Cloning and Characterization of Liver-specific Isoform of Chk1 Gene from Rat*

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We have isolated and characterized an isoform of protein kinase Chk1 gene from rat liver and a rat liver cDNA library by 5'-rapid amplification of cDNA ends. The gene (Cil) contains the C-terminal region of the Chk1 gene, but the 5'-end is derived from a sequence in the intron of Chk1 preceding the C-terminal domain by differential RNA splicing. The kinase domain of Chk1 gene is absent in this isoform. Tissue RNA and protein blot analyses indicated that Cil was specifically expressed only in rat liver, and its expression increased with liver development. Expression of Cil was found to be reduced in three rat hepatoma cell lines examined. A promoter trap experiment suggested that a promoter was located in the intron preceding the C-terminal domain of Chk1, and transcription from this novel promoter generated the new 5' noncoding exon of Cil. Thus Cil was generated by both alternate promoter usage and differential RNA splicing. UV irradiation induced caffeine-sensitive phosphorylation of both Chk1 and Cil at Ser-345 in Chk1 and its equivalent site in Cil, implying a role for ATR kinase in the phosphorylation of both proteins. We demonstrated the interaction between the kinase domain of Chk1 and Cil using a yeast two-hybrid assay and pull-down technique. In contrast to the effect of Chk1, Cil was found to decrease the transactivating function of p53, and the S63A mutation of Cil abolished this effect. These results suggest that Cil may serve as a dominant negative competitor of Chk1 as suggested previously.

Transitions in cell cycle are under the surveillance of regulatory pathways called checkpoints. One of the checkpoints is to ensure the integrity of the genome before entering mitosis (1-6). The mitotic cell cycle checkpoints are conserved from yeast to mammals, and the key target of this surveillance is the Cdc2-cyclin B complex that phosphorylates a number of proteins involved in mitotic processes such as proper chromosome segregation and nuclear disassembly (1, 7-10). When DNA is damaged or DNA replication is unfinished Cdc2-cyclin B is inactivated through inhibitory phosphorylation of Cdc2 by Wee1 and Myt1 kinases (5, 11, 12). The inhibition is reversed by Cdc25 after completion of DNA replication or repair by removing the inhibitory phosphorylation.

One of the effector protein kinases that regulates Cdc25 is Chk1 (13-15). Chk1 was first identified in fission yeast involved in cell cycle arrest when DNA was damaged (16). In mammalian cells a Chk1 homolog was shown to phosphorylate Cdc25A, -B, and -C (13, 17), and this reaction promoted the binding of Cdc25 to 14-3-3 proteins (the mammalian homolog of fission yeast Rad24 and Rad25) to prevent activation of Cdc2. In addition to Cdc25, Wee1 and p53 have also been found to be phosphorylated by Chk1 when checkpoint is activated (18-20).

In human cells, Chk1 is expressed from S to M phase at both RNA and protein levels, and the proteins are localized in the nucleus (21). When DNA is damaged by ionizing radiation, UV, or hydroxyurea, Chk1 is found to be phosphorylated in an ATR-dependent manner (22, 23). However, whether the kinase activity of Chk1 is enhanced in response to DNA damage is still controversial (21, 22, 24, 25).

Chk1 is a highly conserved gene found from yeast to mammalian cells involved in DNA damage checkpoint. Recent studies indicate that Chk1 is involved in both DNA damage and DNA replication checkpoints in mammalian cells (22, 26). Chk1 protein contains a highly conserved N-terminal kinase domain linked to its less conserved C-terminal domain of unknown function through a flexible linker region (27). Functional analysis of kinase activity suggests that the C-terminal domain in human Chk1 may serve as a negative regulator of kinase activity (27).

In the present report we presented evidence for the existence of a rat liver-specific Chk1 isoform (Cil for Chk1 isoform in liver). This isoform was found to be transcribed using a novel promoter residing in the intron of the Chk1 gene preceding the C-terminal domain, and mature mRNA was formed by the joining of the new noncoding exon within the intron to the remaining exons of Chk1 through splicing, deleting the kinase domain of Chk1. We showed that Cil associated with the Chk1 kinase domain using yeast two-hybrid and pull-down assays and that it down-regulated p53-mediated gene transcription. Furthermore, we showed that Cil was phosphorylated at a serine residue corresponding to Ser-345 in Chk1 following UV irradiation and that mutation of this phosphorylation site eliminated its effect on p53 activity.

EXPERIMENTAL PROCEDURES

Oligonucleotide Primers—The primers used were as follows: 2-6-1, GTGTTTCCGCAATAATACGCT; 2-6-3, AGGACTGACGAGGAGGACGACGATC; 2-6-15, TTACTCATGGAACCCAC; 2-6-31, ATACACTCATGGAATCCTGGAACCCAC; Chk1 g-05, GTGTCTGCAGAAGAGAGAGTTG; Chk1 g-06, GAGACTGCAGAAGAGAGAGTTG; Chk1 g-16, GCAGGTCGACGAGGAGGAGGACGACGATC; Chk1 g-19, CAGTCTGAGAGGAGGAGGACGACGATC; Chk1 g-20, GACTGAGGAGGAGGACGACGATC; Chk1 g-21, AGGAGGACGAGGACGACGACGATC; Chk1 g-22, AGGAGGACGAGGAGGAGGACGACGATC; Chk1 g-28, GGTTAAGCTTGCTGTCACCTGAGAGGACGACGATC; Chk1 g-30, GAGAAGCGTAAAGAGGAGGAGGAGGACGACGATC.

Cell Cultures and Transfection—Rat hepatoma cell line FFA-HTC1 (JCRB 0249) was grown in Williams’ medium E supplemented with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin. The
H-4-II-E cell line obtained from Culture Collection and Research Center (CCRC 60209) was cultured in minimum Eagle's medium supplemented with Earle's balanced salt solution with 0.1 mM nonessential amino acids, 10% fetal bovine serum, and 10% calf serum. The MH1C1 (CCRC 60143) cell line was cultured in Ham's F-10 medium, 15% horse serum, and 2.5% fetal bovine serum. Hep3B cells (p53-negative human hepatoma cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Cells were transfected using LipofectAMINE™ (Invitrogen) according to the instructions of the manufacturer. Briefly, cells were grown to 50% confluence in 60-mm dishes. Plasmids and LipofectAMINE were separately diluted in serum-free medium to 300 μl, mixed, and incubated at room temperature for 1 h. DNA-lipid complexes were diluted with 2.4 ml of serum-free medium. Cells were washed twice with phosphate-buffered saline and overlaid with the diluted DNA-lipid complexes.

Cloning of Chk1 Isoform—We used 5'H11032- and 3'H11032-RACE1 (Marathon cDNA Amplification System from CLONTECH) to clone the Cil cDNA from rat liver following the instructions of the manufacturer. Briefly, first-strand cDNA was synthesized using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase. Second-strand synthesis was performed using a mixture of Escherichia coli DNA polymerase I, RNase H, and E. coli ligase. After the T4 DNA polymerase reaction, the double strand cDNA was ligated to the Marathon cDNA adapter. PCR was performed for 30 cycles with a forward primer AP1 and a reverse primer 2-6-3 for 5'H11032-RACE. The 5'H11032-RACE products were then cloned and sequenced. A 5'H11032-RACE clone with a novel DNA sequence was identified. According to the 5'H11032-RACE result, a specific primer for the isoform was designed for the 3'H11032-RACE experiment. Finally, the same cDNA clone of Cil was also isolated from rat liver cDNA library (Stratagene, Inc.) by screening using 32P-labeled Chk1 probe.

Plasmids and Construction of Recombinant Expressing Clones—The mammalian expression vector pCMVTag-2A/B/C and p53-luciferase (p53-Luc) reporter plasmids were purchased from Stratagene. Reporter vector pGL2-Basic and pGL2-Control were purchased from Promega. pCMVp plasmids were purchased from CLONTECH. p53 expression vector (pCMVTag-2C-p53) was constructed by inserting the rat p53 coding region in-frame into pCMVTag-2C. Chk1 and Cil expression plasmids were constructed by inserting PCR products of the coding region in-frame into pCMVTag2B vector (Stratagene) and confirmed by DNA sequencing. Expression of the corresponding proteins was confirmed by Western blot analysis using anti-FLAG M2 antibody (Upstate Biotechnology, Inc.). The reporter plasmids of Cil promoter were constructed by inserting different fragments amplified from the putative promoter region and inserted into the pGL2-Basic plasmid. The primers

1 The abbreviations used are: RACE, rapid amplification of cDNA ends; RT, reverse transcription; GST, glutathione S-transferase.
used for constructing these promoter reporter plasmids were: Chk1 g-05, Chk1 g-06, Chk1 g-16, Chk1 g-19, Chk1 g-20, Chk1 g-21, Chk1 g-22, Chk1 g-29, and Chk1 g-30.

**Western Blot Analysis**—Tissues from Sprague-Dawley rats were homogenized in lysis buffer (50 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 0.1% bromphenol blue, 0.1 mM dithiothreitol, 10% glycerol) using a Polytron homogenizer, cleared by centrifugation at 14,000 × g for 20 min, and stored frozen at -70°C. For Western blot analysis, 20 µl of protein extracts were denatured by boiling for 5 min and loaded in a 12% SDS-polyacrylamide gel in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). After electrophoresis the proteins were transferred to a Hybond-C membrane (Amersham Biosciences, Inc.). The blot was blocked with 5% nonfat milk in TBS-T buffer (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) for 1 h and incubated with primary antibody against the C-terminal portion of Chk1 (custom-produced by Zymed Laboratories Inc. against an oligopeptide in the C-terminal domain of Chk1) for 1 h and washed twice in TBS-T buffer. The immunoblot was then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase and analyzed using the ECL system according to the instructions of the manufacturer (Amersham Biosciences, Inc.). For analysis of Chk1 Ser-345 phosphorylation, Western blots of proteins from cells transfected with Chk1 and/or Cil expression plasmids with or without UV irradiation were probed with antibody specifically against Ser-345-phosphorylated Chk1 (Cell Signaling).

**Reporter Gene Assays**—Typically cells in a 60-mm tissue dish were transfected with 2 µg of reporter plasmids and 1 µg of pCMVβGal (CLONTECH) as internal standard. Cells were harvested 48 h after transfection, washed twice in phosphate-buffered saline, and lysed with 400 µl of reporter lysis buffer in a luciferase assay system (Promega). Cell lysates were collected and centrifuged briefly. For the luciferase activity assay, cell lysates were freeze-thawed and centrifuged at 12,000 × g for 5 min, 10-µl aliquots were loaded into the AutoLumat LB953 (EG&G Berthold), and 100 µl of luciferase substrate were injected into each sample. The luminescence obtained was normalized against β-galactosidase activity. To assay the transfection efficiency, 30 µl of each supernatant were assayed for β-galactosidase activity by adding 3 µl of 100× magnesium solution (0.1 µM MgCl₂, 4.5 mM β-mercaptoethanol), 66 µl of an o-nitrophenyl β-D-galactopranoside solution (4 mg/ml o-nitrophenyl β-D-galactopranoside in 0.1 M sodium phosphate, pH 7.5), and 20 µl of sodium phosphate buffer (0.1 M, pH 7.5). The solution was incubated at 37°C for 30 min or until yellow color was developed. The reaction was stopped by adding 500 µl of 1 M sodium carbonate, and optical absorption at 420 nm was measured.

**Northern Blot and RT-PCR Analysis**—A Multi-Tissue RNA blot containing 2 µg of poly(A) mRNA from various normal rat tissues was purchased from CLONTECH and probed with Chk1 probe according to the procedure of the manufacturer. Total RNA was extracted from rat liver using cultured cells using the RNaseasy Total RNA kit (Qiagen). For RT-PCRs, 1 µg of total RNA was treated with RNase-free DNase (Invitrogen) for 15 min. DNase was inactivated by adding 1 µl of 25 mM EDTA and heated at 65°C for 10 min. 500 ng of oligo(dT)₁₂₋₁₈ was added, and the first-strand cDNA was synthesized using 200 units of SUPERSCRIPT II reverse transcriptase (Invitrogen) for 50 min at 42°C. The reaction was terminated by heating at 70°C for 15 min. Subsequent PCRs were carried out using primers specific to Chk1 or Cil. A rat multiple tissue cDNA panel used as a template for PCR to confirm tissue-specific expression of Cil was purchased from Promega.

**Yeast Two-hybrid Analysis**—The Gal4-based MATCHMAKER Two-hybrid System II (CLONTECH) was used for two-hybrid analysis. Plasmids pAS2-1 and pACT2, encoding the Gal4 DNA-binding domain (Gal4-BD) and Gal4-activating domain (Gal4-AD), respectively, were used as expression hybrid proteins. The yeast strain AH109 was co-transformed with pAS2-1-Chk1KD and pACT2-Cil using the lithium acetate method (28, 29). Transformants were selected on synthetic dropout agar plates lacking tryptophan, leucine, adenine, and histidine. The positive yeast colony was transferred onto Whatman no. 5 paper and processed with β-galactosidase filter assay. Negative controls were performed by co-formation of empty vector pAS2-1 with pACT2, pAS2-1 empty vector, pACT2 empty vector, Cil, pACT2-Cil with pVA3-1, which express fusion protein of GAL4-DB and murine p53 (amino acids 72–390).

**In Vitro Transcription-Translation and GST Pull-down Assay**—In vitro transcription-translation was performed using the TNT Coupled Reticulocyte Lysate System kit from Promega according to the instructions of the manufacturer. For each reaction, 1 µg of plasmid DNA was used in a 50-µl total reaction volume. An amino acid mixture minus methionine was added to each reaction, and reactions were performed in the presence of [35S]methionine (Amersham Biosciences, Inc.). For GST pull-down assays, glutathione-Sepharose beads (Amersham Biosciences, Inc.) were prewashed in NETN (0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol) containing protease inhibitors (Roche Molecular Biochemicals) and 0.5% powdered milk. For GST pull-down assay, the bacterial lysate were purified with the prewashed glutathione-Sepharose beads (25 µl of beads/ml of lysate) by incubation for 1.5 h at 4°C on a rotary mixer. The beads were collected by centrifugation, washed four times with NETN + P (NETN containing protease inhibitors), and resuspended in 1 volume of NETN + P prior to use. 50 µl of beads, containing GST fusion proteins or GST alone (negative control), were incubated overnight at 4°C in tubes containing 935 µl of NETN + P and 5 µl of the in vitro-translated 35S-labeled protein. The beads were then washed four times with NETN, resuspended in 50 µl of 1× protein loading buffer, and boiled for 5 min followed by SDS-PAGE analysis. The gel was dried, and radiolabeled protein was detected by autoradiography.

**RESULTS**

**Cloning of Rat Liver-specific Chk1 Isoform**—We have previously cloned and sequenced rat Chk1 cDNA (Fig. 1). During our analysis of tissue expression of Chk1 gene expression in rat we noticed a second RNA band specifically observed only in the liver tissue (Fig. 2A). We then cloned this shortened Chk1 RNA species from rat liver using 5′- and 3′-RACE techniques based on a primer derived from the rat Chk1 sequence. DNA sequencing of the clones obtained showed that the liver-specific Chk1 isoform, or Cil in short, contained the exon 6 to 3′-untranslated region of rat Chk1 gene linked to a 90-bp unknown sequence at its 5′-end. Sequence analysis of the intron between exons 5 and 6 of the Chk1 genomic sequence in rat showed that the novel 5′
sequence was derived from the internal portion of this intron. The 3'-boundary of the intron-derived sequence abuts the splicing donor consensus sequence GT, consisting with splicing of this sequence with exon 6 (Fig. 3). To confirm the existence of this mRNA species in rat liver we also used the Chk1 sequence to clone the isoform cDNA from the rat liver cDNA library. Clones obtained this way contained a sequence consistent with the RACE-derived clones. A protein product with the molecular weight predicted from the isoform RNA was also observed in Western blot analysis in the adult liver tissue and to a lesser extent in the fetal liver of an 18-day embryo (Fig. 4). A Chk1-related isoform was also observed in the kidney tissue, but its nature is still under investigation.

Analysis of Tissue-specific and Development-specific Expression of Cil—To further confirm liver-specific expression, we examined Cil expression in different rat tissues by RT-PCR. When both Chk1- and Cil-specific primers were used, two RT-PCR products were observed in the liver tissue, corresponding to the expected products of Chk1 and Cil, respectively (Fig. 5A). The other tissues contained only one band corresponding to that of Chk1. This result confirmed the liver-specific expression pattern we first observed in the Northern blot. The identity of the second band in the RT-PCR product as Cil was confirmed by DNA sequencing. We next examined the expression of Cil during embryo development. Total RNA was extracted from rat livers of 18-day fetal liver, liver from days 1 and 5 postnatal liver, adult liver, and adult spleen. RT-PCR analysis showed that Cil was expressed at a low amount in the 18-day fetal liver, but the level of expression increased to adult level after birth (Fig. 5B). Again no Cil isoform was observed in the spleen.

Fig. 3. Panel A, cDNA sequence of rat Cil and its corresponding predicted protein sequence. Panel B, schematic representation of rat Chk1 and Cil. A 90-bp sequence at the 5'-end of Cil is located in intron 5 and spliced to exon 6. E, exon; UTR, untranslated region.
RT-PCR analysis of Chk1 and Cil expression in rat tissues and cultured cells. Panel A, tissue distribution of Cil was examined using the rat multissue cDNA panel (CLONTECH). Polymerase chain reaction was performed for 30 cycles using primers specific for Chk1 (2-6-31 and 2-6-15) and Cil (Cil-2 and 2-6-15), and nested PCR was performed for 20 cycles using nested primers specific for Chk1 (2-6-1 and 2-6-15) and Cil (Cil-2 and 2-6-15). Panel B, RT-PCR analysis of RNA extracted from various rat tissues as indicated in the figure. RT-PCR was performed using cDNA derived from 1.0 μg of total RNA, and PCR was performed for 30 cycles using primers specific for Chk1 (2-6-1 and 2-6-15) and Cil (2-6-15 and Cil-5). Panel C, RT-PCR analysis of Chk1 and Cil expression in three rat hepatoma cell lines. The positions of Chk1 and Cil products are indicated on the right of the panels. E18, embryonic day 18; P1, postnatal day 1; P5, postnatal day 5.

Fig. 4. Chk1 and Cil protein expression in rat tissues. Immunoblotting analysis was performed on adult rat heart, liver, kidney, and embryonic day 18 (E18) fetal liver. The protein blot was probed with antibody against the Chk1 C-terminal domain. Molecular mass markers are in units of kilodaltons.

Analysis of Promoter Activity of Sequences 5’ to the First Exon of Cil—The sequence of Cil suggests a promoter 5’ to the first exon, somewhere in the fifth intron of the Chk1 gene. To examine this possibility we assayed the promoter activity of DNA sequences immediately adjacent to the 5’-end of Cil using the luciferase reporter gene system. As shown in Fig. 6, a 300-nucleotide fragment upstream of the first exon of Cil was found to contain promoter activity when assayed for luciferase reporter gene expression. Deletion analysis indicated that the promoter activity resided in the 3’ half of the 300-bp fragment. Sequence analysis of the 300-bp region revealed three potential transcription factor-binding sites for AP-2, SP1, and the liver-specific LF-A1 transcription factor (30–32). Deletion of the LF-A1 binding sequence reduced the promoter activity by 30–40%.

Phosphorylation of Cil following UV Damage—Since Chk1 has been shown to be activated after DNA damage by the phosphorylation at serines 317 and 345 located in the C-terminal domain (22), we examined whether Cil could also be phosphorylated at serine 63 (corresponding to Ser-345 of Chk1) following UV irradiation. We used the commercial antibody that specifically recognizes the Chk1 peptide phosphorylated at serine 345 to examine the phosphorylation status of Cil in Cil-transfected cells before and after UV irradiation. As shown in Fig. 7, no phosphorylation was found in Chk1 without UV irradiation, but the phosphorylated form became detected after UV irradiation as expected, albeit at a low level. Cil, on the other hand, showed a low level of phosphorylation even before UV irradiation, but the phosphorylated form became detected after UV irradiation as expected, albeit at a low level. Cil, on the other hand, showed a low level of phosphorylation even before UV irradiation, but the phosphorylation was enhanced after UV treatment. These results indicate UV-induced phosphorylation of both Chk1 and Cil at the site corresponding to Ser-345 in Cil. When Chk1 was co-transfected with Cil its phosphorylation was found to be enhanced relative to the transfection of Chk1 alone. This result suggests that Cil enhanced UV-activated phosphorylation of Chk1.

ATR kinase has been shown to be involved in the phosphorylation of Chk1 at Ser-345. Since ATM/ATR kinase is sensitive to the inhibition by caffeine (33, 34), we analyzed whether the phosphorylation of Cil after UV irradiation could be blocked by caffeine. Treatment of cells after UV irradiation with 2 mM caffeine blocked the phosphorylation of both Chk1 and Cil (Fig. 7), consistent with the interpretation that both proteins were phosphorylated by ATR kinase.

Suppression of p53 Transactivation Activity by Phosphorylated Cil—Chen et al. (27) have shown recently that the kinase domain fragment of Chk1 is 20 times more active in kinase activity in vitro than the full-length Chk1. They suggested that...
the C-terminal portion of Chk1 might function as a negative regulator of Chk1 kinase activity. Since Cil contained only the C-terminal sequences of Chk1 without the kinase domain, we suspected that Cil might function as a competitive inhibitor of Chk1. Because Chk1 has been shown to phosphorylate p53 and to regulate the amount of p53 in a co-transfection experiment (20), we tested whether Cil could inhibit the transactivation activity of p53. We used the luciferase p53 transactivation assay to examine this possibility. As shown in Fig. 8, co-transfection of plasmid containing the Chk1 gene in the p53 transactivation assay boosted the p53 transactivation activity 50%. This result is similar to that obtained previously (20). In contrast, Cil was found to reduce the reporter activity by about 50%. Furthermore, the stimulation of reporter gene activity by Chk1 was abolished by co-transfection with Cil, suggesting that Cil was dominant over Chk1 in this activity assay. Since Cil serine 63 (corresponding to Chk1 serine 345) was phosphorylated even without UV treatment, we analyzed the effect of eliminating Cil serine 63 phosphorylation in the inhibition of p53 transactivation activity. As shown in Fig. 8, mutation of Cil serine 63 to alanine resulted in the total loss of the Cil-mediated inhibition of p53 transactivation activity. On the other hand, serine 35 (corresponding to Chk1 Ser-317, another site phosphorylated in vivo) to alanine mutation resulted in a less inhibitory effect on p53 transactivation activity. These results indicated that serine 63 in Cil played an important regulatory role in the function of Cil.

Evidence for the Interaction of Cil with the Kinase Domain of Chk1—Based on crystallographic analysis and in vitro analysis of Chk1 kinase activity, Chen et al. (27) recently suggested that the C-terminal domain of Chk1 could negatively interact with the kinase domain. Because the coding sequence of Cil resides in the C-terminal domain of Chk1, we sought to determine whether Cil could interact with the kinase domain of Chk1. To this end, we cloned the Chk1 kinase domain and analyzed the interaction with Cil by the yeast two-hybrid assay. The Chk1 kinase domain indeed was found to interact with Cil (Fig. 9), whereas no interaction was found between Chk1 and Cil (data not shown). To further demonstrate the interaction between Cil and the Chk1 kinase domain we used an in vitro transcription-
transcription factors AP-2, Sp1, and liver-specific LF-A1. The presence of the liver-specific LF-A1 transcription factor-binding site supports the liver-specific expression of Cil. Deletion of this transcription factor-binding site reduced the promoter activity in rat HTC1 cells. Since this cell line only expressed a low amount of Cil, we believe that this cell line is probably not the best host for studying the regulation of the Cil promoter. Perhaps primary hepatocytes expressing a significant amount of Cil would serve as the appropriate host for studying the regulation of the Cil promoter.

The Cil coding region starts at amino acid 283 of the rat Chk1 protein. This start site is located in the flexible linker between the kinase and the C-terminal domains (27) in the Chk1 protein. Thus Cil is completely devoid of the kinase domain of Chk1 and contains only the C-terminal domain. Since the C-terminal part of Chk1 has been implicated in the negative regulation of Chk1 (27), we analyzed the interaction of Cil and the kinase domain using the yeast two-hybrid assay as well as an in vitro pull-down assay. These analyses indeed showed that Cil could interact with the kinase domain. Chk1 has been shown to enhance the p53 level when overexpressed by transient transfection with or without treatment of ionizing radiation (20). In our studies, overexpression of Chk1 enhanced p53 transactivation activity, but Cil exhibited the opposite effect. These data suggested that Cil might serve as a negative regulator in the Chk1-p53 signaling pathway.

The finding that a possible negative regulator of Chk1 kinase activity is present only in the liver tissue is rather intriguing. Liver differs from other tissues in having a very high proportion of polyploid cells. Rat liver is especially rich in cells with polyploidy (37, 38) with an estimation up to 85% (39–41). On the other hand, fetal and liver tumors are composed mainly of diploid cells (37, 39, 42). Since Chk1 is involved in cell cycle regulation, liver-specific expression of Cil may be related to the unique hepatocyte polyploidization. This possibility remains to be verified in the future. We are constructing inducible plasmids for analyzing the effect of Cil on cell cycle and nuclear polyploidization with or without damage to DNA or stalling of DNA replication.

We have searched a human liver cDNA library for the human counterpart of Cil with no success. Examination of the human intron sequence corresponding to the one in the rat genome containing the Cil promoter showed no obvious sequence similarity between the two species in this part of the gene. RT-PCRs using several possible candidates from human intron sequences with patched homology with the rat sequence and the conserved C-terminal domain sequence as primers did not reveal any Cil-like products in a human cDNA library or in mRNA extracted from human liver. Whether Cil is unique to rat or whether a different isoform in human liver exists will be the subject of future studies.

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**REFERENCES**

1. Walworth, N. C. (2001) Curr. Opin. Cell Biol. 11, 78–82
2. Canman, C. E. (2001) Curr. Biol. 11, R121–R124
3. Clarke, D. J., and Gimenez-Arjona, J. F. (2000) Bioessays 22, 551–563
4. Kitazono, A., and Matsumoto, T. (1998) Bioessays 20, 391–399
5. O’Connell, M. J., Walworth, N. C., and Carr, A. M. (2000) Trends Cell Biol. 10, 296–303
6. Zhou, B. B., and Elledge, S. J. (2000) Nature 408, 433–439
7. Curman, D., Cinel, B., Williams, D. E., Rundle, N., Block, W. D., Goodarzi, A. A., Hutchins, J. R., Clarke, P. R., Zhou, B. B., Lees-Miller, S. P., Andersen, R. J., and Roberge, M. (2001) J. Biol. Chem. 276, 17914–17919
8. Nigg, E. A. (1995) Bioessays 17, 471–480
9. Lew, D. J., and Kurklutsch, S. (1996) Curr. Opin. Cell Biol. 8, 795–804
10. Rhind, N., and Russell, P. (2000) J. Cell Sci. 113, 3889–3896
11. Morgan, D. O. (1997) Ann. Rev. Cell Dev. Biol. 13, 261–291
12. Ohi, R., and Gould, K. L. (1999) Curr. Opin. Cell Biol. 11, 267–273
13. Sanchez, Y., Wong, C., Thoma, R. S., Richman, R., Wu, Z., Piwnica-Worms, H., and Elledge, S. J. (1997) *Science* **277**, 1497–1501
14. Furnari, B., Rhind, N., and Russell, P. (1997) *Science* **277**, 1495–1497
15. Zeng, Y., Forbes, K. C., Wu, Z., Moreno, S., Piwnica-Worms, H., and Enoch, T. (1998) *Nature* **395**, 507–510
16. Walworth, N., Davey, S., and Beach, D. (1993) *Nature* **363**, 368–371
17. Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997) *Science* **277**, 1501–1505
18. Lee, J., Kumagai, A., and Dunphy, W. G. (2001) *Mol. Biol. Cell* **12**, 551–563
19. O’Connell, M. J., Raleigh, J. M., Verkade, H. M., and Nurse, P. (1997) *EMBO J.* **16**, 545–554
20. Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000) *Genes Dev.* **14**, 289–300
21. Kaneko, Y. S., Watanabe, N., Morisaki, H., Akita, H., Fujimoto, A., Tominaga, K., Terasawa, M., Tachibana, A., Ikeda, K., Nakanishi, M., and Kaneko, Y. (1999) *Oncogene* **18**, 3673–3681
22. Zhao, H., and Piwnica-Worms, H. (2001) *Mol. Cell. Biol.* **21**, 4129–4139
23. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donehower, L. A., and Elledge, S. J. (2000) *Genes Dev.* **14**, 1448–1459
24. Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000) *Science* **288**, 1425–1429
25. Yin, M. B., Guo, B., Vanhoefer, U., Azrak, R. G., Minderman, H., Frank, C., Wrozek, C., Slocum, H. K., and Rustom, Y. M. (2000) *Mol. Pharmacol.* **57**, 453–459
26. Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y. A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakaniishi, M., and Nakayama, K. (2000) *Genes Dev.* **14**, 1439–1447
27. Chen, P., Luo, C., Deng, Y., Ryan, K., Register, J., Margosiak, S., Tempczyk, R., Nguyen, B., Myers, P., Lundgren, K., Kan, C. C., and O’Connor, P. M. (2000) *Cell* **100**, 681–692
28. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) *Nucleic Acids Res.* **20**, 1425
29. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
30. Hardon, E. M., Frain, M., Panessa, G., and Cortese, R. (1988) *EMBO J.* **7**, 1711–1719
31. Vautont, S., Puzenat, N., Levrat, F., Cognet, M., Kahn, A., and Raymondjean, M. (1989) *J. Mol. Biol.* **209**, 205–219
32. Ramji, D. P., Tadros, M. H., Harden, E. M., and Cortese, R. (1991) *Nucleic Acids Res.* **19**, 1139–1146
33. Hall-Jackson, C. A., Cross, D. A., Morrice, N., and Smythe, C. (1999) *Oncogene* **18**, 6707–6713
34. Sarkaria, J. N., Buxby, E. C., Bibet, R. S., Roos, P., Taya, Y., Karnitz, L. M., and Abraham, R. T. (1999) *Cancer Res.* **59**, 4375–4382
35. Galgozcy, P., Rosenthal, A., and Platzer, M. (2001) *Gene (Amst.)* **271**, 93–98
36. Aronow, B., Lattier, D., Silbiger, R., Dasing, M., Hutton, J., Jones, G., Stock, J., McNeil, J., Potter, S., and Witte, D. (1989) *Genes Dev.* **3**, 1384–1400
37. Sigal, S. H., Gupta, S., Gehbard, D. F., Jr., Holst, P., Neufeld, D., and Reid, L. M. (1995) *Differentiation* **59**, 35–42
38. Anatskaya, O. V., Vinogradov, A. E., and Kudryavtsev, B. N. (1994) *J. Theor. Biol.* **168**, 181–199
39. Sanz, N., Diez-Fernandez, C., and Cascales, M. (1996) *Biochim. Biophys. Acta* **1315**, 123–130
40. Seglen, P. O. (1997) *Cell Biol. Toxicol.* **13**, 301–315
41. Saeter, G., Lee, C. Z., Schwarze, P. E., Ous, S., Chen, D. S., Sung, J. L., and Seglen, P. O. (1988) *J. Natl. Cancer Inst.* **80**, 1480–1485
42. Seglen, P. O., and Gerlyn, P. (1990) *Environ. Health Perspect.* **88**, 197–205
