Regulation and role of p21 and p27 cyclin-dependent kinase inhibitors during hepatocyte differentiation and growth

Gennady P. Ilyin, Denise Glaise, David Gilot, Georges Baffet, and Christiane Guguen-Guillouzo

Institut National de la Santé et de la Recherche Médicale U522, Hôpital Pontchaillou, 35033 Rennes, France

Submitted 29 July 2002; accepted in final form 13 March 2003

Am J Physiol Gastrointest Liver Physiol 285: G115–G127, 2003. First published March 19, 2003; 10.1152/ajpgi.00309.2002.—Unlike a large number of cell types that undergo terminal differentiation associated with permanent withdrawal from the cell cycle, mature quiescent hepatocytes retain high proliferative potential. We report here a specific behavior of members of the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors during development of the rat liver and proliferation of normal hepatocytes. Expression of p21, p27, and p57 transcripts and proteins was downregulated during the differentiation process to low or undetectable levels in adult liver. In contrast to p27, p21 protein increased in a mitogen-dependent manner in isolated hepatocytes and its expression pattern correlated with that of cyclin D1. In proliferating hepatocytes, p21 was predominantly associated with cyclin D1, these proteins were colocalized in the nucleus and p21-associated retinoblastoma protein (pRb) kinase activity increased in parallel with that of cyclin D1. Overexpression of p21 in mitogen-stimulated hepatocytes reduced DNA synthesis. In contrast, inhibition of p21 expression by antisense or small interfering RNAs oligonucleotides accelerated S phase entry. Finally, expression of p21 and cyclin D1, but not p27 proteins was regulated by MAPK kinase/extracellular signal-regulated kinase and phosphatidylinositol 3-kinase-ferric-reductase/growth factor signaling pathways. In conclusion, these results demonstrate a specific and differential regulation of p21 and p27 during hepatocyte differentiation and proliferation that may contribute to the control of quiescent differentiated hepatic cell proliferating activity.

DEVELOPMENT OF THE LIVER IN mammals is a long-lasting, multistep process involving hepatic specification of foregut endodermal cells, expansion of primitive hepatocytes, hepatoblasts, organogenesis during late fetal life, and finally differentiation of the postnatal liver to acquire a full spectrum of adult hepatic functions. The neonatal period represents one of the critical events of liver development. Indeed, dramatic activation of several liver-specific genes, especially those encoding enzymes involved in gluconeogenesis, occurs at the time of birth or directly after birth (24, 48). Another important period, i.e., maturation of postnatal liver, comprises gradual acquisition of the adult phenotype accompanied by a loss of hepatocyte replicative activity. In normal adult liver, almost all hepatocytes remain in the quiescent state; however, in contrast to a large number of cell types that undergo a terminal differentiation coupled to irreversible cell cycle arrest, they retain a remarkably high proliferative capacity. After partial removal of the organ or acute injury, the growth-arrested hepatocytes can rapidly and synchronously enter the cell cycle (16, 43). The regeneration response of liver can be reproduced in vitro. Isolated rat hepatocytes stimulated with EGF or hepatocyte growth factor enter and progress through the cell cycle (37). It has been previously established that hepatocytes undergo G0/G1 transition during cell isolation and are able to progress in culture from early to mid-late G1 phase regardless of mitogen stimulation but remain blocked at this stage. The addition of growth factors is necessary to trigger G1/S transition and completion of the cell cycle. Cell cycle progression of hepatocytes in vitro-like in vivo models is accompanied by significant and coordinate changes of expression of a large number of genes including oncogenes, transcription factors, and cell cycle-related genes (37, 60).

It is now well established that the control of cell cycle is provided by sequential assembly, activation, and destruction of specific protein complexes, containing cyclins and cyclin-dependent kinases (Cdk) partners. The D-type cyclins and their catalytic subunits Cdk4 and Cdk6 play a key role in cell progression through G1 phase. Both synthesis of D cyclins and assembly in catalytic active complexes is a mitogen-dependent process starting from mid-G1 phase (55). Cyclin-Cdk complex assembly and enzymatic activity are regulated by a number of small proteins termed Cdk inhibitors. To date, two different families of Cdk inhibitors have been described in mammalian cells that differ in structure, mechanism of inhibition, and Cdk target specificity. The Ink family of Cdk inhibitors includes the tumor suppressor protein p16Ink4a, as well as p15Ink4b, p18Ink4c,
and p19Ink4d, that appear to specifically target the G1 phase cyclin D-Cdk4/Cdk6 complexes (56, 57). The structurally and functionally distinct Cip/Kip family comprises three proteins: p21 (also known as Cip1, Waf1, Sdi1) (14, 25, 45, 65), p27Kip1 (50, 63), and p57Kip2 (35, 41). These proteins are able to bind and inhibit with different efficacy the activity of most cyclin/Cdk complexes including Cdk2, Cdk3, Cdk4, and Cdk6 (26, 41).

A wide variety of environmental signals can regulate expression and activity of Cdk inhibitors. Interestingly, either growth arrest resulting from DNA damage, cell senescence, and terminal differentiation or cell cycle entry and progression after stimulation with growth factors were accompanied by p21 gene activation (39, 45, 47, 56) through various transcription factors including p53 (14, 39). Beyond its role as a Cdk inhibitor, at low stoichiometric concentrations, p21 may act as an assembly factor for active cyclin D-Cdk4/6 complexes and could potentially function as an activator of these kinases (7, 34, 57). In contrast to p21, expression of p27 protein generally declines in several cell types in response to mitogenic stimulation (1, 47). Furthermore, inhibitory activity of this protein increases by different antimitogenic signals, such as TGF-β or by contact inhibition (49, 51).

Several reports (11, 21, 39, 41, 56) highlight the important difference that exists between cell types concerning regulation of these Cdk inhibitors, expression, and role during development and differentiation. Particularly, these proteins are involved in coordinate regulation of cell proliferation and differentiation and maintenance of terminally differentiated cells in quiescent state (66). Because of the high proliferative potential of mature hepatocytes at the adult stage, a specific regulation of Cdk inhibitors is intended. Several prior studies (3–5, 12, 30, 33, 42, 52, 61) have documented the pattern of Cdk inhibitor expression in proliferating hepatocytes including p21, p27, and p57. However, although some investigators demonstrated that p21 decreased in regenerating rat liver (61), others reported its upregulation during cell cycle progression of rat hepatocytes in vivo (5, 30, 33). In addition, most reports described expression of Cdk inhibitors in vivo during regeneration of rat or mouse liver. A lack of immunohistochemical analysis could not fully support the conclusion that observed patterns of Cdk inhibitor expression in regenerating liver are fully derived from hepatocytes. Only a very limited number of reports documented patterns of p21 and p27 expression in hepatocytes during rat liver regeneration by immunocytochemistry (30, 52). Although a few reports described some aspects of Cdk inhibitor expression in primary mouse and rat hepatocytes in pure culture (2, 42), the functional role of these proteins in proliferating cells and their regulation by different signal transduction pathways have not yet been investigated.

In this study, to specify the mechanisms that govern the differentiation process of hepatocytes associated with their high proliferative capacity at adult stage and to understand the specific behavior of mitogen-stimulated hepatocytes, we carried out a highly detailed analysis of p21 and p27 expression, association with cyclin-Cdk complexes, regulation, and functional role during development of rat liver and in isolated proliferating hepatocytes.

**MATERIALS AND METHODS**

**Chemicals.** [3H]methylthymidine (5 Ci/mmol) was obtained from Amersham (Les Ulis, France). MEK inhibitor U-0126 and recombinant human EGF were from Promega (Charbonnieres, France). PI3-kinase inhibitor LY-294002 and rapamycin were purchased from Sigma (St. Louis, MO).

**Animals.** Pregnant and normal female Sprague-Dawley rats (weight, 180–200 g) were obtained from Charles River (France). Breeding was done by placing female rats with males of the same strain overnight, and noon of the next day was considered 0.5 days postcoitum. On the appropriate day of gestation, rats were anesthetized, and embryos were removed and their livers were minced and washed briefly with PBS to reduce the number of hemopoietic cells. A partial (two-thirds) hepatectomy was performed as previously described by using female Sprague-Dawley rats (38). The liver samples were minced, frozen in liquid nitrogen, and kept at –80°C until further processing.

**Cell isolation and culture.** Hepatocytes from adult male Sprague-Dawley rats were isolated by a two-step collagenase perfusion procedure. Hepatocytes were seeded in a mixture of 75% MEM and 25% medium 199, supplemented with 10% FCS and (per ml): 100 IU penicillin, 100 mg streptomycin sulfate, 1 mg bovine serum albumin, and 5 μg bovine insulin. After cell attachment (4 h later), the medium was renewed with the same medium deprived of FCS and supplemented with EGF (50 ng/ml), pyruvate (20 mM), and 1.4 × 10−8 M hydrocortisone hemisuccinate or only with hydrocortisone hemisuccinate. It was changed every day thereafter. At indicated times, rapamycin, LY-294002, or U-0126 dissolved in DMSO or only DMSO were added to hepatocyte cultures. Hepatocytes were preincubated with inhibitors or solvent for 1 h before stimulating with EGF.

**[3H]Thymidine and 5-bromo-2-deoxyuridine incorporation.** DNA synthesis in primary culture of hepatocytes was assessed by measuring [3H]thymidine or 5-bromo-2-deoxyuridine (BrdU) incorporation. Cells were pulsed with 2 μCi/ml [3H]methylthymidine for 12–24 h. Radiolabeled cells were washed, scraped, sonicated, and aliquots were taken for protein concentration determination. DNA was precipitated with 15% trichloroacetic acid, washed with 5 and 10% trichloroacetic acid, and dissolved in formic acid. Incorporated radioactivity was detected by liquid scintillation counting. Labeling with BrdU was performed by using a cell proliferation kit (Amersham) according to the manufacturer’s instructions. Labeling reagent was added to EGF-stimulated hepatocytes for the last 24 h that corresponds to 48–72 h of culture.

**RNA isolation and Northern blot analysis.** Total RNA was extracted by the thiocyanate guanidium procedure or by using RNA B reagent (Bioprobe Systems) following manufacturer’s instructions. RNA (10–20 μg) was separated by electrophoresis through 1.2% agarose gel in 2.2 M formaldehyde, 20 mM MOPS (pH 7.0), 10 mM sodium acetate, and 1 mM EDTA and transferred onto a nylon membrane (Hybond N+; Amersham) by capillary blotting. The membranes were prehybridized at 65°C in 3× SSC, 50 mM sodium phosphate buffer (pH 6.8), 5× Denhardt’s solution, 1% sodium dodecyl sulfate, 5% polyethylene glycol (8,000 molecular wt; Sigma), and 200 μg/ml sonicated herring sperm DNA. Hybridization...
was carried out at 65°C for 16–24 h in the presence of
32P-labeled DNA probe produced by using the Rediprime
DNA labeling system (Amersham). The equivalence of RNA
loading was assessed by etidium bromide staining.

cDNA probes. The probes used in this study were as fol-llows: rat wafl cDNA was kindly provided by B. Vogelstein
(15). Rat p27 cDNA was isolated by PCR from reverse-
transcribed rat liver RNA by using the oligonucleotides spe-
cific to highly conserved amino acid sequences at the NH2-
terminal and the COOH-terminal ends of human and mouse
p27: 5′-CCGAATTCGCTCTTCTTTGGGGTCGTGCTTCCACA-3′
and 5′-CCCGATCTGATCAAAGCTGTGTTCA-3′. p27 PCR product was cloned in pTag vector (R&D Systems,
Minneapolis, MN) and DNA sequencing was performed on
both strands. The available sequence appeared identical to the corresponding sequence of rat p27 that was published
when this work was in progress (46). The cloned fragment was released by EcoRI/BamH1 and used as a probe for North-
ern blot analysis. The mouse p57Kip2 probe was provided by
J. Massague (35). Rat a-fetoprotein (AFP) antibodies were from
Zymed (San Francisco, CA) and anti-p21 antibodies were obtained from NeoMarkers (Fremond, CA) and Onco-

Western blot analysis. The following primary antibodies
were used for Western blot analysis: mouse MAb, anti-Kip1
(p27), and anti-cdc2 were purchased from Transduction Lab-
oratories (Lexington, KY); anti-Cdk4 (Ab-1) and anti-PDNA
were obtained from NeoMarkers (Fremont, CA) and Oncogene
Science (Cambridge, MA), respectively. Anti-cyclin D1
MAb were from Zymed (San Francisco, CA) and anti-p21
MAb CP74 was kindly provided by C. Ngwu and E. Harlow.
The rabbit polyclonal anti-cyclin D1, anti-cyclin D3, and anti-green fluorescent protein (GFP) antibodies were from
Clontech (Palo Alto, CA). The rabbit polyclonal anti-Cdk2
and anti-cyclin A were purchased from Santa Cruz Biotech-
ology (Santa Cruz, CA). The monoclonal antibodies were peroxi-

 antisense experiments. Two oligonucleotides containing phosphorothioate backbone and C-5 propyne pyrimidines
were synthesized and purified by Eurogentec (Seraing, Bel-
gium): antisense p21, 5′-ACCAGGATCGCACGTTGCTGCTG-3′
and mismatch p21, 5′-ACCAGGATCGACAGTGCTGCTG-3′. Primary rat hepatocytes were maintained in serum-free Opti-MEM medium supplemented with 5 μg/ml bovine insu-
lin and 1.4 × 10−6 M hydrocortisone hemisuccinate. At 24 h
after seeding, cells were treated with 50 ng/ml EGF and were
transfected with 100 nM oligonucleotides by using liposome-based
reagent lipofectin (GIBCO) in Opti-MEM medium. At 24–72
h later, the cells were washed with PBS, fixed with PBS/4%
paraformaldehyde, and examined by fluorescence micros-

topology. Cells were lysed in IP buffer [(in mM): 50 HEPES (pH
7.5), 10% glycerol, 10 MgCl2, 1 DTT, 0.1 sodium
orthovanadate, 1 NaF, 10 β-glycerophosphate, 0.1 phenyl-
methylsulfonyl fluoride, and 100 μg/ml benzamidine and
protease inhibitor cocktail (5 μg/ml aprotinin, leupeptin,
pepstatin, and soybean trypsin inhibitor) and sonicated in ice.
Lysates were clarified by centrifugation at 10,000 g for 10 min and stored at −80°C. Precleared lysates
(400 μg protein per assay) were incubated with 2 μg of antibodies overnight at 4°C, and immune complexes were
recovered with protein A-agarose beads (Pierce, Rockford,
IL). The following rabbit polyclonal antibodies were used for
IP: anti-cyclin D1 and anti-cyclin D3 were from Clontech;
bodies against cyclin A (H-432), Cdk4, Cdk6, p21 (C-19),
p27 (C-19), and p27 (N-20) were purchased from Santa Cruz
Biotechnology. After being washed with IP buffer, samples
were subjected to SDS-PAGE, protein transfer to nitrocel-

Hepatocyte transfection and fluorescence microscopy anal-
ysis. Hepatocytes were seeded in 35-mm petri dishes (500 ×
104 cells per dish) and after 24 h of culture, were transfected
with GFP-p21 expression vector by using liposome-based
reagent (GIBCO) in Opti-MEM medium. At 24–72
h later, the cells were washed with PBS, fixed with PBS/4%
paraformaldehyde, and examined by fluorescence micros-
copy.
described elsewhere. Briefly, transfection of siRNA or co-transfection of expression plasmid with siRNA was performed in six-well plates in the presence of Lipofectamine 2000 (Invitrogen) per well, 3 μg of expression plasmid and/or 100 nM siRNAs and 5 μl Lipofectamine 2000 was applied in a final volume of 1.2 ml Opti-MEM. Four hours later, the medium was changed to a mixture of 75% MEM and 25% medium 199, supplemented with 5% FCS, serum albumin, insulin, EGF, and hydrocortisone hemisuccinate, as described in Cell isolation and culture and hepatocytes were incubated for further 24–48 h.

RESULTS

Amounts of the Cdk inhibitors decrease during development of rat liver. We determined the kinetics of Cdk inhibitor expression during rat liver development. p27 Transcripts were highly expressed in fetal liver, markedly decreased after birth, gradually declined during the first week of postnatal development, and remained constantly expressed at low levels thereafter. In contrast, the levels of p21 transcripts were low in fetal liver, then strongly and transiently increased shortly after birth, and gradually returned to very low levels in the adult stage. Similarly to p21, p57 was transiently increased shortly after birth and abruptly decreased thereafter (Fig. 1A). No p57 transcripts were detected in adult liver. As expected, AFP, one of the developmentally regulated liver-specific genes, was very highly expressed in fetal and perinatal liver and abruptly shut off after 15 days in accordance with previous studies (48). In contrast, expression of albumin increased after birth and remained at a high level during postnatal development and in adult liver (Fig. 1B).

At protein levels, the kinetics of p27 expression was found similar to that of corresponding transcripts: the amount of p27 protein considerably decreased shortly after birth and gradually declined during the postnatal period. p21 Protein exhibited a transient increased expression during the first 2 wk after birth, whereas its amount in both fetal and adult liver was very low (Fig. 1C).

![Fig. 1](http://ajpgi.physiology.org/)

**Fig. 1.** Pattern of cell cycle-related gene expression during development of rat liver. A and B: RNA blot analysis. Total RNA was extracted from rat livers at different stages of development. Blots were probe for the transcripts indicated, left. C: Western blot analysis. Total liver cell extracts (100 μg) were assayed for the presence of p27 and p21 proteins. H72h, cell extracts prepared from rat hepatocytes stimulated in vitro with EGF and sampled at 72 h of culture. AFP, α-fetoprotein.
**Biphasic induction of p21 in regenerating liver.** The expression and regulation of members of the Cip/Kip gene family during hepatocyte replication was studied by using two models: regenerating rat liver and a primary culture of rat hepatocytes stimulated with EGF. Partial hepatectomy (PHT) triggered a synchronous replication of rat hepatocytes with a dramatic increase of DNA synthesis that occurs from 18 h and peaked at 24 h (38). Examination of Cdk inhibitors expression during the G1-S phase of cell cycle of in vivo proliferating hepatocytes revealed a strong increase of p21 mRNA amounts in early mid-G1 phase with maximum at 4 h after PHT. Then, expression of p21 declined and was upregulated again in the S phase. In contrast, amounts of p27 transcripts modestly decreased from 2 to 12 h after PHT (Fig. 2A). No significant changes of p21 and p27 expression were found in sham-operated animals (data not shown). p57 Transcripts were undetectable in regenerating liver during the observed period.

**Pattern of p21 and p27 mRNA expression during the cell cycle progression of normal hepatocytes.** In accordance to our previous observations (29, 37), adult rat hepatocytes stimulated with EGF 4 h after seeding and throughout culture, started to incorporate [3H]thymidine between 42 and 54 h, reaching a maximum around 72 h (data not shown). In unstimulated cultures, incorporation of the tracer was very low. In contrast to biphasic induction of p21 in regenerating liver in vivo, expression of p21 transcripts in mitogen-stimulated cells was strongly increased shortly after seeding and remained elevated thereafter (Fig. 2B). The level of p27 mRNA did not change significantly in isolated hepatocytes during the first 3 days of culture and then gradually increased. p57 Transcripts were usually undetectable in cultured adult hepatocytes, although a very faint band appeared from 76 h of culture after long-time exposure (data not shown).

**Mitogen-dependent and coordinated induction of p21 and cyclin D1 proteins in EGF-stimulated hepatocytes.** To study the effect of mitogen stimulation on expression of p21 and p27 mRNA and proteins, we determined the levels of these proteins in EGF-stimulated and unstimulated hepatocytes. We found that both p21 and p27 transcripts accumulated with time in culture independently of mitogen stimulation (Fig. 3A). However, expression of p21 protein was uncoupled from that of corresponding transcripts. Indeed, in unstimulated hepatocytes, p21 protein levels remained very low all along the culture period; however, abundance of this protein strongly increased in EGF-treated cells. In contrast, expression of p27 protein in hepatocytes was mitogen independent (Fig. 3B).

We have previously demonstrated that outset and rate of hepatocyte DNA synthesis were dependent on the location of cells in G1 phase at the time of exposure to mitogen and were associated with accumulation of cyclin D1 transcripts (37). To determine whether appearance of p21 protein was associated with mitogen-dependent signaling at the restriction point, rat hepatocytes were exposed continuously to EGF either from 4, 44, or 68 h of culture and amounts of p21 and cyclin D1 proteins were estimated by Western blot analysis in these different cultures. We found that levels of p21 protein were drastically increased in all tested conditions and delayed exposure to mitogen resulted in delayed induction of p21. Indeed, stimulation of rat hepatocytes performed shortly after seeding (4-h culture) resulted in a strong increase of p21 at 54 h, whereas the addition of EGF at 44 and 68 h led to the appearance of significant amounts of p21 at 78 and 92 h, respectively. In addition, delayed EGF stimulation resulted in a shorter induction lag time of p21 over the time of culture. Interestingly, cyclin D1 protein followed the similar pattern of induction, preceding, however, that of p21 (Fig. 3C). Thus these data sug-

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**Fig. 2. Expression of p21 and p27 genes during cell cycle progression of hepatocytes.**

A: total RNA was isolated from regenerating rat liver and subjected to RNA blot analysis by using p27, p21, and albumin cDNA probes. B: total RNA was extracted from hepatocytes treated with EGF as described above and analyzed by hybridization with the p27, p21 and albumin cDNA probes. L, adult rat liver.
gested that induction of both cyclin D1 and Cdk inhibitor p21 is a part of cell response to mitogenic stimulus and is associated with cell progression through the restriction point to the late G1 phase.

Formation and activity of cyclin/Cdk/Cdk inhibitor complexes during cell cycle progression of rat hepatocytes. Coupled IP-Western blot experiments were performed to determine the dynamics of association of p21 and p27 proteins with G1 phase cyclins and Cdk5 during hepatocyte cell cycle progression after EGF stimulation. First, we determined the level of various cyclin and Cdk proteins by Western blot analysis. Expression of cyclin D1, D3, and Cdk4 proteins was very low in freshly isolated hepatocytes and in culture early after seeding. The amount of cyclin D1 protein drastically increased between 24 and 48 h of culture followed by a second wave of induction at 72 h correlating with that of p21. Cyclin D3 was also increased from 48 h; however, its level remained stable at least until 120 h. Similarly to cyclin D1, Cdk4 protein was increased from 48 h of culture (Fig. 4A). p21 Protein increased in parallel to cyclin D1 between 24 and 48 h of culture. Amounts of p27 were relatively stable during the first 3 days in culture and moderately increased thereafter (Fig. 4A).

Second, we examined the association of Cdk inhibitors with cyclins D-Cdk4/Cdk6 complexes. Analysis of the cyclin D1 immunoprecipitates revealed the following observations: 1) Cdk4, p21, and p27 were present in the complex with cyclin D1 from 48 and =120 h; 2) levels of p27 associated with cyclin D1 increased gradually with the time of culture, whereas the amounts of p21 protein complexed with cyclin D1 peaked at 96 h and slightly decreased thereafter (Fig. 4B). Using the same cell lysates, we have performed IP with anti-p21 and anti-p27 antibodies followed by immunoblotting for cyclin D1 protein. Strong association of cyclin D1 with p27 was found at 96 and 120 h of culture, whereas the maximum amount of cyclin D1 complexed with p21 was detected at 72 and 96 h (Fig. 4C).

To determine the relative amounts of p21 and p27 forming a complex with G1 cyclins and Cdk5, the lysates prepared from 96-h cultured hepatocytes, containing the highest levels of both p21 and p27, were immunoprecipitated with COOH-terminal peptide-specific antibodies to cyclin D1, cyclin D3, Cdk4, Cdk6, p21, and p27 followed by immunoblotting with anti-p21 and anti-p27 MAb. From two independent experiments, we found that the majority of p27 was coimmunoprecipitated with cyclin D1, whereas ~30–50% of total p21 was coimmunoprecipitated with cyclin D1 at this point of time (Fig. 5A). In contrast to cyclin D1, only very low amounts of cyclin D3-associated p21 and p27 were found in EGF-stimulated hepatocytes suggesting that...
cyclin D1-containing complexes were the predominant reservoir of p21 and p27 in proliferating liver cells. Interestingly, no p21 was detected in the p27 immunoprecipitates and vs. versa even after long exposure (Fig. 5A). Both p21 and p27 were also detected in Cdk4 and Cdk6 immunoprecipitates. However, p27 was preferentially coimmunoprecipitated with Cdk4. In contrast, approximately equal amounts of p21 were found in both Cdk4 and Cdk6 immunoprecipitates (Fig. 5A).

Furthermore, we studied the relative amounts of cyclin D1, Cdk4, and p21 associated in complex during hepatocyte cell cycle progression. The lysates prepared from EGF-stimulated hepatocytes at 48, 72, and 96 h of culture were immunoprecipitated in parallel with polyclonal antibodies to cyclin D1 and p21 followed by immunoblotting with anti-cyclin D1 or anti-Cdk4 and

![Diagram](image)

Fig. 4. Assembly of cyclin D1-Cdk4-p21/p27 complexes during cell cycle progression of rat hepatocytes. A: cell extracts were prepared from hepatocytes stimulated with EGF from 4 h after seeding. The samples (50 µg each line) were dissolved in 12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with antibodies against the following (from top to bottom): cyclin D1, cyclin D3, Cdk4, p27, and p21. B: the same extracts (400 µg for each time point) were immunoprecipitated with anti-cyclin D1 antibodies. The immunoprecipitates were examined for the presence of cyclin D1, Cdk4, p27, and p21 by immunoblotting. C: the same extracts (400 µg for each time point) were immunoprecipitated with anti-p21 (top) and anti-p27 (bottom) antibodies and immunoprecipitates were assayed for the presence of cyclin D1.

![Diagram](image)

Fig. 5. Analysis of amounts of p21 and p27 associated with cyclins D-Cdk4/Cdk6 in proliferating hepatocytes. A: cell extracts were prepared from mitogen-stimulated hepatocytes collected at 96 h of culture and immunoprecipitated with polyclonal antibodies against p21, cyclin D1, cyclin D3, and p27 (top) and with antibodies against p21, p27, Cdk4, and Cdk6 (bottom). All antibodies used for IP were raised against the COOH-terminal peptides of corresponding proteins. Immunoprecipitates were resolved in 12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-p27 MAb and further with anti-p21 MAb without stripping the blot. Positions of p21 and p27 are indicated. B: extracts were generated from hepatocytes stimulated with EGF and collected at 48, 72, and 96 h of culture. Samples (400 µg for each line) were immunoprecipitated with antibodies against p21, p27, Cdk4, and Cdk6 (bottom) antibodies and immunoprecipitates were assayed for the presence of cyclin D1.

![Diagram](image)

Fig. 6. Immunoprecipitation of cyclin D1-p21/p27 complexes during cell cycle progression of rat hepatocytes. A: cell extracts were prepared from hepatocytes stimulated with EGF from 4 h after seeding. The samples (50 µg each line) were dissolved in 12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with antibodies against the following (from top to bottom): cyclin D1, cyclin D3, Cdk4, p27, and p21. B: the same extracts (400 µg for each time point) were immunoprecipitated with anti-cyclin D1 antibodies. The immunoprecipitates were examined for the presence of cyclin D1, Cdk4, p27, and p21 by immunoblotting. C: the same extracts (400 µg for each time point) were immunoprecipitated with anti-p21 (top) and anti-p27 (bottom) antibodies and immunoprecipitates were assayed for the presence of cyclin D1.
anti-p21 MAb. In accordance with the results presented in Fig. 5A, approximately one-half of total p21 was found associated with cyclin D1, and this ratio did not change significantly from 48 to 96 h of culture. In contrast, p21 immunoprecipitates contained roughly half of the cyclin D1 and Cdk4 compared with that found in cyclin D1 immunoprecipitates at all time point analyzed (Fig. 5B). Using the same approach, we checked the kinetics of association of p27 and cyclin D1. Similar to that found at 96 h (Fig. 5A), most of p27 present in the proliferating hepatocytes was bound to cyclin D1 at 72 and 120 h (Fig. 5C). In addition, examination of cyclin D1 and Cdk4 immunoprecipitates systematically revealed the presence of a faster-migrating band detected with anti-p27 MAb. To identify the nature of this form, lysates of EGF-stimulated hepatocytes were immunoprecipitated with polyclonal antibodies raised against peptides corresponding to amino acids mapping at the amino terminus and the COOH terminus of p27 (N-20 and C-19, respectively) and with anti-cyclin D1 antibody followed by Western blotting with anti-p27 MAb. The novel form of p27, migrating as a 23-kDa protein, was detected only in the samples immunoprecipitated with polyclonal antibodies raised against peptides corresponding to amino acids mapping at the amino terminus and the COOH terminus of p27 (N-20 and C-19, respectively) and with anti-cyclin D1 antibody followed by Western blotting with anti-p27 MAb. The novel form of p27, migrating as a 23-kDa protein, was detected only in the samples immunoprecipitated with anti-cyclin D1 and anti-p27 (N-20) antibodies, but not with anti-p27 (C-19) antibody, suggesting that this protein represents COOH-terminal truncated form of p27 (Fig. 5C). Abundance of this form in rat hepatocytes progressively increased from 96 to 120 h of culture. A similar truncated form resulting from proteolysis of p27 by caspase 3-like protease was demonstrated in myeloma cell line U266 (36). We detected the presence of the processed active form of caspase 3 and a strong increase of caspase 3-mediated cleavage of the fluorogenic DEVD peptide substrate (DEVD-AMC activity) in EGF-stimulated hepatocytes from 96 h of culture, suggesting that caspase 3-like activity is implicated in proteolysis of p27 in hepatocytes (data not shown).

Finally, we have determined the kinase activity of cyclin D1 and p21 immunoprecipitates by using pRb as a substrate. Both cyclin D1- and p21-associated kinase activities increased from 48 h of culture corresponding to the appearance of cyclin D1/Cdk4/p21 complexes and remained elevated at least ≤120 h (Fig. 6).

Intracellular localization and physiological role of p21 in proliferating hepatocytes. To elucidate the role of p21 in hepatocytes, we performed the overexpression of p21 protein by transfection of primary hepatocytes of expression vector encoding p21-GFP fusion protein or inhibition of its expression by using the antisense oligonucleotide or RNA interference (RNAi) technologies. Rat hepatocytes were transiently transfected with p21-GFP expression vector and stimulated with EGF for 24–72 h. Fluorescent microscopy examination of p21-GFP-transfected cells revealed that, in contrast to cells transfected with control vector pEGFP-C3 (Fig. 7A), hepatocytes exclusively exhibited either nuclear homogeneous or punctuated pattern of fluorescence (Fig. 7B). In parallel, staining of EGF-stimulated hepatocytes with anti-cyclin D1 antibody revealed predominant nuclear labeling in most of the hepatocytes (Fig. 7C).
To repress expression of endogenous p21 in mitogen-stimulated hepatocytes, we used antisense oligonucleotides or siRNAs. Treatment of hepatocytes with p21 antisense oligonucleotides resulted at least in 1.4-fold increase of \[^{3}H\]thymidine incorporation compared with control cultures during the first 52 h of culture (Fig. 8A). Thereafter, we observed no significant differences of tracer incorporation between hepatocyte cultures treated with ASp21 and mismatch control oligonucleotides. Similarly, p21 siRNAs specifically reduced the amount of endogenous total and Cdk2-associated p21 (Fig. 8, C and D) and led to a significant rise of \[^{3}H\]thymidine incorporation into hepatocytes between 28 and 52 h of culture (Fig. 8B). This activation of DNA synthesis was accompanied by an increase of Cdk2-mediated histone H1 kinase activity (Fig. 8D), suggesting that p21-deficient hepatocytes entered rapidly into the S phase.

Early association of p21 and p27 with S phase cyclin A. It has been shown previously that p21 exhibits much stronger inhibitory activity on cyclin E/Cdk2 and cyclin A/Cdk2 complexes compared with cyclin D/Cdk4 (57). To further understand the role of p21 and p27 Cdk inhibitors in G1-S transition in these cells, we analyzed their association with S phase cyclin A. Levels of total cyclin A and its partners Cdk2 and PCNA were strongly increased in mitogen-stimulated hepatocytes closely to the G1/S boundary (from 44 h of culture). In accordance with our previous observations, cdc2 protein started to accumulate later, from 56 h when the majority of hepatocytes were entering the S phase (37). The induction of these proteins appeared strictly related to mitogen stimulation, because hepatocytes maintained without growth factor did not express cdc2, cyclin A and PCNA and contained only constant low amounts of Cdk2 (Fig. 9A). Analysis of cyclin A immu-

To assess the consequence of p21 overexpression on cell cycle progression, EGF-stimulated hepatocytes were labeled for the last 24 h with BrdU. Under these conditions, ~70–80% of nontransfected or transfected hepatocytes with GFP expression vector incorporated the tracer as detected by immunocytochemistry. In contrast <5% of p21-GFP-expressing cells were positive for BrdU staining. Furthermore, we demonstrated that expression of p21-GFP in EGF-stimulated hepatocytes resulted in suppression of \[^{3}H\]thymidine incorporation compared with hepatocytes transfected with empty vector (Fig. 7D). Moreover, cotransfection of GFP siRNAs with p21-GFP expression vector led to a nearly complete restoration of hepatocyte proliferation activity (Fig. 7D) accompanied by effective silencing of p21-GFP expression (Fig. 7E).
noprecipitates revealed that both p27 and p21 were associated with cyclin A as early as 44 h of culture with maximum level observed at 44–56 and 56 h for p27 and p21, respectively (Fig. 9B).

**Regulation of p21 protein expression by intracellular signaling pathways.** An essential role of MAPK kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3-kinase)-ferric-reducing ability power (FRAP)/mammalian target of rapamycin (mTOR) pathways in controlling the cell proliferation and expression of cell cycle-related proteins in various cell types are largely recognized (31). Particularly, a temporal biphasic activation of MEK/ERK cascade in rat hepatocytes during liver regeneration in vivo and a critical role of this activation on cell cycle progression of hepatocytes and expression of cyclin D1 has been previously demonstrated (59). In vitro, EGF-stimulated rat hepatocytes but not non-stimulated cells exhibited rapid (within 5–10 min after the addition of EGF) and sustained activation of MAPK pathway as long as for 96 h after cell seeding (53). To analyze the contribution of these different intracellular signaling pathways in the regulation of p21 and p27 expression, primary hepatocytes were synchronized by maintaining them in the absence of growth factors for 48 h and were treated with EGF in the presence or absence of rapamycin, LY-294002, or U-0126, inhibitors of FRAP/mTOR, PI3-kinase, and MEK, respectively. All inhibitors effectively inhibited DNA synthesis of EGF-stimulated hepatocytes as measured by [3H]thymidine incorporation, in accordance with previous data (10). We demonstrated that all three inhibitors strongly suppressed expression of p21 protein and also inhibited accumulation of cyclin D1 synthesis in mitogen-stimulated cells. However, they had only marginal effect on expression of p27 protein (Fig. 10).

**DISCUSSION**

It is generally agreed that Cdk inhibitors play a critical role in regulating cell cycle progression and exit during morphogenesis and differentiation and/or in maintaining cell quiescence (56, 57, 66). Expression of these genes in vivo is frequently correlated with terminally arrested cells. In addition, strong upregulation of p21 was found in several cell lines and tissues undergoing terminal differentiation coupled with irreversible cell cycle arrest (19, 23, 58), and this induction was shown to be important for maintaining the absence of DNA synthesis in these cells even after growth factor stimulation (40). The differentiation process of hepatocytes displays an important specific property, because it is associated with reversible cell growth arrest suggesting particular regulation of cell cycle during hepatic differentiation.

The present study reveals that expression of p21, p27, and p57 genes was differently regulated during rat liver development; p27 was relatively strongly expressed in fetal liver and decreased thereafter, whereas p21 was very low in fetal liver and transiently activated shortly after birth. The induction of both Cdk inhibitors p21 and p57 shortly after the birth suggests an inhibition of hepatocyte proliferation activity at this time. Indeed, the high growth rate of hepatocytes during the late perinatal period was followed by a strong decrease of cell proliferation activity at the time of birth with subsequent recovery of proliferation within 48 h after birth (6, 22).

Differentiated quiescent hepatocytes in adult rat liver contained barely detectable amounts of p21 and p57 and low levels of p27. Similarly, very weak or undetectable expression of p21 and p57 were also observed in mouse and human liver (4, 20, 39, 41). Thus the potency of the negative regulators of the cell cycle that may block Cdk kinase activity seems to be very weak in differentiated quiescent hepatocytes. In this setting, it is tempting to speculate that induction of cyclin/Cdk expression in regenerating liver is sufficient to overcome the weak negative barrier and to cause the cell cycle entry. These observations could explain, at least partly, the high proliferative potential of mature differentiated hepatocytes, even after a long-term quiescence and the capacity of the liver to regenerate in vivo. In line with this possibility, transient expression of cyclin D1 in rodent livers in vivo promoted hepatocyte proliferation (44). In contrast, forced expression of p21 in the livers of transgenic mice halted hepatocyte proliferation after PHT (64). In a similar manner, in vitro overexpression of cyclin D1 in normal rat hepatocytes was sufficient to trigger G1/S phase transition (2). Here we show that overexpression of p21 protein in mitogen-stimulated hepatocytes inhibited DNA synthesis. Furthermore, particular regulation of p21 in

![Fig. 10. Differential effects of cell signaling pathway inhibitors on expression of p21 and p27 proteins. Rat hepatocyte cultures were maintained for 48 h in mitogen-free basal medium and then stimulated with EGF in the absence or presence of 1 nM rapamycin, 15 μM LY-294002, and 50 μM U-0126. Control cultures were maintained without both mitogen and inhibitors. At indicated time after stimulation cultures were arrested and whole cell extracts samples (60 μg each line) were dissolved in 12% SDS-PAGE, transferred to nitrocellulose membrane, and assayed for the presence of p21, p27, and cyclin D1 proteins.]
normal hepatocytes is in agreement with our previous results obtained from analysis of cell cycle gene regulation in human hepatoma cell line HBG that has a property of undergoing a spontaneous differentiation at confluence and has the ability to reverse to active proliferation even after several weeks of quiescence. In this model, p21 protein was highly expressed in proliferating cells but drastically fell down in growth-arrested differentiated cells (21).

The expression and role of Cdk inhibitors in cell cycle progression of normal hepatocytes was analyzed by using a well-characterized in vitro model of primary rat hepatocytes stimulated to proliferate by the addition of EGF (37). Although mouse hepatocytes have the capacity to proliferate in the absence of growth factors in medium, proliferation activity of rat hepatocytes is tightly dependent on stimulation with mitogens. Thus rat hepatocytes in culture is a more appropriate model to study mitogen-dependent entry and progression along the cell cycle.

Whereas it has been agreed that expression of p27 mRNA is generally invariant in growth-arrested or proliferating cells (28, 50), the amount of p27 protein decreases during the G1 to S-phase progression in different types of mitogen-stimulated cells and is elevated at quiescence (1, 8, 9, 21, 47). We found that both p27 transcripts and protein did not decrease but accumulated in EGF-stimulated hepatocytes from 72 h of culture. The fact that the major part of p27 protein presented in proliferating hepatocytes was associated with cyclin D1 leads us to believe that cyclin D1-Cdk4/Cdk6 complexes accumulated in mitogen-stimulated cells from mid- to late-G1 phase sequestered p27, making it inaccessible to cyclin E-Cdk2 and cyclin A-Cdk2 complexes. This particular expression of p27 protein in EGF-stimulated proliferating hepatocytes evokes analysis of cell cycle progression in NIH 3T3 fibroblasts by Cheng et al. (8). Although in cells stimulated to proliferate by the addition of FCS, the level of p27 fell by late G1 phase, induction of MEK in serum-starved NIH 3T3 cells engineered to ectopically expressed cyclin D1 and Cdk4 triggered S phase entry in the absence of p27 decrease. Furthermore, previous studies (4, 5) and our data strongly suggest that p27 is primarily accounting for inhibition of residual activity of cyclin E-Cdk2 and cyclin A-Cdk2 complexes in quiescent liver and for maintaining hepatocytes in the nonproliferative state. Indeed, among three members of the Cip/Kip family of Cdk inhibitors, only p27 was expressed in appreciable quantities in the adult liver. In both unstimulated and EGF-stimulated hepatocytes during the first hours of culture, p27 protein was preferentially associated with Cdk2 and much less strongly with Cdk4 (unpublished observations). Extracts from quiescent adult liver contained an inhibitor of active cyclin E/Cdk2 complex and immunodepletion experiments demonstrated that p27 accounted for a significant proportion of the Cdk inhibitory activity (5). Finally, mice lacking p27 but not p21 gene display liver hyperplasia resulted in increased hepatocyte cell density (17). In accordance with the proposed role of p27 as growth inhibitor in adult liver, hepatocytes isolated from p27 knockout mice exhibited a significantly elevated level of DNA synthesis measured by [3H]thymidine and BrdU incorporation and a strong increase of Cdk2-associated activity both in unstimulated and mitogen-stimulated cells (32).

In contrast to mitogen-independent expression of p27, p21 protein appeared to be dramatically induced only in EGF-stimulated and proliferating hepatocytes. Moreover, we have provided several lines of evidence that coordinated induction of p21 and cyclin D1 proteins is an important component of mitogen-dependent regulatory pathway that controls hepatocyte cell cycle progression. Indeed, induction of both proteins was strongly associated with mitogen stimulation, and their expression pattern and activity of corresponding immune complexes revealed a striking parallelism. Furthermore, p21 was associated with cyclin D1 during the hepatocyte cell cycle progression, and both proteins were colocalized in the nuclei of EGF-stimulated hepatocytes. Finally, expression of both p21 and cyclin D1 was regulated by common intracellular signaling pathways. All of these observations emphasize the specific functional role of p21 in hepatocytes. It is clear that overexpression of this protein both in vivo (64) and in vitro (present study) efficiently inhibited the proliferation activity of hepatocytes. However, available data suggest that at physiological concentrations, p21 could play a positive role in adult hepatocytes by promoting cyclin D1-Cdk4/Cdk6 assembly and targeting them to the nucleus as it was previously shown in fibroblasts (34). This conclusion is further strengthened by observation that livers from mice lacking both p21 and p27 contained significantly fewer cyclins D-Cdk4 and cyclin D-Cdk6 complexes than those from wild-type mice (7). We used the antisense oligonucleotide and RNAi technologies to study the consequence of p21 reduction on cell cycle progression of mitogen-stimulated hepatocytes. Our results indicate that a portion of hepatocytes entered more rapidly into S phase are in accordance with analysis of hepatocyte proliferation in vivo after PHT in p21 knockout and wild-type mice. The hepatocytes isolated from p21(−/−) mice progressed more rapidly through G1 phase and exhibited premature activation of Cdk (4). It was previously demonstrated (34) that direct measurement of cyclin D-Cdk4-p21 kinase activity in vitro did not reveal significant inhibition at 1:1:1 stoichiometry. In contrast, cyclin A-Cdk2 complexes were efficiently inhibited by equimolar concentrations of p21 (27). Here we have found that a portion of p21 was found in cyclin A and Cdk2 immunoprecipitates as early as from G1/S boundary. A decrease of p21 amounts led to earlier activation of the Cdk2-related complex by lowering the inhibitory threshold and, as a consequence, to earlier entry of hepatocytes into S phase.

Our data emphasize the importance of MEK/ERK and PI3-kinase pathways in regulation of p21 expression. Particularly, several lines of evidences argue for the critical role of the MEK/ERK pathway in control of different hepatocyte proliferation activity both in vitro and in vivo. Indeed, although a transient biphasic
MEK/ERK activation was evidenced in proliferating rat hepatocytes in vivo after PTH (59), stimulation of hepatocytes in vitro by EGF triggered a long-lasting activation of this pathway (53). Considering that chronic activation of MAPK cascade activation correlated with increased expression of p21 and an inhibition of hepatocyte DNA synthesis (62), it seems that this mechanism is responsible, at least partly, for reduced proliferation activity of primary hepatocytes in vitro. Taken together, prior studies and our results strongly suggest that in proliferating rodent hepatocytes in primary culture, at least during the first cell cycle, p21 and p27 Cdk inhibitors play distinct roles. Specifically, expression of p21 protein is tightly associated with mitogen stimulation and amount of this Cdk inhibitor in proliferating hepatocytes likely controls the timing of phase S cyclin-Cdk complex activation and entry into the S phase. In contrast, p27 protein expressed in a mitogen-independent manner in cultured rat hepatocytes, seems to be involved in the restriction of the magnitude of DNA synthesis.

In summary, we have demonstrated a characteristic and differential expression and regulation of Cdk inhibitors p21 and p27 during hepatocyte differentiation and proliferation. Specific regulation of these genes in liver cells may account fully or in part for mechanisms involved in the control of the capacity of quiescent mature hepatocyte to enter and progress through the cell cycle.

We acknowledge Drs. E. Harlow, J. Massague, and B. Vogelstein for providing plasmids and antibodies.

This work was supported by the Institut National de la Santé et de la Recherche Médicale, by EEC Grant Biot-CT 960052, and by the Ligue Nationale Contre le Cancer, Comité d’Ille et Vilaine.

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G127

Cdk INHIBITORS p21 AND p27 IN HEPATOCYTES


