. Nevertheless, in all of these experiments, the amount of total S6 ribosomal protein was higher in adult liver than in fetal liver. Although there was also some variability in adult liver S6 content, this could be explained by a stimulatory effect of partial hepatectomy on ribosome biogenesis.

Using phospho-specific antibodies, we examined S6 phosphorylation state (Fig. 8). Under basal conditions, phosphorylation of S6 at Ser235/Ser236, corrected for S6 content, was higher in fetal liver than in liver from a fed adult rat. Although not apparent in Fig. 8, autoradiograms exposed for longer periods demonstrated the same for Ser240/Ser244. These results were consistent with two-dimensional gel electrophoresis data. As expected, we found a marked increase in the phosphorylation of S6 after the two-thirds partial hepatectomy. This stimulation of S6 phosphorylation during adult liver regeneration was potentially inhibited by administration of rapamycin at the time of partial hepatectomy. However, analysis of samples from vehicle-injected versus rapamycin-injected fetuses yielded an unexpected result. S6 phosphorylation at Ser235/Ser236 and Ser240/Ser244 was markedly reduced at both 6 and 24 h after in vivo injection. This result, confirmed in several experiments, led to the conclusion that the rapamycin resistance of fetal hepatocyte proliferation in vivo lies downstream from mechanisms that control S6 phosphorylation.
This resulted in an increase in S6K1 activity as well as a decrease in hepatocytes to enter the cell cycle, a two-thirds hepatectomy. We employed a commonly used method for inducing adult liver proliferation even though S6 phosphorylation was markedly decreased. These results contrasted with those in adult animals.

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We considered S6K2 to be a candidate to account for the phosphorylation of S6 in fetal liver. Basal S6K2 activity was ~2-fold higher in E19 liver than in adult liver, consistent with a role for S6K2 in maintaining S6 phosphorylation in vivo. However, the relatively low S6K2 specific activity that we measured is entirely consistent with the possibility of an alternative mechanism for S6 hyperphosphorylation in fetal liver. One such explanation would be the existence of an alternative S6 kinase. Another possibility is a lower level of S6 phosphatase activity in fetal liver ribosomes. Studies by Olivier et al. (22) indicate that the catalytic subunit of protein phosphatase-1 mediates the dephosphorylation of ribosomal protein S6. Low protein phosphatase-1 expression, decreased localization to ribosomes through decreased expression of associated subunits, or inhibition of ribosomal protein phosphatase-1 activity could serve to maintain S6 in a hyperphosphorylated state, even in the absence of excess kinase activity in fetal liver.

To dissect the pathways accounting for the fetal liver S6 hyperphosphorylation, we administered rapamycin to intact animals. These studies were undertaken to test the hypothesis that fetal hepatocytes utilize an alternative, rapamycin-resistant pathway for S6 phosphorylation. Indeed, we found that rapamycin administration to E19 rats in situ did not inhibit DNA synthesis, despite potent inhibition of S6K1 and S6K2. This led to analysis for the sensitivity of S6 phosphorylation to rapamycin and the unexpected finding that fetal hepatocyte proliferation in vivo could proceed following rapamycin administration even though S6 phosphorylation was markedly decreased. These results contrasted with those in adult animals.

We employed a commonly used method for inducing adult hepatocytes to enter the cell cycle, a two-thirds hepatectomy (30). This resulted in an increase in S6K1 activity as well as a marked increase in S6 phosphorylation. As anticipated, rapamycin effectively blocked in vivo S6 phosphorylation and hepatocyte DNA synthesis following partial hepatectomy. These results are consistent with the recently published report by Jiang et al. (25) showing that rapamycin blocks activation of p70S6K in a dose-dependent manner and inhibits recovery of liver mass after a partial hepatectomy.

There are precedents for the dissociation of S6 phosphorylation and cell proliferation. Kawasome et al. (31) performed studies that point to the existence of S6-independent mechanisms for 5'-TOP mRNA translation. Their S6K1 knockout mouse embryonic stem cells did not show any S6 phosphorylation in vivo. However, 40% of total cellular eukaryotic elongation factor-1α mRNA, a 5'-TOP mRNA, was detected in polysomal fractions, indicating active eukaryotic elongation factor-1α translation. Prior to these studies, S6K1 activation was generally thought to be essential for G1/S transition. This was based on the work of Lane et al. (32), who showed that microinjection of antibodies against S6K1 prevented the entry of cells into the cell cycle. Kawasome et al. (31) found that the S6K1 null embryonic stem cells proliferated well, albeit slower than parental cells, indicating that S6K1 is not essential for G1/S transition. Interestingly, rapamycin inhibited the proliferation of the S6K1 null cells to the same extent as the parental cells, indicating that events independent of S6K1 activation, but probably requiring mTOR activation, are critical for cell proliferation.

This uncoupling of S6K1 activity and proliferation had previously been shown in activated T cells that were already cycling (33) as well as in erythroleukemic cell lines (34). Slavik et al. (35) recently found that p70S6K activation can be uncoupled from proliferation in both freshly isolated CD8+ T cells and CD8+ T cell clones, demonstrating for the first time rapamycin resistance in resting, nontransformed T cells.

In studies by Withers et al. (36) employing Swiss 3T3 cells, p70S6K activation was dissociated from DNA synthesis. Although rapamycin could inhibit both bombesin-mediated p70S6K activation and [3H]thymidine incorporation, the combination of bombesin plus insulin was able to stimulate DNA synthesis and cell cycle progression in the presence of rapamycin despite inhibition of p70S6K activity. These results were interpreted as indicating the presence of a rapamycin-insensitive mitogenic pathway in Swiss 3T3 cells.

Another conclusion from our studies is that the signaling phenotype of fetal hepatocytes is altered when the cells are removed from their in vivo milieu. In vivo, E19 hepatocytes showed a resistance to the S6K-activating effects of insulin and EGF and to the inhibitory effect of rapamycin on DNA synthesis. In contrast, primary cultures of E19 hepatocytes showed an intact response to hormonal activation of S6K1 and S6K2 as well as heightened sensitivity to the growth inhibitory effects of rapamycin. A similar in vivo to in vitro change in mitogenic signaling was seen in our studies on the developmental regulation of hepatic ERK1 and ERK2 (17). We have not established a mechanism for the uncoupling of proximal signaling from S6 kinase activation in developing liver. However, recent studies indicate a similar uncoupling of Akt activation and low levels of key proximal signaling molecules, including insulin receptor substrate-1 and Gab2.

As was the case for signaling to S6 kinases, fetal hepatocyte rapamycin resistance as measured by DNA synthesis was lost when hepatocytes were isolated and cultured. We observed an IC50 for both fetal and adult cells that was near 20 nM. The dose of rapamycin that we chose for our in vivo experiments was

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designed to achieve micromolar concentrations. Studies on “rapamycin-resistant” cancer cell lines have shown an IC50 in the micromolar range (37).

In summary, our results point to an alternative, S6 phosphorylation-independent pathway that supports fetal hepatocyte proliferation during late gestation. Our data indicate that phosphorylation of ribosomal protein S6 is not necessary for fetal hepatocyte proliferation in vivo. Given that translation of 5′-TOP mRNAs is thought to be essential for cellular proliferation, we conclude that fetal hepatocytes possess an alternative pathway for up-regulation of the translation of these genes. We found no evidence that this pathway is operative in adult hepatocytes. This conclusion is consistent with our previous studies (15–17) in that it suggests that mechanisms that mediate the proliferation of late gestation fetal hepatocytes are distinct from those that mediate the proliferation of mature hepatocytes in vivo. Finally, the rapamycin resistance displayed by a spectrum of cancer cells (37) suggests that the mechanism for phospho-S6-independent hepatocyte proliferation in the late gestation fetal rat may prove relevant to liver carcinogenesis.

Acknowledgments—We greatly appreciate the assistance of Theresa Bienieki and Yang-Si Ou in the performance of these studies. We also thank Thomas Radimerski for helpful advice on the two-dimensional gel electrophoresis of ribosomal proteins.

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