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Discordant Hepatic Expression of the Cell Division Control Enzyme p34^{cdc2} Kinase, Proliferating Cell Nuclear Antigen, p53 Tumor Suppressor Protein, and p21^{Waf1} Cyclin-Dependent Kinase Inhibitory Protein after WY14,643 ([4-Chloro-6-(2,3-xylidino)-2-pyrimidinylthio]Acetic Acid) Dosing to Rats

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SUMMARY

The hepatocarcinogen and peroxisome proliferator WY14,643 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid} was examined for its ability to induce changes in the intracellular protein expression of hepatic p34^{cdc2} kinase (CDK1), proliferating cell nuclear antigen (PCNA), p53 tumor suppressor protein, and p21^{Waf1} CDK inhibiting protein. Young, adult male rats were administered 45 mg-kg/day WY14,643 intraperitoneally for 1, 2, 3, 4, or 5 days or fed diets containing 0% or 0.08% WY14,643 for 1, 2, 3, or 4 weeks. WY14,643 dosing increased concentrations of hepatic proteins of 34- and 37-kDa molecular mass, which were identified through immunoprecipitation as CDK1 and PCNA, respectively. Gel filtration of the hepatic S9 fractions determined by enzyme-linked immunosorbent assay confirmed the increased expression of CDK1 and PCNA immunoreactivity in livers from WY14,643-treated rats. Also, gel filtration revealed that the native CDK1 and PCNA in hepatic S9 from WY14,643-treated rats chromatographed as a major peak with an apparent molecular mass of 70 and 76 kDa, respectively. Immunoblotting of the 70-kDa fraction with anti-CDK1 revealed a single band of molecular mass of 34 kDa. Thus, the CDK1 in the major immunoreactive peak of WY14,643-treated rat liver S9 seems to exist as a heterodimer or homodimer. Immunohistochemistry of formalin-fixed liver demonstrated a cytosolic localization of immunoreactive CDK1 and nuclear localization of immunoreactive PCNA in proliferating cells of WY14,643-treated rat livers. WY14,643 increased hepatic CDK1 content by 1.9–6.3-fold through postdosing days 1–5. Hepatic PCNA content was increased 1.9–5-fold over the same period. In the 4-week feeding study, CDK1 and PCNA expression were increased at all weekly time points by an average of 15–50-fold, respectively. Furthermore, the dietary administration of 0.08% WY14,643 resulted in sustained, overexpression of hepatic p53 tumor suppressor protein from week 1 through week 4 and of p21^{Waf1} CDK inhibitory protein from week 3 to week 4.

A family of cell division control enzymes termed CDKs, along with the cyclin and CDK inhibitory proteins, serves to control and coordinate the molecular events of cell division in all eukaryotic cells (1). Although 12 CDKs have been described, the most actively studied is p34^{cdc2} kinase, or CDK1 (1). In resting rat hepatocytes, CDK1 is not expressed, but concentrations of CDK1 increase through G1 and the G1/S transition, reaching maximal levels in the S, G2, and M phases (2). In association with cyclin B, CDK1 is the serine/threonine kinase subunit of M-phase promoting factor; active M-phase promoting factor triggers the G2/M transition in species ranging from yeast to humans (1). Several studies also suggest that CDK1 functions in the control of the G1/S transition and in the initiation of mitosis (3).

The role of CDK proteins is completely dependent on their kinase activity. CDK1 catalytic activity is regulated primarily through post-translational modifications, including cycles of phosphorylation and dephosphorylation (3), interactions with cyclins (3, 4), and intracellular compartment translocation (4). Another form of CDK1 regulation involves the interaction of CDK1 with the tumor suppressor gene product p53 (5) and the CDK inhibitory protein p21^{Waf1} (6).

Abnormal cell proliferation is the most fundamental phenotypic property of cancer (7). In chemical carcinogenesis, the

ABBREVIATIONS: CDK, cyclin-dependent kinase; ELISA, enzyme-linked immunosorbent assay; PCNA, proliferating cell nuclear antigen; S9, 9000 × *g* supernatant fraction; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, Tris-buffered saline/Tween 20; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

induction of sustained cell proliferation seems to be a critical factor in the formation of tumors for many nongenotoxic and weakly genotoxic carcinogens (8). Furthermore, because testing of the genotoxicity and carcinogenicity of chemicals has become routine, many compounds have been found to induce tumors in chronic rat and mouse bioassays while exhibiting negative results in genotoxicity tests (9). One significant category of nonmutagenic carcinogens includes the peroxisome proliferators (10). Within this diverse group of compounds, WY14,643 {[4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]acetic acid} is generally recognized as the prototypic peroxisome proliferator for the purposes of elucidating the mechanisms through which peroxisome proliferators induce rodent tumors (11).

WY14,643 has long been recognized as an inducer of peroxisomes in rodent liver; it has also been demonstrated to be a potent hepatocarcinogen and tumor promoter in rodents (11, 12). WY14,643 toxicity is believed to be mediated by interaction with an intracellular receptor protein complex, the peroxisome proliferator activated receptor, resulting in alteration of gene expression (13). In several respects, the peroxisome proliferator activated receptor is analogous to the aryl hydrocarbon, or dioxin, receptor. First, no known endogenous ligands have been unambiguously identified for either receptor. Second, both receptors form complexes with an additional DNA binding proteins, activate transcription of cytochrome P450 isoenzymes, and interact with DNA response elements that can potentially interfere with estrogen receptor transcriptional activity (14–16). Third, both TCDD (17) and WY14,643 alter tyrosine phosphorylation signal transduction pathways.[1]

Although much is known of the biological responses that occur after WY14,643 administration to laboratory animals, the mechanism of WY14,643 hepatocarcinogenicity is poorly understood. Because none of the carcinogenic peroxisome proliferators interact with or damage DNA, formation of peroxisome proliferator/DNA adducts may not be an essential step in the carcinogenesis of this group of xenobiotics (18). One hypothesis concerning the mechanism of tumor formation of these nongenotoxic carcinogens is termed the "oxidative stress hypothesis" (11, 19). It has been proposed that increased cellular concentrations of hydrogen peroxide, produced by elevations in peroxisomal fatty acid β-oxidation, result in oxidative stress and lead to DNA damage and possibly tumor initiation. However, under conditions similar to those used in tumorigenicity studies, the correlation between peroxisome proliferation and relative hepatocarcinogenicity was poor (19).

In the same study, a strong correlation was observed between the relative hepatocarcinogenicity of WY14,643 and di(2-ethylhexyl)phthalate and their abilities to induce a persistent increase in replicative DNA synthesis. The finding that the carcinogenicity of di(2-ethylhexyl)phthalate and WY14,643 correlated better with sustained DNA replication than peroxisome proliferation suggests that the ability of peroxisome proliferators to induce tumor formation is a result of their ability to induce sustained cellular proliferation (12, 19).

In the current study, we evaluated WY14,643 for its ability to affect changes in the hepatic expression of cell cycle regulatory proteins. The enhanced expression of CDK1 relative to control liver was evidence that the hepatocytes were stimulated to proliferate, whereas the increase in hepatic expression of $p21^{Waf1}$ observed implied a G1/S block and inhibition of cell cycling. These results are a further demonstration that xenobiotics capable of binding to intracellular receptors can function to disregulate the expression of cell division control enzymes and support the inference that one aspect of the mechanism of action of nongenotoxic carcinogens occurs through the discordant expression of signals for cellular proliferation and inhibition (17).

Materials and Methods

Chemicals. WY14,643 (CAS 50892–23-4) was purchased from ChemSyn Science Labs (Lenexa, KY). Bicinchoninic acid was obtained from Pierce Chemical (Rockford, IL). molecular weight standards were supplied through BioRad (Melville, NY). Anti-CDK1 (carboxyl terminus polyclonal), anti-PCNA (PC10 monoclonal), anti-rabbit, and anti-mouse antibodies were obtained from Pederson Biotech (Ithaca, NY). Quantification of CDK1 and PCNA was performed using ELISA kits from Paracelsian, Inc. (Ithaca, NY). One unit of CDK1 or PCNA immunoreactivity was defined as 1 fmol of the CDK1 or PCNA antigen. Antibodies to $p21^{Waf1}$ and p53 were purchased from Oncogene Sciences (Uniondale, NY). Antibodies to CYP4A1 were purchased from Gentest (Woburn, MA). A Zymed Universal Histo Staining Kit (Zymed Labs, San Francisco, CA) was used for immunohistostaining of collected tissues. All other chemicals were purchased from Sigma Chemical (St. Louis, MO) and were of the highest purity available.

Animals. Young, adult male Sprague-Dawley rats (~200 g) were purchased from Charles River Laboratory (Charles River, MA) and acclimated for a minimum of 2 weeks before assignment to treatment groups. Rats were fed Prolab RMH 1000 (Agway, Cortland, NY) and received tap water *ad libitum*. All rats were housed three per cage and maintained at constant temperature (22 ± 2°), humidity (50 ± 5%), and lighting (12/12-hr cycles). Samples of rat liver were obtained at 1, 2, 3, 4, or 5 days after a daily intraperitoneal injection of either corn oil, as a control, or WY14,643 in corn oil at 45 mg-kg/day. The volume of the injections was 0.4–0.5 ml/rat.

For the WY14,643 feeding study, after segregation into treatment groups, the rats were fed Prolab RMH 1000 diet containing WY14,643 at a concentration of 0.08% (prepared by Bioserve, Frenchtown, NJ) or Prolab RMH 1000 diet alone. Based on food consumption estimates of rats of the same age and sex, this dietary percentage of WY14,643 corresponded to an approximate daily dose of 50 mg of WY14,643/kg. Rats were killed each week for 4 consecutive weeks. After CO_2 anesthesia and cervical dislocation, tissues were removed from the rats. The carcasses were weighed before dissection, and the livers were weighed after removal from the bodies. Preparation and storage (at −80°) of the liver homogenates (S9) for SDS-PAGE, immunoblotting, and CDK1 and PCNA quantification were done as previously described (20). For histological and immunohistological evaluation, samples of liver and other tissues were trimmed and immediately immersed in 10× volumes of 10% neutral buffered formalin.

Immnoprecipitation and immunoblotting of CDK1 and PCNA. Fifty microliters of hepatic S9 (1 mg of protein) was solubilized in 950 μl of immunoprecipitation buffer containing 20 mM Tris·HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, and 1 mM sodium vanadate and centrifuged at 13,000 × *g* for 15 min at 4°. The solubilized hepatic S9 proteins were then incubated overnight with 5 μg/ml anti-CDK1 polyclonal or 5 μg/ml anti-PCNA monoclonal antibody at 4°. After the incubation period, 25 μl of protein A/Sepharose was added for each 5 μg of antibody. At 1 hr later, the immune

[1] X. Ma, D. Stoffregen, G. D. Wheelock, J. Rininger, and J. G. Babish, unpublished observations.

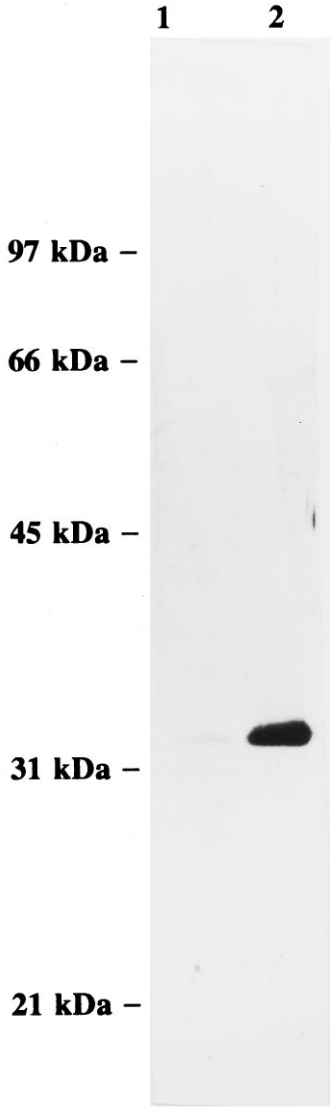

Fig. 1. A representative hepatic CDK1 response demonstrated on day 3 after daily intraperitoneal administration of 45 mg-kg/day WY14,643 to male rats. *Lane 1*, corn oil-treated rat livers; *lane 2*, WY14,643-treated rat livers. See Immunoprecipation and immunoblotting of CDK1 and PCNA for details.

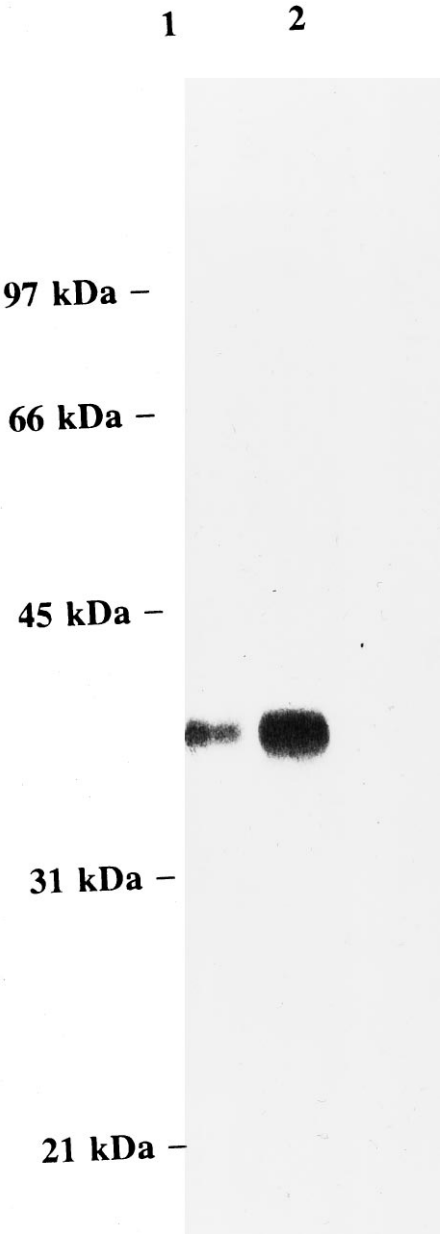

Fig. 2. A representative hepatic PCNA response demonstrated on day 3 after daily intraperitoneal administration of 45 mg of WY14,643/kg-day to male rats. An anti-PCNA immunoprecipitate of control and WY14,643-treated rat hepatic S9 was separated using a 10–11% SDS-PAGE gel as described in legend to Fig. 1. The separated immunoprecipitate was transferred to a blotting membrane and probed with the same anti-PCNA monoclonal antibody. Immunoprecipitation and probing with anti-PCNA detected a single 37-kDa protein in untreated (*lane 1*) and treated (*lane 2*) rat liver samples. The anti-PCNA immunoreactive band in the WY14,643-treated hepatic S9 exhibited considerably thicker and darker staining than the immunoreactive band from control hepatic S9. Thus, WY14,643 treatment for 3 days also increased expression of PCNA compared with control animals.

complexes were collected by centrifugation at 3000 × *g*, washed twice with immunoprecipitation buffer, solubilized in SDS-gel sample buffer (20), and denatured at 100° for 8 min. SDS-PAGE was carried out on the denatured samples as described previously (20) using 11% polyacrylamide gels.

The immunoblotting was carried out as described by Towbin *et al.* (21); however, a Milliblot SDE electroblot apparatus (Millipore, Bedford, MA) was used to transfer proteins from polyacrylamide gels to an Immobilon membrane filter (Millipore). Complete transfers were accomplished within 25–30 min at 500 mA. Membrane filters were blocked by incubation in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) containing 5% commercial nonfat dry milk for 30 min at room temperature and incubation for 2 hr with 5 μg/ml anti-CDK1 or 2 μg/ml anti-PCNA, p53, or $p21^{Waf1}$ in TBST (0.05% Tween 20 in TBS). Molecular weights of immunostained proteins were estimated by adding molecular weight standards to reference lanes and by staining the membrane filters with amido black 10 B.

To visualize the antibody reactions, the membranes were incubated for 2 hr at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG for CDK1 or anti-mouse IgG for PCNA diluted 1:1000 in TBST and developed for 15 min. Detection of p53 and $p21^{Waf1}$ was accomplished using an ECL Western blotting kit (Amersham, Buckinghamshire, UK).

Gel filtration. A 2.6 × 90-cm column was packed with Sephacryl S-200 HR (Pharmacia, Piscataway, NJ) and equilibrated with TBS at 3.5 ml/min. Eluted fractions were monitored at 280 nm. Exclusion volume (V_0) was determined with keyhole limpet hemocyanin (~1000 kDa, elution at 164 ml). The column was further calibrated

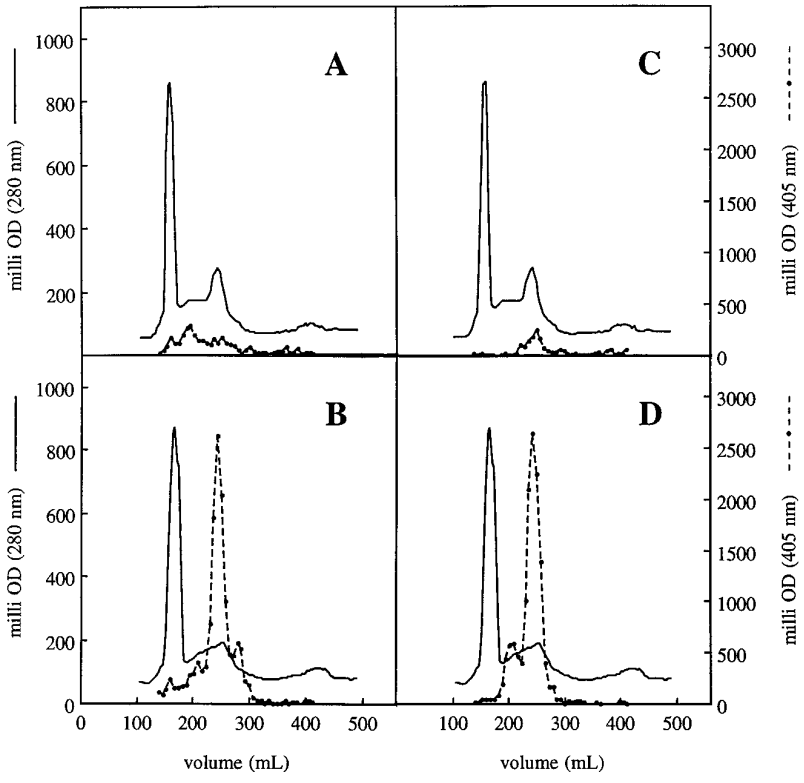

Fig. 3. Gel filtration of rat hepatic S9 showing protein versus CDK1 and PCNA immunoreactivity. See Gel filtration (in Materials and Methods and in Results) for details. *Solid line profile*, elution of protein from the column as measured by absorbance (*OD*) at 280 nm (left axis). A and C, Hepatic S9 from control rats. B and D, Rats dosed with WY14,643 for 4 days. Even-numbered fractions were diluted 80-fold and assayed for CDK1 and PCNA content by ELISA (*dashed lines*; measured at absorbance at 405 nm, *right axis*). The CDK1 ELISA response (A and C) for the most active fraction from the treated rat S9 samples was 25-fold higher than that in the same fraction from control hepatic S9. The elution volume of this immunoreactive peak was consistent with a molecular mass of ~70 kDa. PCNA ELISA (B and D) of the chromatography fractions from WY14,643-treated rat S9 fractions also contained a major peak of PCNA eluting at 245 ml. The PCNA ELISA response for the most active fraction from the treated rat S9 samples was 15-fold higher than that in the same fraction from control hepatic S9. The elution volume of this immunoreactive peak was consistent with a molecular mass of ~76 kDa.

with alcohol dehydrogenase (150 kDa, 217 ml), bovine serum albumin (66 kDa, 245 ml), ovalbumin (45 kDa, 269 ml), carbonic anhydrase (29 kDa, 286 ml), and cytochrome *c* (12.4 kDa, 350 ml). Six milliliters of pooled (4-day post-treatment) rat hepatic S9 from WY14,643-treated rats or corn oil-treated rats were injected onto the column, and eighty 1-min fractions were collected at 38–119 min after injection. Even-numbered fractions were diluted 80-fold and assayed for CDK1 and PCNA content by ELISA.

CYP4A1 induction. CYP4A1 induction was demonstrated by immunoblotting using a Phast-System Electrophoresis Unit (Pharmacia). One microgram of S9 protein was separated on a 12.5% homogeneous gel according to the manufacturer's instructions. Transfer of proteins and immunoblotting were done as described above. WY14,643-treated rats demonstrated dense staining of a single band at 47 kDa, which is consistent with previous reports of CYP4A1 induction with peroxisome proliferators (22). The density of staining seemed to peak at day 2 of dosing and remained constant over the next 3 sampling days. No detectable CYP4A1 protein was observed on the immunoblots for control animals.

Immunohistochemistry. After a minimum of 48–72 hr of fixation in neutral buffered formalin, tissues were routinely trimmed and oriented in specimen cassettes, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at 4 μm. Sections were then stained with hematoxylin and eosin.

Select tissue specimens were cut at 3–4 μm and mounted onto poly-L-lysine-coated Microprobe glass slides (Fisher Scientific, Fair Lawn, NJ). They were then processed on the Microprobe Staining System (Fisher Scientific) according to a modified streptavidin-biotin method. Briefly, tissues were cleared and dehydrated, and endogenous peroxidase was blocked with 0.5% hydrogen peroxide in methanol. The samples were then subjected to antigen unmasking via a microwave antigen retrieval method. Select tissues (liver, heart, and kidney) had endogenous avidin and biotin blocked with a commercial blocking system (Zymed Labs, San Francisco, CA). Samples were then blocked with normal sera (same species as the source of the subsequent secondary antibody). After the blocking step, the tissues were incubated with the polyclonal anti-CDK1 and monoclonal anti-PCNA primary antibodies; CDK1 antibody was used at concentrations of 25 and 50 μg/ml, whereas PCNA was used at 10 and 20 μg/ml. The subsequent biotinylated goat anti-rabbit or goat anti-mouse antibodies, streptavidin/peroxidase conjugate, aminoethyl carbazole chromogen, and Mayer's hematoxylin counterstain were used as supplied and as directed (Zymed Universal Histo Staining Kit). Nonhepatic and control tissues exhibited no detectable induction of anti-CDK1 or anti-PCNA immunoreactivity.

Protein determination. Bicinchoninic acid was used for the spectrophotometric determination of protein concentration as described previously (20).

Statistical analysis. Analysis of variance and regression analysis were performed using JMP version 2 statistical software (SAS Institute, Cary, NC). Statistical significance between treatment and control groups was assessed by the Student's *t* test, using a Bonferroni correction factor for multiple comparisons. Data are reported as mean ± 95% population confidence intervals or mean ± standard deviation for the stated number of animals.

Results

Immnoprecipitation and immunoblotting. To examine the specificity of the anti-CDK1 and anti-PCNA antibodies to native proteins in the hepatic S9, immunoprecipitation of the S9 protein was performed with each antibody. The anti-CDK1 immunoprecipitate of a single control and WY14,643-treated rat hepatic S9 (3-day) was then resolved by SDS-PAGE. The separated immunoprecipitate was transferred to a blotting membrane. Probing with the same anti-CDK1 antibody revealed a single immunoreactive band at 34 kDa (Fig. 1) in immunoprecipitate samples from both corn oil-treated and WY14,643-treated livers. The apparent molecular mass of the band (~34 kDa) and immunoreactivity with anti-CDK1 antibody suggest the identity of the protein as CDK1. Expression of immunoprecipitatable, putative

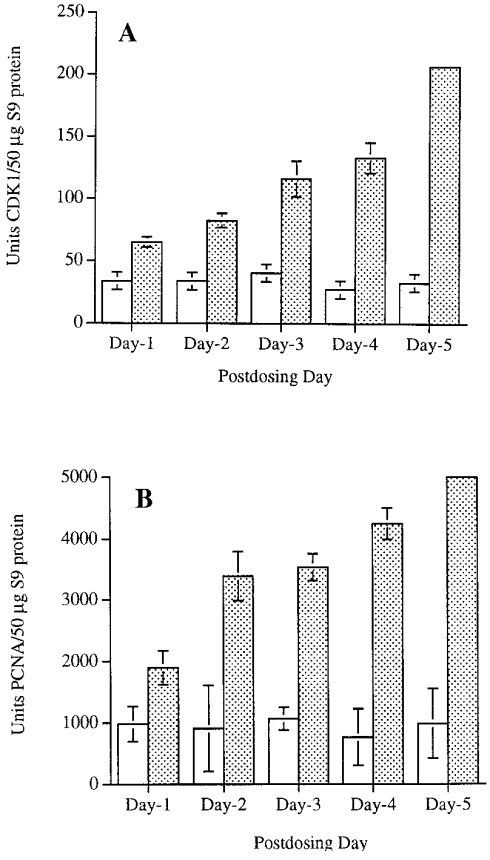

Fig. 4. Time course of rat hepatic CDK1 (A) and PCNA (B) expression with WY14,643 dosing as measured by ELISA. One unit of CDK1 or PCNA immunoreactivity was defined as 1 fmol of the CDK1 or PCNA antigen. Values depicted are mean ± 95% confidence intervals for the control animals (*open bars*) and ± standard deviation for the treated (*shaded bars*) with five observations each for days 1, 2, 3, and 4; eight control and two WY14,643-treated rats were assayed on day 5. WY14,643 treatment demonstrated increased ($p < 0.01$) expression of both biomarkers compared with control animals at all times. Hepatic CDK1 content was increased 1.9-, 2.4-, 3-, 5-, and 6.5-fold relative to control animals, respectively, on dosing days 1, 2, 3, 4, and 5. PCNA content was increased 2.0-, 3.2-, 3.8-, 4.5-, and 5.3-fold relative to control animals over the same period.

CDK1 varied noticeably with WY14,643 dosing. Although the 34-kDa band from the control sample was barely visible, the same band from the treated animal was much heavier. These results indicate that CDK1 was overexpressed in WY14,643-treated rat liver S9 relative to control animals, although the density of the band from the treated sample precluded quantification.

Immunoprecipitation and probing with anti-PCNA detected a single 37-kDa protein in untreated and treated rat liver samples (Fig. 2). This apparent molecular mass is in good agreement with previous description of the known mobility of rat (23) PCNA. The anti-PCNA immunoreactive band in the WY14,643-treated hepatic S9 exhibited considerably thicker and darker staining than the immunoreactive band from control hepatic S9. Thus, the dosing of 45 mg-kg/day WY14,643 for 3 days resulted in an increased expression of hepatic PCNA in the S9 fraction compared with control animals.

Separation and immunoblot detection of CDK1 and PCNA from cytosolic, nuclear, and microsomal compartments illustrated that CDK1 protein concentrations were elevated in all cellular compartments in WY14,643-treated sample preparations relative to control animals (data not shown). PCNA expression was found to be increased only in the cytosolic and nuclear compartments, with negligible staining appearing in the microsomal fraction (data not shown). These data demonstrate that the elevated CDK1 and PCNA concentrations in the hepatic S9 were not due to cellular relocalization.

Gel filtration. Proteins from pooled hepatic S9 from corn oil- or WY14,643-treated rats were separated by gel filtration chromatography. The fractions were assayed by CDK1 and PCNA ELISA as described in Materials and Methods.

CDK1 ELISA of the chromatography fractions from corn oil-treated rat S9 showed little immunoreactivity (Fig. 3A). However, chromatography of the WY14,643-treated rat S9 resulted in a major peak of CDK1 eluting at 245 ml (Fig. 3B) that was absent in the same fractions from corn oil-treated rats. The CDK1 ELISA response for the most active fraction from the treated rat S9 samples was 25-fold higher than in the same fraction from control hepatic S9. The elution volume of this immunoreactive peak was consistent with a molecular mass of ~70 kDa (two determinations), considerably larger than the nominal 34-kDa mass of CDK1 (see Fig. 1). Peak immunoreactive fractions were resolved on SDS-PAGE and immunoblotted using anti-CDK1. The reactive antigen had a molecular mass of 34 kDa (data not shown). Thus, the CDK1 found in the major immunoreactive peak exists as a heterodimer or homodimer.

PCNA ELISA of the chromatography fractions from corn oil-treated rat S9 also showed little immunoreactivity (Fig. 3C). However, WY14,643-treated rat S9 fractions contained a major peak of PCNA also eluting at 245 ml (Fig. 3D). The PCNA ELISA response for the most active fraction from the treated rat S9 samples was 15-fold higher than in the same fraction from control hepatic S9. The elution volume of this immunoreactive peak was consistent with a molecular mass of ~76 kDa, which is considerably larger than the nominal 33–36-kDa mass of PCNA (data not shown).

These results demonstrate that native CDK1 and PCNA can be quantified by ELISA. Furthermore, treatment of rats *in vivo* with WY14,643 results in a dramatic increase in CDK1 and PCNA complexed as heterodimers or homodimers. Immunoprecipitation experiments revealed that a portion of the heterodimers formed was a complex consisting of CDK1 and PCNA (data not shown).

Time course of CDK1 and PCNA expression. Within 24 hr, WY14,643 dosing resulted in a 100% increase ($p < 0.01$) in hepatic CDK1 content (Fig. 4A). WY14,643 increased hepatic CDK1 content by 1.9-, 2.4-, 3.0-, 5.0-, and 6.3-fold relative to control animals on postdosing days 1, 2, 3, 4, and 5, respectively. CDK1 concentrations in control hepatic S9 was constant over the 5 sampling days.

The time course of rat hepatic PCNA expression with WY14,643 administration demonstrated a pattern similar to that observed with hepatic CDK1 expression (Fig. 4B). Relative to concurrent control groups, the increases observed in hepatic S9 PCNA content were 1.9-, 3.7-, 3.3-, 5.5-, and 5.0-fold, respectively, for dosing days 1, 2, 3, 4, and 5 ($p < 0.01$ for all sampling times).

Immunohistochemistry. The intracellular localization of CDK1 and PCNA in WY14,643-treated rat liver was examined by immunocytochemical assay in liver sections from

Fig. 5. A and B, Control Sprague-Dawley rat liver immunostained with the anti-CDK1 antibody at a concentration of 50 μg/ml. Note that there are no immunostained cells (compare with 4-day WY14,643-treated liver) and the paucity of mitotic figures in this control liver sample. C and D, WY14,643-treated Sprague-Dawley rat liver immunostained with nonimmune rabbit sera in lieu of primary antibody. Notice the diffuse generalized brown-red coloration of all cells in the field. An occasional mitotic figure is present and does not stain selectively. *Bar length*, 50 μm for A and C (low-power magnification) and 100 μm for B and D (higher-power magnification). Controls did not exhibit detectable hepatocyte staining with either anti-CDK1, anti-PCNA antibodies, or preimmune rabbit sera that served as a reaginic control.

rats after four daily doses of corn oil or WY14,643. Staining was not observed in control rats with either anti-CDK1 or anti-PCNA antibodies or with preimmune rabbit sera (Fig. 5). There were no obvious apoptotic cells in either control or WY14,643-treated liver sections.

Anti-CDK1 immunostaining of WY14,643-treated rat livers demonstrated a marked orange-brown cytoplasmic staining limited to the randomly oriented hepatocytes (Fig. 6) that seemed to exhibit cytomegaly, karyomegaly, and mitotic changes. Those mitotic figures without an evident nuclear membrane had diffuse cellular staining; neighboring nonreactive hepatocytes were not stained. These results show that WY14,643-induced CDK1 expression is cytoplasmic. Furthermore, increased CDK1 expression was limited to proliferating hepatocytes, and the increased expression of CDK1 was correlated with the proliferative stimulus of WY14,643 treatment.

Immunostaining with anti-PCNA demonstrated that the affected hepatocytes had deep-red nuclear staining, with distribution of staining varying from homogeneous to marginated along the inner aspect of the nuclear membrane. Those mitotically active hepatocytes without the presence of a nuclear membrane had course globules of red stain located throughout the cell. Thus, in contrast to the results from immunostaining with anti-CDK1, staining with anti-PCNA revealed a predominately nuclear localization of PCNA. However, PCNA staining was present in the cytoplasm of cells without a nuclear membrane.

Four-week feeding study. CDK1 and PCNA ELISA analyses revealed that expression of both CDK1 and PCNA proteins was elevated at all time points by 15- and 50-fold, respectively, relative to control animals (Fig. 7, A and B). These results were corroborated by immunoblotting (data not shown).

Expression of p53 tumor suppressor protein and $p21^{Waf1}$ CDK inhibitory protein was detected by immunoblot analysis (Fig. 8). Control rat hepatic S9 samples exhibited little p53 cross-reactivity through the 4 weeks (Fig. 8A, *lanes 1–3*); however, p53 expression was induced in WY14,643-treated animals (Fig. 8A, *lanes 4–6*). Probing with anti-p53 detected faint, single bands at 53 kDa at 1 week in WY14,643-treated rat liver samples. At 2 and 3 weeks of WY14,643 administration, the 53-kDa band became thicker and more pronounced and stained as a doublet in four of the six rats. By 4 weeks, all three rats exhibited the doublet pattern of staining of p53.

The anti-$p21^{Waf1}$ band in the WY14,643-treated hepatic S9 did not become apparent until week 2 (Fig. 8B, *lanes 4–6*). At weeks 3 and 4, the 21-kDa band that cross-reacted with the anti-$p21^{Waf1}$ antibody exhibited dark and diffuse staining in all six rats. No cross-reactivity was observed with anti-$p21^{Waf1}$ antibodies in any of the control lanes at any time period (Fig. 8B, *lanes 1–3*). Overall, dietary administration of

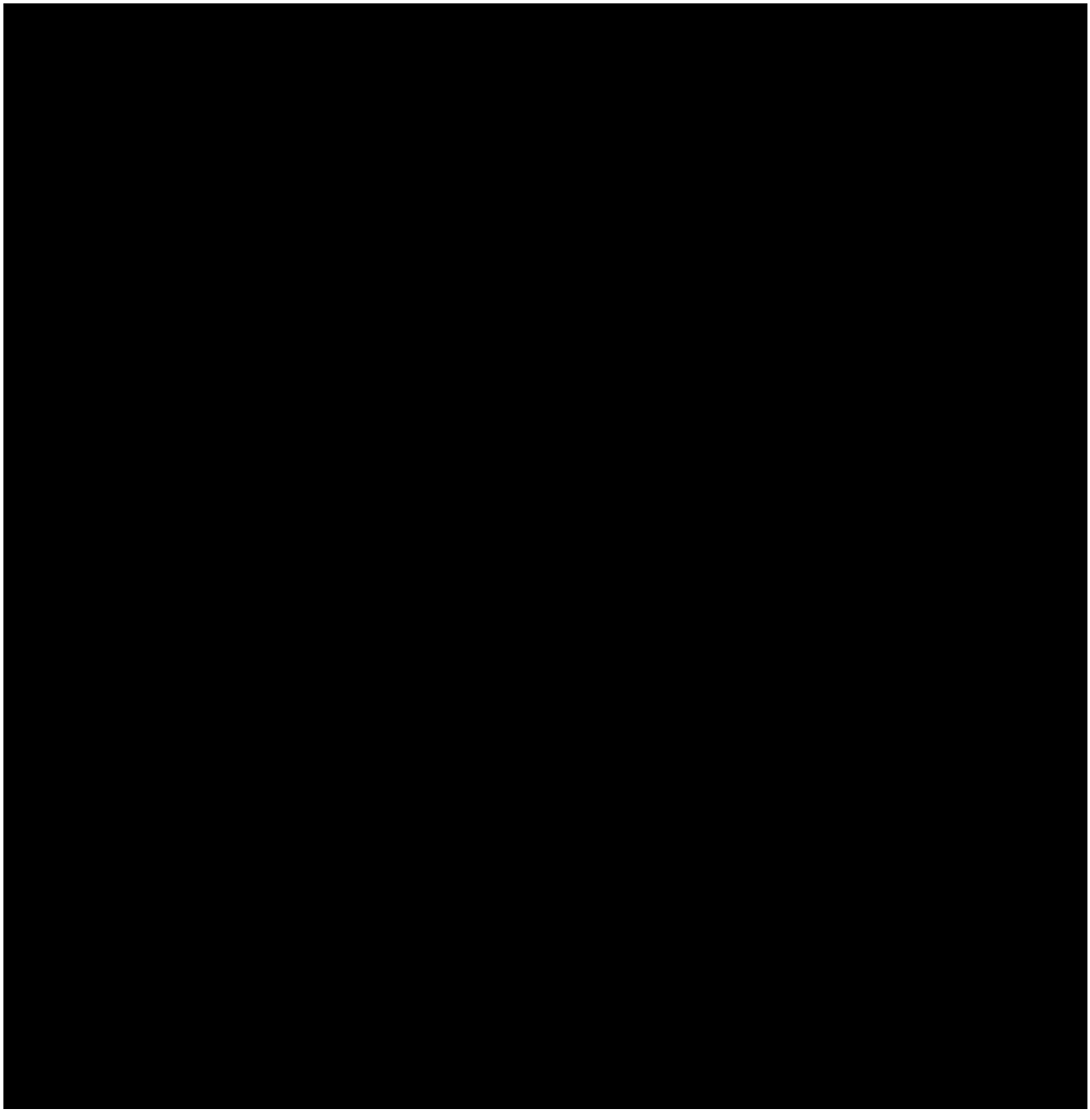

Fig. 6. A and B, Hematoxylin and eosin staining of WY14,643-treated rat liver (4 days). C and D, Immunohistochemical demonstration of the cytosolic localization of CDK1. E and F, Nuclear localization of PCNA. *Bar length*, 50 μm for A, C, and E (low-power magnification) and 100 μm for B, D, and F (higher-power magnification). C and D, Anti-CDK1 immunostaining of WY14,643-treated rat livers demonstrated a marked orange-brown cytoplasmic staining limited to scattered hepatocytes that seemed to exhibit cytomegaly, karyomegaly, or mitotic changes. Mitotic figures without an evident nuclear membrane had diffuse cellular staining; neighboring, nonproliferating hepatocytes were not stained. E and F, Immunostaining with anti-PCNA demonstrated that the affected hepatocytes had deep-red nuclear staining, with distribution of color varying from homogeneous to marginated along the inner aspect of the nuclear membrane. Mitotically active hepatocytes without the presence of a nuclear membrane had course globules of red stain located throughout the cell. Thus, in contrast to the results from immunostaining with anti-CDK1 (C and D), staining with anti-PCNA revealed a predominately nuclear localization of PCNA.

0.08% WY14,643 resulted in sustained expression of hepatic p53 tumor suppressor protein from week 1 through week 4 and of $p21^{Waf1}$ CDK inhibitory protein from week 3 to week 4 relative to corn oil-treated control animals. In addition, the observed increase in $p21^{Waf1}$ expression was in concert with p53 expression, a known transcriptional regulator of $p21^{Waf1}$ (24), and with reduction in the rate of DNA synthesis after WY14,643 exposure (19).

Discussion

The results of the current study demonstrated that the hepatocarcinogen WY14,643 produced increases in hepatocyte concentrations of the cell division control enzyme CDK1, the auxiliary protein of DNA polymerase δ-PCNA (25), the tumor suppressor protein p53, and the CDK inhibitory protein $p21^{Waf1}$. The increases of CDK1 and PCNA in concert

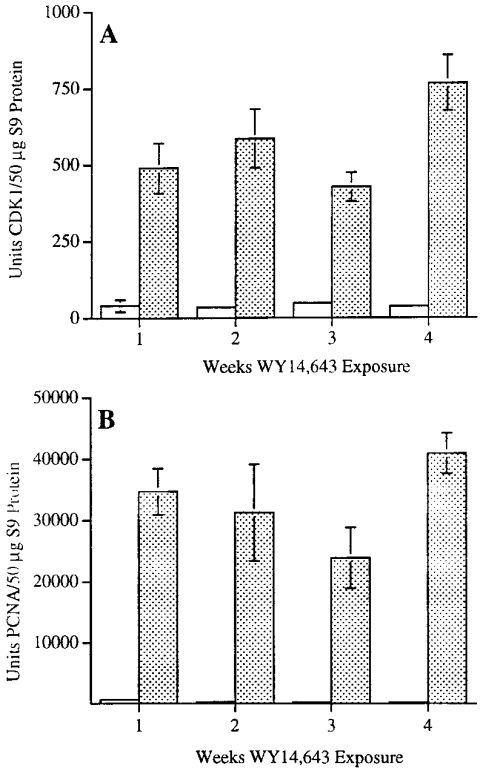

Fig. 7. Time course of rat hepatic CDK1 (A) and PCNA (B) expression after dietary administration of 0.08% WY14,643 for 1, 2, 3, and 4 weeks as measured by ELISA. One unit of CDK1 or PCNA immunoreactivity was defined as 1 fmol of the CDK1 or PCNA antigen. Values are mean ± standard deviation of six animals per group for control animals (*open bars*) and four animals per test group (*shaded bars*). WY14,643 treatment produced increased ($p < 0.001$) expression of both cell cycle proteins compared with control animals at all times. Hepatic CDK1 and PCNA protein concentrations were elevated an average of 15- and 50-fold, respectively, relative to control animals, indicating continued signaling for cell cycle progression.

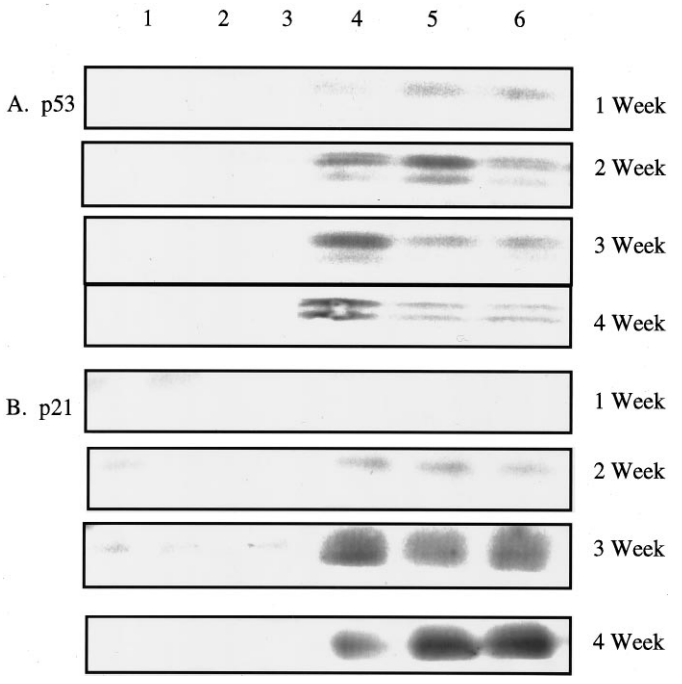

Fig. 8. Immunoblot demonstrating the time course of (A) rat hepatic p53 tumor suppressor protein and (B) $p21^{Waf1}$ CDK inhibitory protein expression after dietary administration of 0.08% WY14,643 over 4 weeks. Hepatic 9000 × *g* supernatant fractions (S9) of control and WY14,643-treated rats were separated using an 11% SDS-PAGE gel as described in Materials and Methods. The separated proteins were transferred to a blotting membrane and probed with (A) anti-p53 or (B) anti-$p21^{Waf1}$ antibodies. Each lane (100 μg of hepatic S9 protein) represents a single animal. *Lanes 1–3*, corn oil-treated control animals. *Lanes 4–6*, 0.08% dietary WY14,643-treated group. Probing with anti-p53 detected faint, single bands at 53 kDa at 1 week in WY14,643-treated rat liver samples. At 2 and 3 weeks of WY14,643 administration, the 53-kDa band became thicker and more pronounced and stained as a doublet in four of the six rats. By 4 weeks, all three rats exhibited the doublet pattern of staining of p53. No cross-reactivity was observed with anti-p53 antibodies in any of the control lanes at any time period. The anti-$p21^{Waf1}$ band in the WY14,643-treated hepatic S9 did not become apparent until week 2. At weeks 3 and 4, the 21-kDa band that cross-reacted with the anti-$p21^{Waf1}$ antibody exhibited dark and diffuse staining in all six rats. No cross-reactivity was observed with anti-$p21^{Waf1}$ antibodies in any of the control lanes at any time period. Overall, dietary administration of 0.08% WY14,643 resulted in sustained expression of hepatic p53 tumor suppressor protein from week 1 through week 4 and of $p21^{Waf1}$ CDK inhibitory protein from week 3 to week 4 relative to corn oil-treated control animals.

with WY14,643 dosing suggest a relationship of the two proteins to the proliferative stimulus generated by the xenobiotic. Numerous research articles appearing over the past decade have substantiated PCNA as a biomarker of proliferating cells (26). More recently, however, the central role of CDK1 in the regulation of the mammalian cell cycle has been described (1). Increasing evidence supporting the relationship of aberrant CDK1 expression and cancer (27–30), combined with the central role of CDK1 in cell cycle control, suggests that chemically induced abnormal entry into the cell cycle resulting in the expression of CDK1, and probably other cell cycle regulatory proteins, could be a critical factor in chemical carcinogenesis induced via chronic chemical exposure.

WY14,643-induced cellular proliferation has been quantified by several laboratories using bromodeoxyuridine or [^{3}H]thymidine incorporation into hepatic DNA. The administration of diets containing 50 or 1000 ppm WY14,643 to F344 rats for 21 days resulted in a 2.7- and 5.4-fold increase, respectively, in cell proliferation relative to control animals (31). It was also reported that similar hepatocyte proliferation rates were found in Crl:CDBR(CD) rats using the same dietary concentrations of WY14,643, specifically a 3.7-fold increase relative to control animals at each dietary concentration. Eldridge *et al*. (32) described a 15-fold increase in hepatic [^{3}H]thymidine incorporation and a 25-fold increase in hepatic bromodeoxyuridine labeling in male F344 rats 5 days after the animals received a diet containing 1% WY14,643. Marsman *et al*. (19) found that labeling of hepatocyte nuclei with a single injection of [^{3}H]thymidine resulted in a 2–4-fold burst in replicative DNA synthesis in WY14,643-fed rats, with a return to control levels by dosing day 4. With osmotic pumps implanted in rats, the same research group observed a 5–10-fold relative increase in replicative DNA synthesis in rats receiving WY14,643 for 39–365 days. The results of these studies are in excellent agreement with the relative increases in expression of CDK1 observed in the current study, which would be expected for independent measurements of cell cycle activation correlated with DNA synthesis. Recently, the correlation of CDK1 expression and [^{3}H]thymidine incorporation was reported in neurogenesis in rat brain (33).

CDK1 or its kinase activity has been reported to be elevated in transformed cells, in clinical cancer, and after acute and subchronic exposure to TCDD. Overexpression of CDK1 was noted in 90% of breast tumor cell lines examined (29) and in all clinical gastric and colon carcinomas studied (28). Primary human keratinocytes immortalized by the entire genome or only by the E6/E7 genes of human papillomavirus types 16 and 17 displayed a 5–20-fold increase in the abundance of CDK1 (27). TCDD dosing has been shown to induce a rapid increase in expression of murine hepatic CDK1 (20) that can be sustained over 90 days with continuous exposure to the xenobiotic (34). Furthermore, proliferation-associated tyrosine and threonine/serine kinase activities have recently been demonstrated to be activated by TCDD interacting with the aryl hydrocarbon receptor (35). Considering that both TCDD and WY14,643 toxicities are believed to be receptor mediated, it is tempting to infer that the stimulus for increased expression of CDK1 provided by TCDD and WY14,643 is initiated via xenobiotic/receptor interaction and that this interaction functions to provide the cell with a continuous proliferative signal that mimics the aberrant signaling pathways that exist in transformed cells.

The formation of CDK1 and PCNA complexes is supported by the column chromatography and immunohistochemistry data in this study. Estimates of the molecular masses of CDK1 and PCNA indicate the existence of heterodimeric or homodimeric complexes with molecular masses of ~70 kDa. This could represent a variety of combinations of CDK1, PCNA, cyclins, and $p21^{Waf1}$. The detection of PCNA in the hepatic S9 supernatant fraction and the failure to visualize cytosolic PCNA with immunohistochemistry could be explained by the masking of the epitope for the anti-PCNA monoclonal antibody in the complex existing in the cytosol. When present in the nucleus, the functioning PCNA has exposed epitope and is clearly visualized with immunostaining. A recent report estimated that only ~20-30% of PCNA is capable of immunohistochemical detection (36). Clearly, PCNA is abundant outside the nucleus, where it most likely exists in a complexed form.

At early time points after xenobiotic exposure, cellular proliferation can be prevented through functioning inhibitory pathways such as p53 (37). CDK1 has been reported to phosphorylate p53 (5). Phosphorylated p53 can then function as a transcriptional regulatory protein for $p21^{Waf1}$ expression (24). Inhibition of proliferation is accomplished through the formation of $p21^{Waf1}$ complexes with CDK1, CDK2, CDK4, and PCNA (6, 38). The result of the formation of these complexes is a block of the progression of the cell cycle at the G1/S interphase. Recent evidence demonstrates that $p21^{Waf1}$ can directly inhibit DNA replication *in vitro* (lacking CDK) by binding to PCNA (38). Thus, $p21^{Waf1}$ may play a dual role in response to the overexpression of CDK1: it prevents entry into S phase by inhibiting CDKs and may directly block ongoing DNA replication by inhibiting PCNA.

The results of a G1/S block can be seen in DNA labeling studies in which the labeling index approaches control levels within several days of WY14,643 administration (19). However, the existence of this G1/S block does not indicate an attenuation of the signals for cellular proliferation. Evidence from this study with WY14,643 and previously work with TCDD (34) indicates that expression of CDK1 protein remains elevated while the animals are exposed to the carcinogen. Therefore, proliferative signals are sustained in the presence of inhibitory signals, as demonstrated by the lack of continued nuclear labeling and the induced expression of p53 and $p21^{Waf1}$. Expression of inhibitory signals in concert with proliferative signals results in a signaling conflict within the cell. It is our hypothesis that maintenance of signaling conflicts via continued xenobiotic exposure will eventually result in cell transformation or apoptosis (39). Which of these events will occur is no doubt a function of a variety of factors; the more obvious of these would include the stimulating xenobiotic administered, dose, cell damage, cell type, and additional chemical exposure.

It is now generally recognized that neither hepatomegaly nor peroxisome proliferation is an adequate predictor of the carcinogenicity of the class of chemicals termed peroxisome proliferators (40). This and previous studies (20, 34) suggest that the proliferative signal of increased CDK1 expression may function as a mechanistic biomarker of the carcinogenic potency of peroxisome proliferators such as WY14,643 and other chemicals representing the group of nongenotoxic carcinogens. Further studies elaborating dose-response and structure-activity relationships and receptor function for the peroxisome proliferators and dioxins are under way in our laboratory. A comparison of the expression of CDKs and cyclins in normal regenerative systems (2) with the expression in xenobiotic-treated animals should provide a valuable model for the identification of aberrant responses characteristic of chemical carcinogens.

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