Transcript Expression of Cyclin D1 Is Sufficient to Promote Hepatocyte Replication and Liver Growth in Vivo

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ABSTRACT

Cyclin D1 regulates mitogen-dependent progression through G1 phase in cultured cells, and its overexpression in malignant cells is thought to contribute to autonomous proliferation in vivo. However, previous studies in cell lines have not demonstrated that cyclin D1 is sufficient to trigger cell replication. In this study, we found that transient transfection of adult hepatocytes with cyclin D1 stimulated assembly of active cyclin D1/cdk4 complexes, robust hepatocyte proliferation, and liver growth in the intact animal. After several days, hepatocyte proliferation was inhibited despite the persistence of high levels of cyclin D1 and cyclin E, suggesting that endogenous antiproliferative mechanisms were induced. Our data suggest that this antiproliferative response includes the marked up-regulation of p21, which in turn inhibits cyclin D1/cdk4 and cyclin E/cdk2 complexes. This study offers further evidence that cyclin D1 plays a pivotal role in the regulation of hepatocyte proliferation in the liver. Furthermore, this model may offer a unique system to study the normal cellular response to cyclin D1 expression in vivo.

INTRODUCTION

In cell culture systems, mitogen-stimulated cells encounter a critical checkpoint in mid-to-late G1 phase, after which they generally become committed to replicate, even if growth factors are withdrawn (1). Passage through this G1 checkpoint, called the restriction point, allows cells to progress through the cell cycle in an autonomous and mitogen-independent manner. Progression through G1 phase is regulated by holoenzyme complexes consisting of D- and E-type cyclins and their cdk partners (2, 3). In many cell types, induction of cyclin D1 protein by extracellular signals appears to be a key intracellular event that regulates passage through G1 phase. Activation of the cyclin D1/cdk4 kinase, which phosphorylates Rb, may represent the biochemical step at which cells become competent to proceed through replication in the absence of mitogen (2–4). However, in several cell lines, transfection with cyclin D1 does not promote cell cycle progression in the absence of growth factor, presumably because other mitogen-dependent events (such as activation of Mek1) are required to stimulate formation of active cyclin D1/cdk4 complexes (2, 3, 5).

Deregulation of the cyclin D-Rb pathway, which occurs in most malignancies, is thought to promote cell proliferation that is independent of normal mitogenic stimuli (3). This can occur though diverse mechanisms, including deletion of tumor suppressor genes that normally repress this pathway, such as Rb or the ink4 proteins. Alternatively, cyclin D1 is overexpressed in numerous cancers, either as a result of chromosomal rearrangements involving the cyclin D1 gene, or through deregulation of signaling pathways that normally control the expression of this protein. Transgenic mice with targeted overexpression of cyclin D1 in different cell types demonstrate enhanced proliferation during development, and in some cases a predisposition toward malignancy, providing experimental evidence that cyclin D1 can act as an oncogene (6–9). However, these prior studies have not determined whether induction of cyclin D1 is sufficient to trigger proliferation of quiescent, differentiated cells in the adult animal. Furthermore, transgenic models with constitutive protein expression can be confounded by developmental abnormalities and compensatory changes in gene expression that may affect the cellular response.

In normal adult liver, hepatocytes are highly differentiated and rarely undergo cell division, but they retain a remarkable ability to proliferate in response to acute or chronic injury, resulting in diminished functional liver mass (10). Previous studies have suggested that cyclin D1 is a critical mediator of G1 progression in hepatocytes (10). For example, transfection of cultured primary hepatocytes with cyclin D1 induced entry of these cells into S-phase (11), although we were unable to demonstrate mitosis or cell replication in that study. The present experiments were initiated to examine whether transient overexpression of cyclin D1 was sufficient to promote replication of adult hepatocytes in the normal animal. The results suggest that cyclin D1 alone can drive cell cycle progression of differentiated hepatocytes in vivo. This system may provide a useful model to study the normal cellular response to cyclin D1.

MATERIALS AND METHODS

Animal Studies. Male BALB/c mice (7–8 weeks of age) were housed in an American Association of Laboratory Animal Care-approved facility, and animals were treated in accordance with institutional and NIH guidelines. PH was performed as described previously (12). CsCl-purified adenoviruses were injected via the tail vein, at a dose of 5 × 10^9 pfu/animal unless otherwise noted. The construction of the adenoviruses is reported elsewhere (11, 13, 14). For control experiments, an equivalent adenovirus encoding β-galactosidase was used. Two h before harvest, animals were given an i.p. injection of BrdUrd (12). At the indicated time points, animals were sacrificed, and liver tissue was harvested, as described previously (12, 13). At the time of harvest, liver and body weights were recorded. BrdUrd immunohistochemistry and hepatocyte mitotic rates were performed as described elsewhere (13).

Biochemical Studies. For Western blot, immunoprecipitation, and column chromatography, liver tissue was homogenized in a 0.1% Tween 20 buffer as described (12). Nuclear and cytoplasmic extracts were obtained using methods outlined previously (15). The techniques and antibodies used for Western blot and immunoprecipitation are the same as described previously, with the exceptions outlined below (12, 13). Liver extracts were subjected to gel filtration chromatography using a Bio-Sil SEC-250 column (Bio-Rad, Hercules, CA). Three hundred-µl of Tween 20 extract (8 mg/sample) were loaded onto the column, followed by gel filtration buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM β-mercaptoethanol] at a flow rate of 0.5 ml/min. The columns had been calibrated with molecular mass standards (Bio-Rad) including thyroglobulin (670,000), gamma-globulin (158,000), and ovalbumin (44,000). Three hundred-µl fractions were collected, and 25 µl were subjected to Western blot analysis. Immunoprecipitation/kinase assays were performed using a modification of the method of Jinno et al. (16). In brief, 500 µg of liver lysate were immunoprecipitated with antibodies to cdk4 or cyclin E (Santa Cruz Biotechnology, Inc.).

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3 Then abbreviations used are: cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; PH, 70% partial hepatectomy; BrdUrd, bromodeoxyuridine; pfu, plaque-forming unit(s).

4 Immunoprecipitation/kinase assays were performed using a modification of the method of Jinno et al. (16). In brief, 500 µg of liver lysate were immunoprecipitated with antibodies to cdk4 or cyclin E (Santa Cruz Biotechnology, Inc.).
Synchronous fashion, with peak DNA synthesis occurring at

RESULTS

Earlier studies have documented that cyclin D1 is not expressed in normal mouse liver but is markedly induced during compensatory hyperplasia following PH (12). In the mouse PH model, a large number of hepatocytes progress though the cell cycle in a relatively synchronous fashion, with peak DNA synthesis occurring at ~1.5 days, at which time cyclin D1 expression is readily detectable (Fig. 1A). To determine whether transient expression of cyclin D1 could promote hepatocyte proliferation in vivo, we transfected these cells with an adenovirus encoding the human cyclin D1 protein (ADV-D1; Ref. 11). Previous studies have shown that >95% of i.v.-injected adenoviruses target the liver, and most hepatocytes are transduced using this method (14, 18). Mice were injected with increasing doses of the ADV-D1 or control virus (Fig. 1A). As described for other adenoviruses (14), there was a threshold dose at which cyclin D1 protein expression was detected, which may be the result of overcoming pathways that normally degrade this protein in quiescent cells (2, 3). Cyclin D1 protein expression was detectable as early as 16 h after transfection and was persistent at 6 days, whereas the control adenovirus did not induce cyclin D1 expression (Fig. 1B). At 30 days after transfection, cyclin D1 expression was nearly undetectable (data not shown), consistent with other studies documenting that adenoviruses promote only transient transgene expression in the liver (14).

Transfection of growth factor-deprived cell lines with cyclin D1 does not promote cell cycle progression because of the inactivity of mitogen-dependent pathways that regulate intracellular localization and promote assembly of active cyclin D1/cdk4 complexes (2, 5). To determine whether the transfected cyclin D1 was transported to the nucleus, we examined its expression in nuclear and cytoplasmic extracts. Previous studies have shown that in regenerating rat liver after PH, cyclin D1 is more abundant in the cytoplasm than in the nucleus (15). In mouse liver after PH, cyclin D1 was similarly more abundant in the cytoplasm than in the nuclear extract (Fig. 1C). After transfection with ADV-D1, cyclin D1 was readily detectable in both the nuclear and cytoplasmic extracts (Fig. 1C). To confirm that cyclin D1 assembled efficiently into complexes in this system, liver extracts were fractionated by gel filtration on a column that had been calibrated with molecular weight markers as shown in Fig. 2A. In quiescent liver, no cyclin D1 was detected, and cdk4 was most abundant in lower molecular weight fractions. After PH, most cyclin D1 was found in fractions of ~100–170 kDa, which comigrated with a substantial proportion of cdk4. These fractions also comigrated with the majority of p21 and p27 after PH (data not shown). This is compatible with studies in cell culture systems, which have shown that most native cyclin D/cdk4 is contained in complexes of this molecular weight range (19). On a Western blot with a darker exposure, cyclin D1 was also noted in higher molecular weight complexes of ~600 kDa (Fig. 2A, bottom panel). In extracts of liver after transfection with ADV-D1, cyclin D1 was similarly expressed in ~100–170 kDa complexes. In addition, cyclin D1 was prominently expressed in higher molecular weight complexes that did not appear to contain a proportional amount of cdk4 (or cdk6; data not shown).

These results indicate that after transfection of quiescent hepatocytes in vivo, cyclin D1 is efficiently transported to the nucleus, and virtually all of this protein is recruited into protein complexes. Furthermore, a substantial portion of the transfected cyclin D1 forms complexes in the 100–170 kDa range, similar to the pattern seen after PH.

To determine whether transfection with cyclin D1 led to activation of cdk4 kinase activity, immunoprecipitation studies were performed (Fig. 2B). At 1–2 days after ADV-D1 injection, cdk4 kinase activity was induced to levels higher than that seen maximally after PH. In mice transfected with the control adenovirus, no similar induction of cdk4 kinase activity was noted. At 6 days after ADV-D1 transfection, abundant cyclin D1 expression and cyclin D1/cdk4 complex formation were noted. However, the activity associated with this kinase complex was markedly diminished, suggesting the presence of inhibitors. Coprecipitation studies showed that more p21 (but not p27) was associated with cyclin D1/cdk4 at this time point, suggesting that p21 may be responsible for inhibition of kinase activity.

Transfection with cyclin D1 led to evidence of substantial cell cycle progression during the first 1–2 days. The expression of genes associated with the progression into S and M phase (cyclins E, A, and B) was up-regulated to levels similar to that seen maximally after PH (Fig. 3A). In addition, there was moderate up-regulation of cdk2 and cdk4. No effect on cdk6 or cyclin D3 was observed (data not shown). The expression of p27 was not substantially altered, but p21 was highly induced after transfection with cyclin D1, particularly at 6 days. Hepatocyte DNA synthesis, as measured by BrdUrd immunohistochemistry, was markedly induced by ADV-D1 at 1–2 days and then declined at 6 days (Fig. 3B, D, and E). As reported previously (13, 20), transfection with the control adenovirus led to a low level of hepatocyte DNA synthesis. Transfection with an adenovirus encoding human cyclin E (ADV-E) at a similar dose promoted little hepatocyte DNA synthesis (Fig. 3B). This dose of ADV-E induced readily detectable cyclin E expression and formation of inactive cyclin E/cdk2/p27 complexes (data not shown and Ref. 13). Hepatocyte replication was confirmed by the presence of numerous mitotic hepatocytes at 2 days after cyclin D1 transfection. Furthermore, liver size was increased by ~50% in the ADV-D1-treated mice. Thus, cyclin D1 transfection was sufficient to induce expression of downstream cell cycle control genes, robust hepatocyte DNA synthesis and mitosis, and liver enlargement in vivo.

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Fig. 1. Expression of cyclin D1 in the liver after transfection. Mice were injected i.v. with an adenovirus (ADV) encoding cyclin D1 or a control ADV, and liver extract was subjected to Western blot. A, dose-response curve. Liver was harvested 1.5 days after PH or 1 day after ADV-D1 at the indicated doses (× 10⁵ pfu/animal). Two separate animals are shown for each dose. B, time course. Mice injected with 5 × 10⁵ pfu of ADV-D1 or control ADV, and cyclin D1 expression was determined at time points from 16 h to 6 days. C, expression of cyclin D1 in nuclear and cytoplasmic extracts in specimens of normal liver (0), 1.5 days after PH, or 1 day after injection with the indicated ADV.
At 6 days after transfection with ADV-D1, hepatocyte DNA synthesis had diminished, although cyclin E expression was markedly up-regulated (Fig. 3). As was the case for cyclin D1/cdk4, cyclin E/cdk2 possessed little kinase activity, although the abundance of these complexes was maximal at 6 days (Fig. 4). The amount of p27 associated with cyclin E did not increase at this time point, but substantially more p21 bound to the complex. Closer examination of the Western blots revealed that the amount of faster migrating cdk2 [presumably representing the CAK-activated Thr-160-phosphorylated form (21)] associated with cyclin E had increased between 1 and 6 days (data not shown). This suggests that the diminished cyclin E-associated kinase activity was not the result of dephosphorylation of cdk2. In both cyclin E and cdk2 immunoprecipitates, no Tyr-15-phospho-cdk2 was detected by Western blot using a commercially available antibody (Cell Signaling, Inc.; data not shown), indicating that inhibitory phosphorylation of this residue was not responsible for diminished kinase activity. Thus, at 6 days after transfection, inhibition of cyclin D1/cdk4 and cyclin E/cdk2 activity and diminished hepatocyte DNA synthesis correlated with marked recruitment of p21 into these complexes.

DISCUSSION

In this report, we demonstrate that short-term transfection of cyclin D1 was sufficient to promote proliferation of quiescent hepatocytes and increased liver size in the adult animal. In addition, we found that proliferation induced in this manner eventually provoked mechanisms to down-regulate cell cycle progression despite the persistence of abundant cyclin D1/cdk4 and cyclin E/cdk2. Thus, the current studies support the hypothesis that induction of cyclin D1 is a key regulatory step required for progression of hepatocytes through the restriction point during liver regeneration or hyperplasia (10). Furthermore, consistent with recent studies in transgenic plants and Drosophila demonstrating that cyclin D1 enhances organ growth (22-24), the current results indicate that this protein is able to induce both cell proliferation and liver growth.
proliferation and the biosynthetic pathways necessary for liver growth.

Because cyclin D1 is up-regulated in many human malignancies [including some hepatocellular carcinomas (25)], the response to its overexpression has been studied extensively in cell culture systems. In general, cyclin D1 alone does not promote cell cycle progression in the absence of mitogen, but it shortens the duration of G_{1} phase in response to growth factors (2). Cyclin D1 is post-translationally regulated through diverse mechanisms downstream of growth factor receptors. For example, in NIH 3T3 fibroblasts, cyclin D1/cdk4 complex assembly and nuclear translocation requires activation of Mek1 (5). Thus, the failure of cyclin D1 to induce mitogen-independent cell cycle progression in these other systems may reflect differences in the basal activity of signal transduction proteins in “quiescent” cells. We had shown previously that cyclin D1 was sufficient to induce cultured primary hepatocytes to enter S-phase in the absence of growth factors (11). Other studies have shown that isolation of primary hepatocytes to allow this protein to trigger cell cycle progression could involve activation of cell cycle checkpoints or homeostatic mechanisms that control liver size (10). The regulation of p21 in this model is the subject of ongoing studies and may provide insight into the normal cellular response to cyclin D1 overexpression.

In summary, the current studies suggest that induction of a single G_{1}-regulatory protein, cyclin D1, is sufficient to trigger cell replication of adult hepatocytes. These results support the concept that cyclin D1 is the crucial intracellular mediator of mitogenic signals that control hepatocyte proliferation in the regenerating liver (2, 10, 11, 26). Furthermore, sustained expression of this protein induced an antiproliferative response that inhibited cell cycle progression, despite persistent expression of cyclin E/cdk2. This model may therefore offer insight into cell cycle checkpoints that are activated as a result of cyclin D1 overexpression. Because constitutive expression of cyclin D1 plays a role in the development of neoplasia (3), further study of this antiproliferative response may provide relevant information about cellular mechanisms that resist the development of malignancy. Finally, this model offers a unique system to examine cell cycle control proteins in the in vivo context.

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