Chemical transformation of mouse liver cells results in altered cyclin D–CDK protein complexes

Andrea J. Gonzales1,3,6, Thomas L. Goldsworthy2,4 and Tony R. Fox2,5

1 Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC 27599-7270 and 2Chemical Industry Institute of Toxicology, 6 Davis Drive, PO Box 12137, Research Triangle Park, NC 27709, USA

Present addresses: 3Department of Cancer Research, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105, 4Integrated Laboratory Systems, PO Box 13501, Research Triangle Park, NC 27709 and 5Glaxo-Wellcome Inc., TOX-T1130, 5 Moore Drive, Research Triangle Park, NC 27709, USA

To whom correspondence should be addressed

Introduction

Chemical carcinogenesis is a multistage process characterized in part by gradual loss of controls regulating cell growth and division. Many chemical carcinogens involved in this process are classified as genotoxic agents, which are capable of directly interacting with and disrupting the integrity of genomic DNA. These agents are believed to play a role in the carcinogenic process by causing mutations in key proto-oncogenes or tumor suppressor genes (1). To date a complete understanding of the cellular components altered as a result of direct or indirect gene mutations by these agents is still lacking. We propose that proteins regulating cell cycle progression represent viable targets that may be altered and involved in the multistep process of genotoxic chemical carcinogenesis.

Normal cell cycle progression is dependent on the activity of serine/threonine kinases known as cyclin-dependent kinases (CDKs) (2). These kinases associate with a class of proteins called cyclins to form holoenzymes in which the CDK functions as the catalytic domain and the cyclin acts as the regulatory domain (3,4). In higher eukaryotes multiple cyclins and CDKs are categorized into two families: the p21 family and the p16 family. Members of the p21 family include p21WAF1, p27KIP1, PCNA, a well-characterized molecule that serves as an auxiliary protein to DNA polymerase δ functions as a DNA replication and repair factor (14). The associating 21 kDa protein has since been found to be a universal inhibitor of CDKs (15–17) that can be transcriptionally activated by the p53 gene product (18–20). Numerous CDK inhibitors (CDIs) have been discovered since the finding of p21WAF1 (21–25). These CDIs have been categorized into two families: the p21 family and the p16 family. Members of the p21 family include p21WAF1, p27KIP1 (26,27) and p57KIP2 (28,29). These proteins bind to cyclin–CDK complexes and inhibit kinase activity in vitro. Overexpression of these molecules in vivo leads to G1 arrest. The p16 family members p15INK4b (30), p16INK4a (31,32), p18INK4c (33) and p19INK4d (34) preferentially bind to CDK4 or CDK6 to form binary complexes. They function in part to sequester these CDKs and prevent formation of active G1 cyclin D1–CDK protein complexes.

Dysregulated cell proliferation is one phenotypic change associated with neoplasia. Key protein complexes involved in regulating cell division are composed of cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (CDI). Many virally transformed cells in culture exhibit disrupted cyclin–CDK–CDI complexes, suggesting that such changes may play a mechanistic role in viral transformation. To determine whether similar alterations may be involved in chemical carcinogenesis we characterized cyclin D1–CDK–CDI protein complexes in a non-tumorigenic mouse liver cell line and investigated whether complexes were altered after transformation with the genotoxic carcinogens N-methyl-N′-nitro-N-nitrosoguanidine (MNG) or 3-methylcholanthrene (MC). In non-tumorigenic mouse liver cells cyclin D1 associated with CDK6, CDK4 or CDK2 to form binary (cyclin D1–CDK), tertiary (cyclin D1–CDK–p21WAF1) or quaternary (cyclin D1–CDK–p21WAF1–PCNA) complexes. After chemical transformation of mouse liver cells with either MC or MNG, select cyclin D1–CDK–CDI protein complexes were altered. In MC-transformed cells formation of various binary, tertiary and quaternary cyclin D1–CDK–CDI protein complexes was reduced, resulting in decreased CDK4 kinase activity. Interestingly, CDK6 kinase activity was dramatically elevated due to high levels of cyclin D3 in association with CDK6. In MNG-transformed cells select cyclin D1–CDK6–CDI and cyclin D1–CDK2–CDI protein complexes were altered but CDK6 and CDK4 kinase activity remained unaffected. Distinct changes in cyclin D1–CDK–CDI complexes found between the two chemically transformed mouse liver cell lines suggest that each cell line harbored unique mutations or alterations that differentially contributed to stabilization of cyclin D1–CDK–CDI holoenzymes. p53 gene mutations were not detected in the MC- or MNG-transformed mouse liver cell lines and thus were not involved in disrupting cyclin D1–CDK–CDI protein complexes. In summary, this study presents evidence that D-type CDK protein complexes can be altered physically and functionally after chemical transformation with genotoxic carcinogens, suggesting that components of the cell cycle machinery can be targeted during chemical carcinogenesis.

© Oxford University Press
Analyses of quaternary complexes in virally transformed human fibroblasts and epithelial cells revealed that these holoenzymes are disrupted or rearranged after transformation (35). Cyclin D1–CDK4–p21WAF1–PCNA complexes are rearranged and replaced with a CDK4–p16INK4a binary complex. p21WAF1 and PCNA completely dissociate from other quaternary complexes, leaving binary complexes of cyclin A–CDK2 and cyclin B1–CDC2 intact. Subsequent studies showed that expression of specific viral oncoproteins (SV40 T antigen or HPV E6 and E7) can inactivate the tumor suppressor gene products p53 and retinoblastoma protein (pRb) and lead to similar changes in quaternary complexes prior to neoplastic transformation of normal human fibroblasts (36,37). These findings suggest that perturbations in biochemical pathways regulating formation of CDK protein complexes may play a causal role in cellular transformation by inducing dysregulated cell cycle progression and genomic instability.

Currently little is known about the cellular components that can be altered as a consequence of genetic mutations caused by genotoxic chemical carcinogens. Therefore, the present study was conducted to investigate whether transformation of cells by 3-methylcholanthrene (MC) and N-nitro-N-nitrosoguanidine (MNN) can lead to alterations in formation of CDK protein complexes. We focused on investigating changes in cyclin D1–CDK protein complexes since these holoenzymes play an important role in integrating growth factor pathways with progression into the cell cycle (4). We also chose to use mouse liver cells in our study since this cell type is the most common target in rodents for chemical carcinogenesis (38), and a significant amount of research has been conducted on chemically induced hepatocarcinogenesis. Initial studies involved characterizing D1-like type CDK protein complexes in a non-tumorigenic mouse liver cell line (BNL CL.2). We found that cyclin D1 associated with CDK6, CDK4 or CDK2 to form several types of cyclin D1–CDK–CDI protein complexes. After transformation of the BNL CL.2 mouse liver cell line with either MC or MNN we observed that the composition of select cyclin D1–CDK–CDI protein complexes was altered, and in some cases kinase activity was affected.

Materials and methods

Cell culture

A mouse liver cell line (BNL CL.2) established from BALB/c embryonic liver cells was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and used for characterization of CDK protein complexes. 3-Methylcholanthrene epoxide-transformed (BNL 1ME A.7R.1), MNN-transformed (BNL 1NG A.2) and SV40-transformed (BNL SV A.8) liver cells, all of which were derived from BNL CL.2 cells, were also available from ATCC and used to assess changes in CDK protein complexes after chemical and viral transformation. All four cell lines were established and characterized by Patek et al. (38). The BNL CL.2 cells are immortalized mouse liver cells which show no tumor cell phenotype, as measured by anchorage-independent growth and in vivo tumorigenicity. MC-, MNN- and SV40-transformed liver cells, however, are capable of growing in soft agar and forming tumors in immunodeficient mice (39,40). All cell lines were grown at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 0.3% yeast extract (Gibco BRL).

Antibodies

Affinity-purified rabbit polyclonal anti-CDK2 (M2), anti-CDK4 (C-22), anti-CDK6 (C-21), anti-p21WAF1 (C-19), anti-p15INK4b (M-20), anti-cyclin D1 (72/13G), anti-cyclin D3 (C-16) and mouse monoclonal anti-PCNA (PC10) antibodies were obtained from Santa Cruz (Santa Cruz, CA) for use in immunoprecipitation reactions and Western blotting procedures. The rabbit polyclonal anti-cyclin D (Upstate Biotechnologies Inc., Lake Placid, NY) and anti-p27kip1 (M-197; Santa Cruz) were used only as immunoprecipitating antibodies, and the mouse monoclonal anti-cyclin D1 (72/13G; Santa Cruz) and anti-p27kip1 (Transduction Laboratories, Lexington, KY) were used exclusively in Western blotting procedures.

25S metabolic labeling, immunoprecipitation of CDK protein complexes and Western blotting

Initial studies involved labeling cellular proteins with [35S]methionine so that immunoprecipitating proteins could be visualized. Subconfluent cells in culture (60–80%) were preincubated in methionine/cysteine-free DMEM (Gibco) supplemented with 0.2% dialyzed fetal bovine serum for 30 min at 37°C in 5% CO2 before [35S]methionine (PRO-MIX 35 S cell labeling mix, sp. act. ~1000 Ci/ml; Amersham) was added directly to the medium (final concentration 1000 Ci/ml). Cells were incubated at 37°C in a 5% CO2 chamber. After labeling, plates were placed on ice, the medium was removed and the cells were washed three times with cold phosphate-buffered saline (PBS). Lysates were prepared and concentration of protein in the lysates was determined (BioRad Bradford Assay). Two milligrams of protein lysate were incubated with primary antibody for 2 h at overnight at 4°C. ImmunoPure immunobilized protein A–agarose beads (Pierce Chemical Co., Rockford, IL) were added to immunoprecipitation reactions to adsorb the antigen–antibody complexes. After a 1 h incubation immunoprecipitates were collected by centrifugation (500 g) and washed four times (50 mN Tris base, pH 7.4, 150 mM NaCl, 0.5% NP-40, 50 mM NaF; 1 mM Na3VO4; 1 mM DTT and 1 mM PMFS). Antigen–antibody complexes were dissociated by resuspending beads in SDS sample buffer (2.5% SDS, 25% glycerol, 25 mM Tris base, pH 8.0, 2.5 mM EDTA, 0.1 M DTT and pyronin Y). Samples were boiled at 100°C for 5 min and separated on 17.5% SDS–polyacrylamide gels. Gels containing radiolabeled proteins were fixed for 30 min (10% glacial acetic acid and 25% isopropanol) then enhanced for 30 min with a fluorographic reagent (Amphlity; Amersham Life Science Inc., Arlington Heights, IL). Gels were dried and exposed to Hyperfilm-MP (Amersham) at –80°C.

To verify the identity of bands visualized from the [35S]methionine-labeled immunoprecipitates, reactions were performed without radiolabeling the proteins. These precipitates were also separated on a 15.7% SDS–polyacrylamide gel but then transferred to a polyvinyl membrane (PVDF, Immobilon-P; Millipore) for Western blotting. Transfer of proteins to a polyvinyl membrane was achieved using a BioRad Trans-Blot Cell. PVDF membranes were blocked for 1 h to overnight with 5% non-fat dry milk in TBS containing 0.1% Tween 20. After blocking membranes were washed with PBS/Tween 20 (PBS-T) then incubated with primary antibody (0.1–1 µg/ml primary antibody diluted in PBS-T) for 1 h to overnight. Membranes were washed and exposed to the appropriate secondary antibody (1:10 000 dilution of either sheep anti-mouse or donkey anti-rabbit immunoglobulin coupled to horseradish peroxidase) (Amersham). Specific proteins were detected using an enhanced chemiluminescence system from Amersham. At least three independent experiments were conducted for all radiolabeled immunoprecipitation reactions and immunoprecipitations coupled with Western blotting. Almost identical results were produced each time.

CDK6/CDK4 kinase assay

Subconfluent cells in culture (60–80%) were rinsed three times with cold PBS while on ice. Kinase assay lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 10, 10 mM glycerophosphate, 1 mM NaF; 0.1 mM Na3VO4; 1 mM DTT, 1 mM PMFSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 2 µg/ml peptatin A) was added directly to the plates (2 ml/150 mm dish). Cells were then scraped, transferred to a chilled microfuge tube and frozen immediately in liquid nitrogen. Frozen lysates were thawed and placed on ice for 2 h. Samples were spun for 15 min at 15 000 g to pellet cellular debris and chromosomal DNA. Supernatants were removed and incubated with protein A beads (Sigma). Samples were rotated for 1 h at 4°C, they were spun for 3 min at 500 g to pellet the beads. Supernatants were collected and protein concentrations were determined (Pierce BCA Protein Assay). To immunoprecipitate CDK6 or CDK4 protein complexes from collected cellular lysates collected from 500 µl of lysate, 500 µl of lysis buffer was incubated with 2 µg of either anti-CDK6 or anti-CDK4 rabbit polyclonal antibodies. Immunopure immunobilized protein A–agarose beads (Pierce Chemical Co., Rockford, IL) were added to immunoprecipitation reactions to adsorb the antigen–antibody complexes. After a 1 h incubation immunoprecipitations were collected by centrifugation (500 g) and analyzed pleated for kinase activity in kinase assay lysis buffer, then once in kinase assay reaction buffer (50 mM
HEPES, pH 7.5, 10 mM MgCl₂, and 1 mM DTT). After removing the final wash, 12 µl kinase reaction mix (5 µl [γ-β³²P]ATP, 10 µM ATP and 1 µg GST–pRb; a gift from Onyx Pharmaceuticals, Richmond, CA) was added to each tube. Samples were mixed briefly then spun gently before incubating the tubes at 30°C for 30 min. Kinase reactions were terminated by addition of SDS sample buffer. Samples were boiled for 5 min, cooled, then loaded onto a 15% SDS–polyacrylamide gel. Gels were dried for 1.5 h at 80°C then exposed to a phosphor screen (Molecular Dynamics). Images were quantitated using ImageQuant v.3.3 (Molecular Dynamics). At least three independent experiments were conducted to assess kinase activity of CDK6 and CDK4. A two-tailed paired Student's t-test was conducted to determine whether the mean kinase activity for chemically and virally transformed liver cells differed from the mean values obtained for the non-tumorigenic liver cell line BNL CL.2.

Mouse p53 gene sequencing

Total RNA was isolated from BNL CL.2, BNL 1ME A.7R.1 and BNL 1NG A.2 liver cell lines using the RNAzol™ B method. Reverse transcription reactions were performed using Superscript™ II reverse transcriptase (Gibco BRL) and followed the manufacturer's instructions. One microliter of the cDNA reaction was used as template to amplify reactions were performed using PCR. Reactions consisted of 50 pmol each MC01 and MC02 primers, described in Sun et al. (40), PCR buffer (Perkin Elmer), 200 µM each dNTP (Gibco BRL) and 1.25 U AmpliTaq DNA polymerase (Perkin Elmer). The PCR reaction was heated to 94°C for 2 min before 30 cycles of melting (94°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 45 s) were completed. A final incubation was at 72°C for 7 min was performed to complete any extensions. The PCR reaction yielded a 1246 bp fragment that flanked the entire coding region of the p53 cDNA. PCR reactions were spun through Centricon-100 concentrator columns (Perkin Elmer) to remove primers. Two microfilters of the purified and concentrated fragment were then used in a cycle sequencing reaction using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit. The following primers were used in separate cycle sequencing reactions for each cell line: MC01, MC02, MC08, MC03, MC04, MC05 and MC06 (see 40 for primer sequences). Cycle sequencing reactions were carried out on a Perkin Elmer Cetus GeneAmp PCR System 9600. Each reaction generated a product that was purified by ethanol precipitation and resolved in loading buffer (50 mM EDTA). Samples were run on an ABI 373 DNA Sequencer. Data were analyzed using Factura and Autoassembler (Applied Biosystems Divisions).

Population doubling times and percentage in S phase determinations

Population doubling times were determined by plating 2×10⁴ cells/100 mm culture dish. Every 24 h cells were trypsinized and counted using a hemocytometer. Values were plotted over time (5 days), and population doubling times were calculated using the portion of the curve representing log phase growth.

The percentage of cells in S phase was determined using the flow cytometric techniques described in White et al. (41). Newly synthesized DNA was labeled in log phase growing cells with 10 µM bromodeoxyuridine (BrdU; Sigma) for 3 h prior to fixation in PBS:ethanol (1:2 v/v). Nuclei were then isolated by pepsin digestion. Incorporated BrdU was detected using an anti-BrdU–fluorescein isothiocyanate conjugated antibody (Becton Dickinson), and total DNA was stained with 50 µg/ml propidium iodide. Cell cycle analysis was performed using a Becton Dickinson FACScan Vantage instrument. Data were acquired and analyzed using Lysis II software (Becton Dickinson). Percentage of cells in G1 or G2 could not be determined due to the presence of diploid and tetraploid populations in the mouse liver cell lines. A two-tailed paired Student’s t-test was conducted to determine whether the mean population doubling time and proportion in S phase for chemically and virally transformed liver cells differed from the mean values obtained for the non-tumorigenic liver cell line BNL CL.2.

Results

Characterization of D₁-type CDK complexes in non-tumorigenic mouse liver cells

Since little is known about the composition of CDK protein complexes in mouse liver cells, initial studies involved characterizing these complexes in the non-tumorigenic mouse liver cell line BNL CL.2. The results discussed below were reproduced in three independent experiments.

CDK6 was present in binary and tertiary complexes. To characterize CDK6 complexes in non-tumorigenic mouse liver cells, an anti-CDK6 polyclonal antibody was used to immuno-

Fig. 1. Characterization of CDK protein complexes from BNL CL.2 mouse liver cells. (A) [³⁵S]Methionine-labeled BNL CL.2 cell lysates were immunoprecipitated with one of eight antisera specific to either cyclin D, CDKs, CDIs or PCNA (as indicated above each lane) and resolved by SDS–PAGE. The mobilities of protein molecular weight standards and relevant proteins are indicated. (B) Non-radiolabeled cell lysates prepared from BNL CL.2 cells were immunoprecipitated with a variety of antisera (indicated above each lane). Following SDS–PAGE and protein transfer the blot was sequentially probed with antisera indicated in the left-hand column. The figure contains representative data obtained from three independent experiments.

precipitate protein complexes from [³⁵S]labeled BNL CL.2 cellular lysates. Precipitated CDK6 complexes were separated by SDS–PAGE and detected by autoradiography. Significant bands migrating with molecular weights of ~38, 31 and 14 kDa were visualized (Figure 1A, lane 4). To determine the identity of the recovered proteins, immunoblotting techniques were employed subsequent to non-radiolabeled immunoprecipitations. CDK6, cyclin D₁, p27KIP1 and p15INK4b were detected by Western blotting in the anti-CDK6 immunoprecipitates (Figure 1B, lane 4). CDK6 and p15INK4b corresponded to the 38 and 14 kDa proteins, respectively, visualized by autoradiography (compare lanes 4 from Figure 1A and B). Interestingly, CDK6-associating p27KIP1 detected by Western blotting had been found at times to migrate with a mass of 27 kDa and at other times with a molecular weight of 31 kDa, the latter being consistent with that observed in radiolabeled anti-CDK6 immunoprecipitations. This observation suggests that CDK6-associating p27KIP1 may be post-translationally modified in proliferating cells.

To determine whether cyclin D₁, CDK6, p27KIP1 and p15INK4b proteins associated as one protein complex reciprocal, [³⁵S]labeled immunoprecipitations were performed using either an anti-cyclin D₁ or anti-p27KIP1 rabbit polyclonal antibody. In both precipitates proteins migrating with molecular weights of 38, 36 and 27 kDa were detected along with additional bands that will be discussed later (Figure 1A, lanes 1 and 6).

Interestingly, the 14 kDa protein (p15INK4b) was not recovered in either of the anti-cyclin D₁ or anti-p27KIP1 immune complexes, showing that p15INK4b associated with CDK6 in the absence of cyclin D₁ and p27KIP1. Thus CDK6 could associate with cyclin D₁ to form binary (cyclin D₁–CDK6) or tertiary (cyclin D₁–CDK6–p27KIP1) complexes. In the absence of cyclin D₁ and p27KIP1 binding, CDK6 could also associate with p15INK4b to form binary (CDK6–p15INK4b) complexes in proliferating mouse liver cells.

1095
CDK4 protein complexes were immunoprecipitated from 35S-labeled BNL CL.2 cellular lysates using a rabbit polyclonal anti-CDK4 antibody. Proteins migrating with molecular weights of 36, 33, 27, 21 and 14 kDa were recovered (Figure 1A, lane 3). Non-radiolabeled immunoprecipitations followed by Western blotting confirmed that these proteins were p36cyclin D1, p33CDK4, p27KIP1, p21WAF1 and p15INK4b, respectively (Figure 1B, lane 3). PCNA was also detected by Western blotting in anti-CDK4 immunoprecipitates (Figure 1B, lane 3), but the protein was difficult to visualize in radiolabeled immunoprecipitates.

To determine whether all five proteins coexisted as one complex, reciprocal radiolabeled and non-radiolabeled immunoprecipitations were carried out with anti-cyclin D, anti-p21WAF1 and anti-p27KIP1 rabbit polyclonal antibodies. Anti-cyclin D precipitates contained PCNA (visualized only in Figure 1B, lane 1), cyclin D1, CDK4, p27KIP1 and p21WAF1, but not p15INK4b (Figure 1A and B, lanes 1). Anti-p21WAF1 co-precipitated PCNA (visualized only in Figure 1B, lane 5), cyclin D1, CDK4 and p21WAF1, but not p27KIP1 or p15INK4b (Figure 1A and B, lanes 5). Anti-p27KIP1 antibody recovered p21WAF1 (visualized only in Figure 1B, lane 5), cyclin D1, CDK4 and p21WAF1, but not p27KIP1 or p15INK4b (Figure 1A and B, lanes 5). Anti-p27KIP1 antibody recovered cyclin D1, CDK4 and p27KIP1, but not PCNA, p21WAF1 or p15INK4b (Figure 1A and B, lanes 6). Dissection of these results revealed that p27KIP1 and p21WAF1 existed in separate CDK complexes, since they did not coexist in anti-p27KIP1 or anti-p15INK4b precipitates. In addition, since none of the antibodies recovered p15INK4b (Figure 1A and B, lanes 1, 5 and 6), this protein must have associated with CDK4 only when cyclin D1, p27KIP1 and p21WAF1 were absent. Hence, several types of CDK4 complexes existed in proliferating mouse liver cells: binary (cyclin D1–CDK4), tertiary (cyclin D1–CDK4–p27KIP1) and quaternary (cyclin D1–CDK4–p21WAF1–PCNA) complexes. In addition, CDK4 could associate with p15INK4b to form binary (CDK4–p15INK4b) complexes in the absence of cyclin D1, p27KIP1 and p21WAF1 binding.

CDK2 formed binary, tertiary and quaternary complexes. CDK2 protein complexes in the non-tumorigenic BNL CL.2 mouse liver cells were also characterized. An anti-CDK2 rabbit polyclonal antibody used in immunoprecipitation reactions coupled with Western blotting recovered PCNA, cyclin D1, CDK2, p27KIP1 and p21WAF1 (Figure 1B, lane 2). Most of these proteins (with the exception of PCNA) could also be easily visualized in 35S-labeled anti-CDK2 precipitates (Figure 1A, lane 2).

Reciprocal immunoprecipitations using anti-cyclin D, anti-p21WAF1 and anti-p27KIP1 were performed to determine whether all of the proteins existed in one complex. Protein complexes isolated with anti-cyclin D looked similar to the anti-CDK2 precipitates in that they both recovered p40PCNA (visualized in Figure 1B, lane 1 only), p36cyclin D1, p32CDK2, p27KIP1 and p21WAF1 (Figure 1A and B, lanes 1). Anti-p21WAF1 precipitates contained PCNA (seen in Figure 1B, lane 5 only), cyclin D1, CDK2 and p21WAF1, but not p27KIP1 (Figure 1A and B, lanes 5). Anti-p27KIP1 pulled down cyclin D1, CDK2 and p21WAF1, but not p21WAF1 or PCNA (Figure 1A and B, lanes 6). Again, p27KIP1 and p21WAF1 appeared to exist in separate complexes since they did not co-precipitate with each other in anti-p27KIP1 or anti-p21WAF1 precipitates. From these data several types of complexes emerged: CDK2 could associate with cyclin D1 to form binary (cyclin D1–CDK2), tertiary (cyclin D1–CDK2–p27KIP1) or quaternary (cyclin D1–CDK2–p21WAF1–PCNA) complexes.

Alteration of cyclin D1–CDK protein complexes after chemical transformation of mouse liver cells

To determine potential alterations in CDK protein complexes after chemical transformation, complexes were isolated from two mouse liver cell lines transformed with either MC or MNNG. CDK complexes from the chemically transformed cell lines were compared with their non-tumorigenic counterpart BNL CL.2 cells. An SV40-transformed mouse liver cell line (derived from BNL CL.2 cells) was used as a positive control throughout the study. The results discussed below were reproduced in three independent experiments.

Alteration of cyclin D1–CDK6 protein complexes in chemically transformed mouse liver cells. CDK6 protein complexes from non-tumorigenic and tumorigenic mouse liver cells transformed with chemical carcinogens or SV40 were collected by immunoprecipitating techniques and separated by SDS–PAGE. Cyclin D, CDK6 and p27KIP1 proteins were visualized by Western blotting, and levels were compared among the cell lines (Figure 2). CDK6 protein levels were similar among the four cell lines studied (Figure 2, row 1). However, CDK6-associating cyclin D1 exhibited a much more dramatic decrease in cyclin D1 than MNNG-transformed cells (BNL 1NG A.2), and SV40-transformed cells (BNL SV A.8) had undetectable levels of cyclin D1 (Figure 2, row 2). Association of p27KIP1 with CDK6 was also decreased after chemical transformation and exhibited the same pattern as cyclin D1 (Figure 2, row 3). In conclusion, CDK6 protein complexes were altered after chemical (and viral) transformation of mouse liver cells resulting in formation of fewer binary (cyclin D1–CDK6) and tertiary (cyclin D1–CDK6–p27KIP1) complexes.

**Fig. 2.** Alteration of CDK6 protein complexes after chemical and viral transformation of mouse liver cells. Cell lysates prepared from non-tumorigenic (BNL CL.2) mouse liver cells as well as MC- (BNL ME A.7R.1), MNNG- (BNL 1NG A.2) and SV40-transformed (BNL SV A.8) tumorigenic mouse liver cells were used to immunoprecipitate CDK6 protein complexes. Following SDS–PAGE and protein transfer, blots were sequentially probed with anti-CDK6, anti-cyclin D1 and anti-p27KIP1, as indicated in the left-hand column. The composition of CDK6 protein complexes in the chemically and virally transformed cell lines was compared with the non-tumorigenic BNL CL.2 cell line. The figure contains representative data obtained from three independent experiments.
Cyclin–CDK alterations in transformed liver cells

Fig. 3. Alteration of CDK4 protein complexes after chemical and viral transformation of mouse liver cells. Cell lysates prepared from non-tumorigenic (BNL CL.2) mouse liver cells as well as MC- (BNL ME A.7R.1), MNNG- (BNL 1NG A.2) and SV40-transformed (BNL SV A.8) tumorigenic mouse liver cells were used to immunoprecipitate CDK4 protein complexes. Following SDS–PAGE and protein transfer, blots were sequentially probed with anti-CDK4, anti-PCNA, anti-cyclin D1, anti-p27KIP1 and anti-p21WAF1, as indicated in the left-hand column. The composition of CDK4 protein complexes in chemically and virally transformed cell lines was compared with the non-tumorigenic BNL CL.2 control cell line. The figure contains representative data obtained from three independent experiments.

Alteration of cyclin D1–CDK4 protein complexes in chemically transformed mouse liver cells. Detection and comparison of CDK4 protein complexes were achieved by immunoprecipitating and immunoblotting reactions (Figure 3). As was the case with CDK6, CDK4 protein levels remained consistent between cell lines (Figure 3, row 1). However, recovery of CDK4-associating cyclin D1 was reduced relative to the control in MC-transformed cells (Figure 3, row 3). In the same cell line CDK4-associating PCNA and p21WAF1 were significantly decreased, but p27KIP1 levels remained similar to control levels (Figure 3, rows 2, 5 and 4 respectively). Interestingly, CDK4 complexes recovered from MNNG-transformed cells looked similar to the control cell line BNL CL.2 (Figure 3, lanes 1 and 3). CDK4 complexes isolated from SV40-transformed cells did not contain any cyclin D1, PCNA, p27KIP1 or p21WAF1 (Figure 3, lane 4). In summary, only the MC- and SV40-transformed cell lines exhibited a significant decrease in formation of binary (cyclin D1–CDK4), tertiary (cyclin D1–CDK4–p27KIP1) and quaternary (cyclin D1–CDK4–p21WAF1–PCNA) protein complexes.

Alteration of cyclin D1–CDK2 protein complexes in chemically transformed liver cells. Differences in CDK2 protein complexes from the four cell lines were determined using immunoprecipitating and Western blotting techniques (Figure 4). CDK2 protein levels were slightly elevated in the chemically and virally transformed cells relative to the BNL CL.2 control cells (Figure 4, row 1), whereas CDK2-associating PCNA, cyclin D1 and p21WAF1 were significantly decreased in all tumorigenic cell lines (Figure 4, rows 2, 3 and 5 respectively). Interestingly, p27KIP1 levels in association with CDK2 were significantly increased in the MC- and SV40-transformed cells (Figure 4, row 4). In summary, formation of binary (cyclin D1–CDK2), tertiary (cyclin D1–CDK2–p27KIP1) and quaternary (cyclin D1–CDK2–p21WAF1–PCNA) complexes were decreased after chemical and viral transformation of mouse liver cells.

Changes in total cellular protein levels of cyclin D1, p21WAF1 and PCNA in chemically transformed mouse liver cells

Total cellular levels of cyclin D1, p27KIP1, p21WAF1 and PCNA proteins were evaluated in the non-tumorigenic and tumorigenic mouse liver cell lines by immunoprecipitating and immunoblotting techniques to determine whether the decreased formation of cyclin D1–CDK protein complexes was due in part to changes in total cellular levels of CDK-associating proteins. Total cellular levels of cyclin D1 protein were decreased in MC- and SV40-transformed cell lines, however, they remained unchanged in MNNG-transformed liver cells (Figure 5, row 2). Total cellular levels of p21WAF1 were also increased in the MC-, MNNG- and SV40-transformed mouse liver cells.
Fig. 5. Changes in total cellular protein levels after chemical and viral transformation of mouse liver cells. Cell lysates prepared from non-tumorigenic (BNL CL.2) mouse liver cells as well as MC- (BNL ME A.7R.1), MNNG- (BNL 1NG A.2) and SV40-transformed (BNL SV A.8) tumorigenic liver cells were used to immunoprecipitate either PCNA, cyclin D1, p27KIP1 or p21WAF1. Following SDS–PAGE and protein transfer, proteins were detected using immunoblotting techniques. Total cellular levels of each protein in chemically and virally transformed cell lines were compared with the non-tumorigenic BNL CL.2 cell line. The figure contains representative data obtained from three independent experiments.

liver cells as compared with the non-tumorigenic BNL CL.2 cell line (Figure 5, row 4). Interestingly, total cellular levels of p27KIP1 protein remained unaltered in the chemically transformed mouse liver cell lines but was slightly decreased in SV40-transformed cells (Figure 5, row 4). Furthermore, cellular levels of PCNA protein were elevated in all tumorigenic cell lines relative to BNL CL.2 mouse liver cells (Figure 5, row 1).

Sequencing of the mouse p53 gene
To determine whether mutations in the p53 gene contributed to the observed changes in cellular levels of p21WAF1, seven separate fragments of p53 cDNA that overlapped one another and spanned the entire coding region were sequenced. No mutations in the p53 gene were observed in the non-tumorigenic control or in the MC- or MNNG-transformed tumorigenic liver cells.

Association of p15INK4b with CDK6 and CDK4 in chemically transformed mouse liver cells
As presented earlier, CDK6 and CDK4 formed binary complexes with p15INK4b in the non-tumorigenic cell line BNL CL.2. Since p15INK4b competes with cyclin D1 for CDK6 and CDK4 binding, increased levels of p15INK4b in association with these CDKs may contribute to the decreased formation of cyclin D1–CDK6 or –CDK4 protein complexes observed in the chemically (and virally) transformed mouse liver cell lines. To investigate this possibility the amount of p15INK4b in association with CDK6 and CDK4 was evaluated in chemically transformed cells and compared with levels observed in the non-tumorigenic BNL CL.2 control cells. Results showed that the amount of p15INK4b protein in association with CDK6 was decreased in the chemically (and virally) transformed mouse liver cell lines as compared with their non-tumorigenic counterpart (Figure 6). p15INK4b protein was also decreased in association with CDK4 in the MNNG- and SV40-transformed mouse liver cells but was slightly elevated in MC-transformed cells.

Alteration of CDK protein kinase activity after chemical transformation of mouse liver cells
Kinase activity of CDK6 and CDK4 protein complexes was evaluated in the non-tumorigenic and tumorigenic mouse liver cell lines to determine whether decreased formation of cyclin D1–CDK protein complexes resulted in decreased function of the CDKs. CDK2 activity was not evaluated since this kinase can be activated by association with non-D-type cyclins, such as cyclin E and cyclin A. Specifically, CDK6 and CDK4 protein complexes were isolated using immunoprecipitating techniques. Kinase activity was measured by assessing the ability of the CDK to phosphorylate a portion of pRb. Results indicated that CDK6-associated kinase activity was elevated 2.7-fold in the MC-transformed mouse liver cell, whereas activity in MNNG-transformed cells remained similar to the control (Figure 7 and Table 1). CDK4-associated kinase activity
Cyclin–CDK alterations in transformed liver cells

### Table II. Population doubling time and S phase fraction during log phase growth of mouse liver cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Population doubling time (h)</th>
<th>S phase fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNL CL.2</td>
<td>17.0 ± 1.7</td>
<td>21.8 ± 1.2</td>
</tr>
<tr>
<td>BNL 1ME A.7R.1</td>
<td>15.0 ± 2.0</td>
<td>26.5 ± 2.6*</td>
</tr>
<tr>
<td>BNL 1NG A.2</td>
<td>15.3 ± 0.5</td>
<td>30.3 ± 1.7*</td>
</tr>
<tr>
<td>BNL SV A.8</td>
<td>11.3 ± 2.1*</td>
<td>20.3 ± 0.5</td>
</tr>
</tbody>
</table>

*Significant difference from the non-tumorigenic control cell line BNL CL.2 (two-tailed paired Student’s t-test; *P* < 0.025).

The amount of cyclin D1 associated with the CDKs was also evaluated. Although cyclin D1 did not bind to CDK2 (data not shown), increased levels were found in association with CDK6 and CDK4 within the MC-transformed mouse liver cell line as compared with the non-tumorigenic control cell line. Levels of cyclin D1–CDK protein complexes did not appear to be altered in the MNNG- or SV40-transformed cells (Figure 8). Thus in at least the MC-transformed cell line, cyclin D1–CDK protein complexes appeared to be elevated relative to the control cell line.

### Population doubling times and percent of cells in S phase

Population doubling times were calculated for each cell line during log phase growth. The percentage of cells in S phase was determined for each cell line at the time lysates were collected for isolation of CDK protein complexes. Results are listed in Table II. No significant difference in population doubling time was observed between the immortalized and chemically transformed cell lines. However, the virally transformed cells exhibited a significantly shorter population doubling time relative to the non-tumorigenic control. Interestingly, the percentage of cells in S phase was significantly greater in the chemically transformed liver cells compared with the non-tumorigenic counterpart, but no difference was observed between control and virally transformed liver cells.

### Discussion

The present study was undertaken to determine whether transformation of mouse liver cells by the genotoxic carcinogens MC and MNNG could lead to alterations in the cyclin D1–CDK–CDI protein complexes, which play an important role in integrating external growth signals with cell cycle progression through G1.

In the non-tumorigenic immortalized mouse liver cell line BNL CL.2, cyclin D1 associated with CDK6, CDK4 or CDK2 to form binary (cyclin D1–CDK), tertiary (cyclin D1–CDK–p27KIP1) or quaternary (cyclin D1–CDK–p21WAF1–PCNA) protein complexes. Analysis of these holoenzymes in MC-, MNNG- and SV40-transformed mouse liver cells revealed that lower levels of various types of cyclin D1–CDK6–CDI, cyclin D1–CDK4–CDI (MC- and SV40-transformed only) and cyclin D1–CDK2–CDI protein complexes existed after chemical and viral transformation of the BNL CL.2 mouse liver cells (summarized in Figure 9). In addition, distinct changes in cyclin D1–CDK–CDI holoenzymes existed among the chemically and virally transformed mouse liver cell lines, suggesting that each line harbored unique mutations or alterations that differentially

---

**Table I. Relative kinase activity of cyclin-dependent kinases during log phase growth of mouse liver cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Relative kinase activity of CDK6</th>
<th>Relative kinase activity of CDK4</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNL CL.2</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>BNL 1ME A.7R.1</td>
<td>109 ± 20.9</td>
<td>62 ± 3.8</td>
</tr>
<tr>
<td>BNL 1NG A.2</td>
<td>130 ± 30</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>BNL SV A.8</td>
<td>130 ± 80</td>
<td>58 ± 8</td>
</tr>
</tbody>
</table>

*Significant difference from the non-tumorigenic control cell line BNL CL.2 (two-tailed paired Student’s t-test; *P* < 0.13).

*Significant difference from the non-tumorigenic control cell line BNL CL.2 (two-tailed paired Student’s t-test; *P* < 0.025).
of cyclin D1–CDK4 protein complexes in MC (and SV40-) transformed cells. These changes translated into decreased activity of the CDK4 enzyme, suggesting that CDK4 kinase activity was predominantly modulated by cyclin D1 binding in these cells. Thus MC (and SV40)-transformed mouse liver cells appeared to have evolved a mechanism to decrease their dependence on functional cyclin D1–CDK4 protein complexes. Interestingly, CDK6 activity was elevated 2.7-fold in MC-transformed cells, despite significant disruption of cyclin D1–CDK6 protein complexes. We later found that cyclin D1 protein levels were dramatically elevated in MC-transformed cells relative to the control cell line and that this cyclin associated significantly with CDK6. Thus transforming events in the MC-transformed cell line resulted in decreased cyclin D1 protein levels, but aberrant levels of cyclin D1 and significant formation of active cyclin D1–CDK6 protein complexes.

Cyclin D–CDK6 or –CDK4 protein complexes have been shown to possess a distinct substrate specificity for pRb (10,42,43). Alterations in these holoenzymes may therefore affect the activity of pRb tumor suppressor protein, which controls transition into S phase and regulates transcription of genes needed for DNA synthesis (10). Although both cyclin D1–CDK and cyclin D1–CDK4 protein complexes have been shown to phosphorylate pRb in vitro, it has not been determined whether additional substrates exist for either of the protein complexes. Thus some biochemical functions may still be lacking in cells with lower levels of cyclin D1 and/or functional cyclin D1–CDK protein complexes, even when they contain highly active cyclin D1–CDK protein complexes.

The role of cyclin D1–CDK2 protein complexes is not as well defined. They have been detected in the Y11 protein complex that binds to the mouse thymidine kinase gene (TK) promoter during early G1 (44). The Y11 complex appears to regulate TK gene expression, which is tightly coupled with the onset of S phase (45,46). Altered cyclin D1–CDK2–CDI protein complexes after chemical (or viral) transformation could affect transcription of specific genes (such as TK) needed for DNA synthesis and normal S phase progression. Interestingly, it appeared that the MC- and MNNG-transformed liver cells were spending more time in S phase relative to the non-tumorigenic control, which may have been due to premature entry into S phase or delayed S phase progression. Alterations in the cyclin D1–CDK–CDI protein complexes may have contributed to the observed changes in cell cycle kinetics.

Characterization of cyclin D–CDK–CDI protein complexes in mouse liver cell lines also led to the detection of several negative regulators of cell proliferation, namely p27kip1, p21WAF1 and p15INK4b. These proteins were found in association with various CDKs and have been shown to affect stability and activity of cyclin D–CDK protein complexes (30–34,47). These CDIs are likely to play an important role in controlling cell cycle progression within mouse liver cells, and biochemical pathways regulating expression of these proteins may represent additional pathways vulnerable to attack during chemical transformation of mouse liver cells.

In our studies we found that cellular levels of p21WAF1 were decreased after chemical and viral transformation, possibly contributing to the observed changes in quaternary (cyclin D1–CDK–p21WAF1–PCNA) complexes within these cell lines. The p53 tumor suppressor protein has been shown to transcriptionally activate p21WAF1 (48), and p21WAF1 is believed to stabilize the quaternary complexes when present at low levels (47). Sequencing of the p53 gene, however, revealed that the p53 gene was not mutated in either of the chemically transformed cell lines. These findings suggest that other genetic alterations may be responsible for lowering p21WAF1 protein levels within the chemically transformed cell lines. The dramatic decrease in p21WAF1 protein levels and disruption of quaternary (cyclin D1–CDK–p21WAF1–PCNA) protein complexes in SV40-transformed liver cells were likely due to the presence of large T antigen, which is capable of binding to and inactivating p53 (49).

We also evaluated levels of p16-type CDK inhibitors in association with CDK6 or CDK4, since these proteins can compete with cyclin D for binding with CDK6 or CDK4 and prevent formation of active D-type CDK protein complexes (30–34). In the mouse liver cell lines used in our studies only p15INK4b could be detected. Levels of p15INK4b in association with CDK6 were dramatically decreased in the chemically and virally transformed cell lines, implying that disruption of cyclin D1–CDK6–CDI protein complexes was not due to increased association of CDK6 with p15INK4b. Instead, the dramatic disruption of cyclin D1–CDK6–CDI complexes observed in the MC-transformed cells was likely due to overexpression of cyclin D1 (as discussed previously), which led to increased association of cyclin D1 with CDK6 and abundant kinase activity of the CDK6 enzyme. Alternatively, p15INK4b levels were elevated in association with CDK4 in MC-transformed
mouse liver cells, correlating with decreased formation and activation of cyclin D1–CDK4 protein complexes in this cell line.

Some of the cell cycle protein complexes did not change after chemical transformation. Quaternary (cyclin D1–CDK4–p21WAF1–PCNA) complexes isolated from MNNG-transformed liver cells remained intact, and the amount recovered from this transformed cell line was similar to that seen with the non-tumorigenic control. In addition, tertiary (cyclin D1–CDK4–p27Kip1) complexes isolated from both the MC- and MNNG-transformed cell lines remained unchanged (data not shown). Some investigators have hypothesized that all human tumors and tumorigenic cell lines are likely to possess disrupted cyclin D1–CDK4–CDI protein complexes (35). Our studies showed that this effect does not always occur after cellular transformation, suggesting that the transformation process in rodent and human cells may involve different molecular changes or that disruption of cyclin D1–CDK4–CDI protein complexes may not be obligatory for all cases of chemical transformation.

In summary, our study characterized D-type CDK protein complexes in mouse liver cells and provided the first example of these complexes, which function to integrate growth factor pathways with cell cycle progression, can be altered physically and functionally after chemical transformation with the genotoxic carcinogens MC and MNNG. These complexes were differentially altered among the MC-, MNNG- and SV40-transformed liver cells, suggesting that each cell line may have harbored distinct molecular alterations that contributed to the rearrangement of cyclin D1–CDK–CDI protein complexes. Although MC and MNNG are not likely to mutate the same gene each time during chemical transformation, this study demonstrates that components of the cell cycle machinery have the potential of being targeted and altered during genotoxic chemical carcinogenesis. Identifying cellular and molecular targets involved in genotoxic chemical carcinogenesis is an important first step toward providing a biological basis for phenotypic changes observed after chemical transformation of mouse liver cells.

Acknowledgements

The authors would like to thank Linda Pluta and Rekha Shah for technical assistance with p53 sequencing and flow cytometric sample collection, respectively. We would also like to thank Drs Julian Preston, Patrice Ferriola, Russell Cattley and Barbara Kuyper for critical reading of the manuscript. A.J.G. was supported by a Ford Foundation Predoctoral Fellowship.

References


Received on January 31, 1997; revised on January 16, 1998; accepted on January 29, 1998