Construction of a Cyclin D1-Cdk2 Fusion Protein to Model the Biological Functions of Cyclin D1-Cdk2 Complexes*

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Cyclin D1 is frequently overexpressed in human breast cancers, and cyclin D1 overexpression correlates with poor prognosis. Cyclin D1-Cdk2 complexes were previously observed in human breast cancer cell lines, but their role in cell cycle regulation and transformation was not investigated. This report demonstrates that Cdk2 in cyclin D1-Cdk2 complexes from mammary epithelial cells is phosphorylated on the activating phosphorylation site, Thr160. Furthermore, cyclin D1-Cdk2 complexes catalyze Rb phosphorylation on multiple sites in vitro. As a model to investigate the biological and biochemical functions of cyclin D1-Cdk2 complexes, and the mechanisms by which cyclin D1 activates Cdk2, a cyclin D1-Cdk2 fusion gene was constructed. The cyclin D1-Cdk2 fusion protein expressed in epithelial cells was phosphorylated on Thr160 and catalyzed the phosphorylation of Rb on multiple sites in vitro and in vivo. Kinase activity was not observed if either the cyclin D1 or Cdk2 domain was mutationally inactivated. Mutational inactivation of the cyclin D1 domain prevented activating phosphorylation of the Cdk2 domain on Thr160. These results indicate that the cyclin D1 domain of the fusion protein activated the Cdk2 domain through an intramolecular mechanism. Cells stably expressing the cyclin D1-Cdk2 fusion protein exhibited several hallmark features of transformation including hyperphosphorylation of Rb, resistance to TGFβ-induced growth arrest, and anchorage-independent proliferation in soft agar. We propose that cyclin D1-Cdk2 complexes mediate some of the transforming effects of cyclin D1 and demonstrate that the cyclin D1-Cdk2 fusion protein is a useful model to investigate the biological functions of cyclin D1-Cdk2 complexes.

Cyclin D1 provides a critical link between signaling initiated by extracellular growth factors and cell cycle regulation. Cyclin D1 levels in the mammary gland are regulated by steroid hormones and polypeptide growth factors, suggesting a unique role for cyclin D1 in mammary development (1). Consistent with an important role for cyclin D1 in mammary gland function, mice lacking cyclin D1 fail to undergo normal lobuloalveolar development during pregnancy (1, 2). Cyclin D1 regulates mammary development through the formation of catalytically active complexes with the cyclin-dependent kinase Cdk4

1 The abbreviations used are: Cdk, cyclin-dependent kinase; GFP, green fluorescent protein; HEK, human embryonic kidney; MOPS, 4-morpholinepropanesulfonic acid; Rb, retinoblastoma tumor suppressor protein; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PCNA, proliferating cell nuclear antigen; TGF, tumor growth factor.

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Complexes are phosphorylated on the activating CASK phosphorylation site of Cdk2, Thr\(^{160}\). Isolated cyclin D1-Cdk2 complexes phosphorylate histone H1 and Rb on sites preferred by Cdk2, as well as on sites preferred by Cdk4. Furthermore, we have constructed a cyclin D1-Cdk2 fusion protein to model the biological functions of cyclin D1-Cdk2 complexes. The cyclin D1-Cdk2 fusion protein phosphorylates Rb in vitro on sites preferred by Cdk2 and Cdk4. Stable expression of the cyclin D1-Cdk2 fusion protein confers anchorage-independent cell proliferation and resistance to the growth inhibitory effects of TGFβ. These data suggest that cyclin D1-Cdk2 complexes mediate some of the transforming effects of cyclin D1 overexpression in cancers and may be a useful target for anti-cancer therapeutics.

MATERIALS AND METHODS

Cell Culture, [\(^{1}H\)]Thymidine Incorporation Assays, and Soft Agar Assays—NMuMG, MDA-MB-231, and HaCaT cells were propagated as described previously (15). HEK 293, HCT116, and MDA-MB-361 cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Life Technologies, Inc.) supplemented with 10% fetal bovine serum. p21-null HCT116 cells were obtained from Dr. Bert Vogelstein, Johns Hopkins, Baltimore, MD, and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. [\(^{1}H\)]Thymidine incorporation assays and soft agar assays were performed as described previously (15). Images of soft agar colonies were captured using an Olympus CK40 inverted microscope and an Olympus DP10 digital camera.

Construction of Recombinant Adenoviruses, Recombinant Retroviruses, Stable Cell Lines, and Transient Transfections—Adenoviruses were introduced by PCR using the primer sets K114E: 5′-TTGGTACGCTTTTTTAATC-3′ and cloned into the EcoRI and BamHI sites of pSP72 (Promega). Cdk2 was amplified using the primers 5′-TTTTGAGTCTTAAGGTTGAC-3′ and cloned into the EcoRI and XhoI sites of pcDNA3 (Invitrogen, Life Technologies, Inc.) and analysis of intact Cdk2 complexes. An adenovirus encoding hexahistidine-tagged Cdk2 (Cdk2-His\(_{6}\)) was constructed with mass accuracies of less than 20 ppm, allowing for 1 missed cleavage, complete carbamidomethylation of cysteine sulfhydryls, and partial oxidation of methionine residues.

RESULTS

Characterization of Cyclin D1-Cdk2 Complexes from Human Carcinoma and Mouse Mammary Epithelial Cells—We previously showed that treatment of mouse mammary epithelial cells and human mammary carcinoma cells with TGFβ + rapamycin alters the subunit composition of Cdk2 complexes and Cdk2 intracellular localization (15, 20). To systematically study TGFβ and rapamycin regulation of Cdk2 complexes we constructed a recombinant adenovirus encoding hexahistidine-tagged Cdk2 (Cdk2-His\(_{6}\)) to allow the rapid purification and analysis of intact Cdk2 complexes. An adenovirus encoding GFP was used as a control for the purifications. Cdk2 complexes isolated from human mammary MDA-MB-231 carcinoma cells consistently contained cyclin D1 (Fig. 1A). The levels of cyclin D1-Cdk2 complexes were not altered by TGFβ + rapamycin treatment. Similar results were obtained with human mammary MDA-MB-361 carcinoma cells (data not shown).
shown). A previous study demonstrated the presence of cyclin D1-Cdk2 complexes in human mammary carcinoma cell lines (13). However, whether cyclin D1-Cdk2 complexes play a role in cell cycle regulation in mammary carcinoma cells or mammary epithelial cells has not been explored.

To determine whether endogenous cyclin D1-Cdk2 complexes are present in mouse mammary epithelial cells, coinmunoprecipitation experiments were performed in mouse mammary NMuMG cells (Fig. 1B). These experiments demonstrated that cyclin D1-Cdk2 complexes were present in NMuMG cells and that their levels were unaffected by TGFβ, rapamycin, or TGFβ + rapamycin treatment. Strikingly, Cdk2 associated with cyclin D1 was phosphorylated on the cyclin-dependent kinase activating kinase site, Thr160, suggesting that cyclin D1-Cdk2 complexes may be catalytically active.

In addition to acting as a kinase, cyclin D1-Cdk2 complexes might also regulate the cell cycle indirectly by sequestering p21 and p27. There is extensive evidence that cyclin D1-Cdk4 complexes regulate the cell cycle through this mechanism; however, cyclin D1-Cdk2 complexes have not been shown to perform a similar function. To determine the subunit structure of cyclin D1-Cdk2 complexes, NMuMG cells were co-infected with recombinant adenoviruses encoding FLAG-epitope-tagged cyclin D1 and Cdk2-His6. Cyclin D1-Cdk2 complexes were purified by sequential chromatography using TALON resin to bind His6-tagged Cdk2 and anti-FLAG-agarose resin to bind cyclin D1-containing complexes. Purified cyclin D1-Cdk2 complexes from cells treated with TGFβ + rapamycin (lanes 3) or left untreated (lane 2), or a control purification using GFP adenovirus (lane 1), were resolved by SDS-PAGE and visualized by colloidal Coomassie staining (Fig. 1C). Individual bands were excised, subjected to trypsin digestion, and the peptides analyzed by MALDI-TOF mass spectrometry. These analyses indicated that the cyclin D1 complexes contained proliferating cell nuclear antigen (PCNA) and p21 in a near stoichiometric ratio as judged by Coomassie staining. Interestingly, TGFβ + rapamycin treatment dramatically increased the amount of p27 present in cyclin D1-Cdk2 complexes. This result is consistent with our previous study (15), but extends these results by showing that TGFβ + rapamycin-induced p27 binding is independent of the cyclin (E, A, or D1) associated with Cdk2. The observation that cyclin D1-Cdk2 complexes bind both p21 and p27 suggests that, like cyclin D1-Cdk4 complexes, cyclin D1-Cdk2 complexes sequester p21 and p27 and may indirectly activate cyclin E-Cdk2 and cyclin A-Cdk2 complexes.

**p21 Stabilizes, but Is Not Required for the Formation of Cyclin D1-Cdk2 Complexes**—The experiment in Fig. 1C involved overexpression of both cyclin D1 and Cdk2, so we examined NMuMG cells and cancer cell lines for the presence of endogenous cyclin D1-Cdk2 complexes. Co-immunoprecipitation experiments demonstrated that cyclin D1-Cdk2 complexes are present at relatively high levels in human HCT116 colorectal carcinoma cells and NMuMG cells, and at lower levels in T98G glioblastoma cells or SW480 colorectal carcinoma cells.

**FIG. 1.** Cyclin D1-Cdk2 complexes are present in human mammary carcinoma cells and mouse mammary epithelial cells. A, MDA-MB-231 cells were infected with an adenovirus encoding His6-tagged Cdk2 (Cdk2-His6) or a control adenovirus encoding GFP. Twenty-four hours post-infection the cells were treated for an additional 24 h with normal growth medium (C) or 10 ng/ml TGFβ + 100 nM rapamycin (T + R). Proteins from cell extracts were partially purified by TALON affinity chromatography, and the bound material analyzed by immunoblotting with antibodies specific for Cdk2, Cdk2 phosphorylated on Thr160 (P-Cdk2(T160)), or cyclin D1. B, protein extracts from NMuMG cells treated for 24 h with 10 ng/ml TGFβ (T), 100 nM rapamycin (R), or 10 ng/ml TGFβ + 100 nM rapamycin (T + R) were immunoprecipitated with antibodies to Cdk2 (left panel) or cyclin D1 (right panel). The immunoprecipitates were analyzed by immunoblotting with antibodies specific for Cdk2, Cdk2 phosphorylated on Thr160 (P-Cdk2(T160)), or cyclin D1. IgG and mouse IgG1 represent normal rabbit IgG and IgG1 isotype control immunoprecipitation reactions, respectively. C, NMuMG cells were infected with adenoviruses encoding GFP (lane 1), or FLAG-Cyclin D1 + Cdk2-His6, (lanes 2 and 3), and incubated 24 h. The cells were treated with normal growth medium (lanes 1 and 2) or with 10 ng/ml TGFβ + 100 nM rapamycin (lane 3) for an additional 24 h. Cyclin D1-Cdk2 complexes were purified by sequential chromatography on TALON and anti-FLAG-agarose resins. The proteins were resolved by SDS-PAGE, and the gels stained with colloidal Coomassie. The indicated bands were excised and identified by MALDI-TOF mass spectrometry. IgG LC is mouse immunoglobulin light chain derived from the FLAG antibody.
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FIG. 2. p21 stabilizes, but is not required for, the formation of cyclin D1-Cdk2 complexes. A, extracts from the indicated cell lines were immunoprecipitated (IP) with antibodies specific for Cdk2, E2F4, or normal rabbit IgG (Rab. IgG) as a control. The immunoprecipitates were analyzed by immunoblot (IB) with the indicated antibodies. B, cell extracts were prepared from rapidly growing HCT116 cells (+ Serum) or HCT116 cells deprived of serum for 14 h (− Serum) that were either wild type for p21 (p21+/+ ) or p21-null (p21−/− ). Equal amounts of protein were immunoprecipitated (IP) with antibodies to Cdk2, or normal rabbit serum (Rab. IgG) as a control, and the crude extracts and Cdk2 immunoprecipitates corresponding to each sample were analyzed by immunoblot with the indicated antibodies.

(Fig. 2A). In addition to cyclin D1, Cdk2 complexes from NMuMG and HCT116 cells contained high levels of p21 and PCNA. In contrast, Cdk2 complexes from T98G and SW480 cells contained low levels of cyclin D1, p21, and PCNA. These results are consistent with the results in Fig. 1C, and suggest that cyclin D1-Cdk2 complexes in rapidly growing NMuMG and HCT116 cells are part of a quaternary complex containing cyclin D1, Cdk2, p21, and PCNA. Interestingly, Cdk2 immunoprecipitated from all four cell lines associated with similar amounts of E2F4, p107, and p130. This observation suggests that different subsets of Cdk2-containing complexes may be differentially regulated by cellular levels of p21, p27, or PCNA.

p21 and p27 act as assembly factors for the formation of cyclin D1-Cdk4 complexes (25, 26). However, it is unclear whether p21 or p27 is required for the formation of cyclin D1-Cdk2 complexes. To address this question, extracts from parental or p21-null HCT116 cells were examined for the presence of cyclin D1-Cdk2 complexes in coimmunoprecipitation experiments (Fig. 2B). Cyclin D1-Cdk2 complexes were present in both parental and p21-null HCT116 cells that were either rapidly growing or rendered quiescent by serum deprivation. The levels of cyclin D1-Cdk2 complexes were slightly decreased in p21-null HCT116 cells suggesting that although p21 is not absolutely required for the formation of cyclin D1-Cdk2 complexes, p21 may stabilize these complexes. Cdk2 complexes from neither parental nor p21-null HCT116 cells contained significant amounts of p27, indicating that p27 did not substitute as a cyclin D1-Cdk2 assembly factor in the absence of p21 (data not shown). p21 deletion prevented PCNA binding to cyclin D1-Cdk2 complexes. This was expected based on previous studies showing that p21 binds Cdk2 through its N terminus while p21 binds PCNA through its C terminus and serves to bridge Cdk2 and PCNA (27, 28). p21 deletion did not alter the levels of Cdk2/p107/E2F4 complexes, supporting the notion that basal levels of p21 in HCT116 cells regulate the subunit composition of cyclin D1-Cdk2 complexes but not E2F4/Cdk2 complexes.

Cyclin D1 and Cdk2 Cooperate to Induce Rb Phosphorylation and Activate E2F-dependent Transcription—Cyclin D1-Cdk2 complexes might regulate cell cycle progression indirectly by sequestering p21 and p27 as discussed above or might regulate cell proliferation directly by phosphorylating important cell cycle regulators such as the pocket proteins Rb, p130, and p107. To assay the kinase activity of cyclin D1-Cdk2 complexes, NMuMG cells were infected with adenovirus encoding Cdk2-His6, and cyclin D1-Cdk2 complexes were isolated by chromatography on TALON resin followed by immunoprecipitation with anti-cyclin D1 antibodies. These complexes exhibited kinase activity when assayed using Histone H1 as the substrate and exhibited Cdk2 phosphorylation on Thr160 (Fig. 3A).

To determine whether cyclin D1 and Cdk2 expression cooperates to induce Rb phosphorylation in vivo, HEK 293 cells were transfected with cyclin D1, the stabilized mutant of cyclin D1(T286A) (29), FLAG-tagged cyclin D1(T286A), or Cdk2-His6, either alone, or in the indicated combinations. Immunoblotting with an antibody recognizing Rb phosphorylated on Ser780/Ser792 (Fig. 3B, upper panel) indicated that the stabilized constitutively nuclear mutant of cyclin D1, and the FLAG-tagged derivative, cooperated with Cdk2 expression to increase endogenous Rb phosphorylation on Ser780 Thr252. To support the notion that these effects were due to increased kinase activity of cyclin D1-Cdk2 complexes, extracts from the transfected cells were immunoprecipitated with the FLAG antibody, and the immunoprecipitates subjected to in vitro Rb kinase assays (Fig. 3B, lower panels). High levels of kinase activity were only observed where Flag-cyclin D1(T286) and Cdk2-His6 were co-expressed. Rb phosphorylation was observed on Ser780 and Ser811 using phosphospecific antibodies. These sites are preferred by Cdk4 and Cdk2, respectively, suggesting that
cyclin D1-Cdk2 may phosphorylate Rb on sites considered to be Cdk4 sites and Cdk2 sites.

We reasoned that if cyclin D1 and Cdk2 cooperate to induce Rb phosphorylation in vivo then they should also cooperate to activate E2F-dependent transcription. Cyclin D1, Cdk2, and Cdk4 each activated E2F-dependent transcription when expressed alone (Fig. 3C). Interestingly, a kinase-dead mutant of Cdk2 (Cdk2(KD)) also activated E2F-dependent transcription severalfold over the empty vector (pcDNA3). This may be caused by the formation of complexes between cyclins and Cdk2(KD) that sequester p21 and p27. Co-expression of cyclin D1 with Cdk2 or Cdk4 additively activated E2F-dependent transcription. Co-expression of cyclin D1 with Cdk2(KD)-activated E2F-dependent transcription more than either construct alone, but not as much as co-expression of cyclin D1 and Cdk2. The simplest interpretation of these results is that cyclin D1-Cdk2 complexes drive Rb phosphorylation and stimulate E2F-dependent transcription by two mechanisms: (1) by sequestering p21 and p27 through a kinase-independent mechanism, and (2) by phosphorylating key cell cycle substrates such as Rb.

Construction of a Cyclin D1-Cdk2 Fusion protein That Is Phosphorylated on Regulatory Sites and Binds the Same Proteins as Endogenous Cyclin D1-Cdk2 Complexes—It is likely that the biological function of cyclin D1-Cdk2 complexes have not been investigated because of the inherent technical difficulties. Experiments involving overexpression of cyclin D1 and Cdk2 together are difficult to interpret. Cyclin D1 binds a number of partners including Cdk4, Cdk6, and Cdk2. Cdk2 binds cyclin E, cyclin A, and D-type cyclins. Likewise, knocking down or knocking out either cyclin D1 or Cdk2 would alter the function of multiple cyclin-dependent kinase complexes.

We constructed a cyclin D1-Cdk2 fusion gene to specifically study the functions of cyclin D1-Cdk2 complexes. This construct was patterned after a cyclin D1-Cdk4 fusion gene described previously (30), and encodes from N terminus to C terminus the FLAG epitope tag, cyclin D1, a flexible linker domain, Cdk2, and the His6 affinity tag (Fig. 4A). To determine whether the cyclin D1-Cdk2 fusion gene is expressed and the protein phosphorylated on the same regulatory sites as endogenous Cdk2, HaCaT cells were cotransfected with the indicated plasmids and an E2F-luciferase reporter plasmid. Luciferase assays were performed and the results plotted as relative luminescence units per microgram of cellular protein extract (RLU/µg) as the average of triplicate determinations ± S.D.

FIG. 3. Cyclin D1 and Cdk2 form catalytically active kinase complexes that phosphorylate Rb and activate E2F-dependent transcription. A, NMuMG cells were infected with adenoviruses (Ad) encoding GFP or Cdk2-His6 as indicated and incubated 48 h. Cell extracts were prepared and purified over TALON resin. Proteins eluted from the TALON resin were immunoprecipitated (IP) with antibodies to cyclin D1 or a nonspecific isotype control antibody (NS IgG1). Immunoprecipitates were assayed for kinase activity using histone H1 as the substrate. Kinase assay results were visualized using a phosphorimagery. The kinase reactions were also analyzed by immunoblot with antibodies specific for cyclin D1, Cdk2, or Cdk2 phosphorylated on Thr160 (P-Cdk2(T160)). B, HaCaT cells were transfected with empty vector (pcDNA3), or pcDNA3 encoding Cdk2-His6, cyclin D1 (D1), cyclin D1(T286A) (D1(T286A)), or FLAg-tagged cyclin D1(T286A) (Flag-D1(T286A)) in the indicated combinations. Cell extracts were prepared and the proteins analyzed by immunoblotting (top panel) with antibodies specific for Rb, Cdk2, cyclin D1, actin, or Rb-phosphorylated on Ser249/Thr252 (P-Rb(S249/T252)). The same cell extracts were immunoprecipitated with antibody to the FLAG epitope tag, and the immunoprecipitates assayed for kinase activity using histone H1 or GST-Rb as the substrate (lower panel). Kinase reactions were also analyzed by immunoblot with antibodies specific for the FLAG epitope, Cdk2, which recognizes both the endogenous (Endog. Cdk2) and His6-tagged Cdk2 (Cdk2-His6), Rb phosphorylated on Ser780 (P-Rb(S780)), and Rb phosphorylated on Ser780/Ser811 (P-Rb(S780/S811)). C, HaCaT cells were cotransfected with the indicated plasmids and an E2F-luciferase reporter plasmid. Luciferase assays were performed and the results plotted as relative luminescence units per microgram of cellular protein extract (RLU/µg) as the average of triplicate determinations ± S.D.
Tyr15) Cdk2 phosphorylation sites. This result suggests that the cyclin D1-Cdk2 fusion protein is regulated similarly to native Cdk2 complexes.

To provide further support for the notion that the cyclin D1-Cdk2 fusion protein behaves as a native cyclin-dependent kinase complex, we isolated cyclin D1-Cdk2-, cyclin D1-kinase-dead Cdk2-, cyclin D1-Cdk4-, and cyclin D1-Cdk2- containing complexes and examined whether the fusion protein complexes contained the same components as the other Cdk complexes (Fig. 4C). This experiment demonstrated that the cyclin D1-Cdk2 fusion bound the same proteins present in the other Cdk complexes, including PCNA, p21, and p27. In B and C, asterisks (*) represent bands corresponding to the cyclin D1-Cdk2 fusion protein. Dashes (—) represent bands corresponding to endogenous proteins.

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HEK 293 cells transfected with these mutant constructs were analyzed by immunoblot to examine the regulatory phosphorylation sites on the Cdk2 domain, and to examine the ability of the mutants to induce the phosphorylation of endogenous Rb (Fig. 5A). Interestingly, inactivation of the cyclin D1 domain prevented phosphorylation of the regulatory sites on the Cdk2 domain. This result indicates that in the fusion protein the cyclin D1 domain controls the phosphorylation state of the Cdk2 domain through an intramolecular mechanism. In support of this hypothesis, neither cyclin E nor cyclin A were detected in immunoprecipitates of the cyclin D1-Cdk2 fusion protein (data not shown). Expression of the active cyclin D1-Cdk2 fusion protein (Cyclin D1-Cdk2) strongly induced the phosphorylation of endogenous Rb on Ser249/Thr252 and Ser807/Ser811. In contrast, the fusion protein containing an inactivated cyclin D1 domain did not increase Rb phosphorylation, likely due to the severely diminished phosphorylation of the Cdk2 domain on the activating site, Thr160. The fusion protein containing an inactive Cdk2 domain also did not significantly increase Rb phosphorylation. This result rules out the possibility that the cyclin D1 domain of the fusion protein acts independently of the Cdk2 domain to regulate Rb phosphorylation by interacting with endogenous Cdks. Together, the results are most consist-
ent with the cyclin D1 domain of the fusion protein regulating the phosphorylation and kinase activity of the Cdk2 domain of the fusion protein through an intramolecular mechanism.

To verify that the cyclin D1-Cdk2 fusion protein directly phosphorylates Rb, extracts from cells transfected as in Fig. 5A were immunoprecipitated using antibodies to the FLAG tag and the immunoprecipitates were assayed for kinase activity using Rb as the substrate (Fig. 5B, top panels). The results obtained were consistent with the results obtained in Fig. 5A and indicated that the cyclin D1-Cdk2 fusion protein directly phosphorylated Rb, and that mutation of either the cyclin D1 or Cdk2 domains of the fusion protein severely diminished Rb kinase activity. To examine site-specific phosphorylation of Rb by the cyclin D1-Cdk2 fusion protein, the kinase assay reactions were subjected to immunoblot analysis with Rb phosphospecific antibodies. These experiments indicated that the fusion protein phosphorylated Rb on Ser780, Ser795, and Ser807 or Thr160 (cyclin D1-Cdk2 (T160)). The results indicated that the cyclin D1-Cdk2 fusion protein directly inhibited Rb kinase activity, but not to bind p21 or activate E2F-dependent transcription.

The experiments in Figs. 4 and 5 suggest that the cyclin D1-Cdk2 fusion protein mimics the function of native cyclin D1-Cdk2 complexes in terms of its regulatory phosphorylation and protein-protein interactions. The cyclin D1-Cdk2 fusion protein may therefore serve as a physiologically relevant model of the biological functions of cyclin D1-Cdk2 complexes.
biological effects of constitutively expressing the cyclin D1-Cdk2 fusion protein. Cells expressing the cyclin D1-Cdk2 fusion protein, but not cells transformed with an empty retroviral vector (Puro. Cont.), formed colonies when plated in soft agar. These results indicate that the cyclin D1-Cdk2 fusion protein is capable of inducing cell transformation. In contrast, expression of Cdk2 or cyclin D1 separately did not promote anchorage-independent cell proliferation (data not shown).

In addition to anchorage-independent proliferation, another hallmark of transformed cells is the loss of sensitivity to growth inhibitory signals (31). We showed previously that a number of oncogenes override TGFβ-induced cell cycle arrest, but that rapamycin partially restores TGFβ cell cycle responses (15). We hypothesized therefore that since expression of the cyclin D1-Cdk2 fusion protein transformed Mv1Lu cells, it would also block TGFβ-induced growth arrest. As expected, TGFβ sensitivity was severely diminished in two cell lines stably expressing the cyclin D1-Cdk2 fusion protein as compared with the control cell line (Fig. 6B). Rapamycin alone had little effect on the proliferation of the control Mv1Lu cells, but cooperated with TGFβ to induce cell cycle arrest. Expression of the cyclin D1-Cdk2 fusion protein partially reversed TGFβ+ rapamycin-induced cell cycle arrest.

Expression of the Cyclin D1-Cdk2 Fusion Protein Overrides TGFβ− and TGFβ+ Rapamycin-induced Cell Cycle Responses—We performed immunoblot analyses on cell extracts from control Mv1Lu cells or Mv1Lu cell lines stably expressing the cyclin D1-Cdk2 fusion protein to understand mechanistically how expression of the cyclin D1-Cdk2 fusion protein overrides TGFβ− and TGFβ+ rapamycin-induced cell cycle arrest (Fig. 7A, left panel). In the control cells, TGFβ, and to a greater extent TGFβ+ rapamycin, decreased cyclin E and E2F1 protein levels. TGFβ also blocked Cdk2 phosphorylation on the regulatory sites Thr160 and Tyr15. Fig. 5 shows that Cdk2 phosphorylation is dependent on cyclin binding; thus, the loss of Cdk2 regulatory phosphorylation may result from decreased Cdk2 binding to cyclin E. Strikingly, expression of the cyclin D1-Cdk2 fusion protein largely reversed TGFβ− and TGFβ+ rapamycin-induced down-regulation of cyclin E and E2F1 protein levels and Cdk2 phosphorylation.

The genes encoding E2F1 and cyclin E are regulated by E2F-dependent transcription, which is regulated in turn by Rb phosphorylation. We therefore examined the effect of TGFβ and TGFβ+ rapamycin on Rb phosphorylation in the control cells and cells expressing the cyclin D1-Cdk2 fusion protein (Fig. 7A, right panel). In the control cells TGFβ strongly suppressed the phosphorylation of Rb at several sites including Ser780, Thr298, Ser807/Ser811, and Ser780. TGFβ and rapamycin cooperated to inhibit the phosphorylation of Ser780. Thr298 phosphorylation was only weakly affected by TGFβ and rapamycin. Expression of the cyclin D1-Cdk2 fusion protein increased the basal level of Rb phosphorylation on Ser780/Thr298, Ser807/Ser811, and largely reversed TGFβ− and TGFβ+ rapamycin-induced suppression of Rb phosphorylation on Ser780/Thr298, Ser807/Ser811, Ser780, and Ser780. These results are consistent with the idea that the cyclin D1-Cdk2 fusion protein induced Rb phosphorylation in a TGFβ− and TGFβ−+ rapamycin-resistant manner to constitutively activate E2F-dependent transcription and cell cycle progression. If this hypothesis is correct then the cyclin D1-Cdk2 fusion protein immunoprecipitated from TGFβ+ rapamycin-treated cells should exhibit kinase activity. Immune-complex histone H1 kinase assays in which the cyclin D1-Cdk2 fusion protein was immunoprecipitated with the FLAG antibody demonstrated that TGFβ treatment had little effect on the kinase activity of the cyclin D1-Cdk2 fusion protein (Fig. 7B). Rapamycin and TGFβ+ rapamycin treatment only weakly inhibited the kinase activity of the cyclin D1-Cdk2 fusion protein. These data are consistent with the idea that the cyclin D1-Cdk2 fusion protein confers anchorage-independent cell proliferation and resistance to TGFβ-induced growth arrest by driving constitutive Rb phosphorylation and activation of E2F-dependent transcription.

**DISCUSSION**

Cyclin D1-Cdk2 complexes are present in multiple human mammary carcinoma cell lines (Ref. 20 and the present study), but the biochemical activities of these complexes, and their potential role in cell cycle regulation has not been investigated in detail. Our results indicate that cyclin D1-Cdk2 complexes from mammary epithelial cells are phosphorylated on the activating site of Cdk2, Thr160, and catalyze the phosphorylation of Rb and histone H1 in vitro. Overexpression of cyclin D1 cooperated with overexpression of Cdk2 to increase Rb phosphorylation, consistent with a previous study (32), and to activate E2F-dependent transcription (Fig. 3C).

The observation that nontransformed NMuMG cells exhibit
high levels of cyclin D1-Cdk2 complexes questions why NMuMG cells are not transformed if cyclin D1-Cdk2 complexes contribute to transformation. It should be noted however that NMuMG cells form colonies in soft agar at a low frequency, and cells from the soft agar colonies form metastatic carcinomas when injected into nude mice (33). Epidermal growth factor and transforming growth factor-β (TGFβ) induce anchorage-independent growth of NMuMG cells in soft agar in a dose-dependent manner (34). These observations suggest that NMuMG cells lie somewhere in between a nontransformed and a fully transformed phenotype. It is also likely that just as cyclin D1 is expressed in many cell types and is only oncogenic when overexpressed, cyclin D1-Cdk2 complexes may have to exceed a threshold level to confer cell transformation. As discussed below, the transforming activity of cyclin D1-Cdk2 complexes also depends on the absolute and relative levels of p21 and p27 (Fig. 8).

Experiments in p21-null HCT116 cells indicated that p21 is not required for the formation of cyclin D1-Cdk2 complexes. However, p21-null cells exhibited lower levels of cyclin D1-Cdk2 complexes that did not contain PCNA. Thus, while p21 deletion does not influence the levels of E2F4/p107/Cdk2 complexes. E2F4/p107/Cdk2 complexes are disrupted by overexpression of p21 (36–38), and we have shown that TGFβ and rapamycin cooperate to disrupt E2F4/p107/Cdk2 complexes through increased p21 and p27 binding to Cdk2 (15). Together these observations suggest

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**FIG. 7.** The cyclin D1-Cdk2 fusion protein reverses TGFβ + rapamycin-induced Rb dephosphorylation and cyclin E downregulation. A, cell lines described in Fig. 6B were treated for 24 h with 10 ng/ml TGFβ (T), 100 nm rapamycin (R), or 10 ng/ml TGFβ + 100 nm rapamycin (T+R), or left untreated (control, C) and cell extracts prepared. Extracts were analyzed by immunoblot with antibodies specific for cyclin D1, cyclin E, E2F1, the FLAG epitope tag, Cdk2, Cdk2 phosphorylated on Thr160 (P-Cdk2(T160)) or Tyr15 (P-Cdc2/Cdk2(Y15)), actin, Rb, and Rb phosphorylated on Ser408/Thr412 (P-Rb(S408/T412)), Ser450/Ser464 (P-Rb(S450/S464)), Ser383 (P-Rb(S383)), or Thr398 (P-Rb(T398)). Asterisks (*) represent bands corresponding to the cyclin D1-Cdk2 fusion protein. Dashes (—) represent bands corresponding to endogenous proteins. B, extracts prepared as in A were immunoprecipitated (IP) with the FLAG antibody to monitor immunoprecipitation of the cyclin D1-Cdk2 fusion protein.

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Cyclin D1-Cdk2 complexes purified from mammary epithelial cells contained cyclin D1, Cdk2, PCNA, and p21 in a near equimolar ratio. We also demonstrated that human colorectal carcinoma HCT116 cells contain relatively high levels of cyclin D1-Cdk2 complexes, and this correlates with high levels of Cyclin D1-Cdk2 fusion protein.
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that different subsets of Cdk2 complexes are regulated in distinct manners, and that each subset may have differing thresholds of sensitivity to the levels of p21 and p27. Our interpretation of these results is that basal levels of p21 in HCT116 cells stabilize cyclin D1-Cdk2/p21/PCNA complexes. However, these basal p21 levels are insufficient to disrupt E2F4/p107/Cdk2 complexes. If the basal levels of p21 present in HCT116 cells regulated the levels of E2F4/p107/Cdk2 complexes, then higher levels of E2F4/p107/Cdk2 complexes should be observed in p21-null HCT116 cells relative to the levels of E2F4/p107/Cdk2 complexes observed in parental HCT116 cells.

Studying the biological functions of cyclin D1-Cdk2 complexes is complicated by the fact that cyclin D1 interacts with multiple Cdns, and Cdk2 interacts with multiple cyclins. Thus, the effects of co-expressing cyclin D1 and Cdk2 might not be due specifically to cyclin D1-Cdk2 complexes, but rather to other Cdk complexes. We have used two approaches to circumvent this problem. First, we co-expressed affinity-tagged cyclin D1 and cyclin-dependent kinase constructs and performed sequential purifications based on the binding of the differentially tagged cyclin a cyclin-dependent kinase proteins. This methodology allowed specific cyclin-dependent kinase complexes to be isolated and analyzed biochemically. Second, we constructed and expressed a gene encoding a cyclin D1-Cdk2 fusion protein to specifically study the function of cyclin D1-Cdk2 complexes.

The cyclin D1-Cdk2 fusion protein exhibited many of the same biological and biochemical activities as cyclin D1-Cdk2 complexes including: (a) histone H1 and Rb kinase activity, (b) activation of E2F-dependent transcription, (c) binding to p21, p27, PCNA and several other proteins (data not shown), and (d) cyclin-dependent control of the phosphorylation of the Cdk2 domain on stimulatory and inhibitory sites. These properties, along with the observation that cyclin E, cyclin A, Cdk4, and Cdk6 do not copurify with the cyclin D1-Cdk2 fusion protein, strongly support our supposition that the cyclin D1-Cdk2 fusion protein mimics the biochemical and biological functions of cyclin D1-Cdk2 complexes. This suggests that the cyclin D1-Cdk2 fusion protein will serve as a useful tool to explore the biological functions of cyclin D1-Cdk2 complexes.

Cell lines stably expressing the cyclin D1-Cdk2 fusion protein proliferated in soft agar, indicating that cyclin D1-Cdk2 complexes may promote anchorage-independent cell division. This is in agreement with a report demonstrating that cyclin D1 overexpression induced anchorage-independent cell cycle progression, but that cyclin E overexpression did not (39). Our cyclin D1-Cdk2 fusion protein was based on the design of a previously reported cyclin D1-Cdk4 fusion protein (30). The cyclin D1-Cdk4 protein cooperated with activated Ha-Ras to transform primary rat embryo fibroblasts (REFs) as measured by anchorage-independent cell proliferation. Interestingly in their study, as well as in ours (data not shown), overexpression of cyclin D1 alone did not confer transformation. These results suggest that in some cell types the levels of a cyclin partner for cyclin D1 (Cdk2 or Cdk4) may be limiting and blunt cyclin D1-induced transformation. Consistent with this interpretation, in addition to cyclin D1 overexpression, Cdk2 and Cdk4 overexpression is frequently observed in human breast cancers (40).

The cyclin D1-Cdk2 fusion protein also conferred another hallmark of cell transformation, loss of responsiveness to TGFβ cytostatic actions. TGFβ causes cell cycle arrest by inducing the expression of p15 and p21. p15 binds and inhibits Cdk4 and Cdk6, and releases p27 bound to cyclin D-Cdk4 complexes. The released p27, along with the p21 induced by TGFβ, bind and inhibit cyclin E-Cdk2 and cyclin A-Cdk2 complexes (41). The cyclin D1-Cdk2 fusion protein bypasses the TGFβ cell cycle arrest program through at least two mechanisms. First, cyclin D1-activation of Cdk2 is resistant to inhibition by p15, so the cyclin D1-Cdk2 complexes may promote Rb phosphorylation in a p15-insensitive manner. The hypothesis that the cyclin D1-Cdk2 fusion protein functionally substitute for cyclin D1-Cdk4 complexes is supported by the observation that the cyclin D1-Cdk2 fusion protein phosphorylates Rb on multiple sites, including sites preferred by Cdk2 (e.g. Ser807/Ser811) and sites preferred by Cdk4 (e.g. Ser249/Thr252, Ser780, Ser785) (42–45).

Second, our results indicate that under basal conditions cyclin D1-Cdk2 complexes bind high levels of p21, and that upon TGFβ + rapamycin treatment cyclin D1-Cdk2 complexes also bind high levels of p27. These observations are consistent with the hypothesis that cyclin D1-Cdk2 complexes and the cyclin D1-Cdk2 fusion protein override TGFβ and rapamycin actions in part by sequestering p21 and p27 and preventing p21- and p27-dependent inhibition of cyclin E-Cdk2 and cyclin A/Cdk2 kinase activities. It should be noted that in our studies cyclin D1-Cdk2 complexes and cyclin D1-Cdk4 complexes were not equivalent in their abilities to bind p21 and p27. Cyclin D1-Cdk2 complexes exhibited a dramatic preference for p21 binding over p27 binding. In contrast, cyclin D1-Cdk4 complexes exhibited a strong preference for p27 binding over p21 binding. This observation suggests that the relative effect of cyclin D1-Cdk2 complexes and cyclin D1-Cdk4 complexes on cell cycle progression will vary depending on the absolute and relative levels of p21 and p27 in a given cell type. In short, cyclin D1-Cdk2 complexes likely regulate TGFβ sensitivity and the cell cycle through both their kinase activity and their ability to sequester p21 and p27. This is supported by the observation that expression of catalytically inactive Cdk2 still activates E2F-dependent transcription, but does not cooperate with cyclin D1 to activate E2F-dependent transcription to the same extent as catalytically active Cdk2 (Fig. 3C).

In summary, the data presented here show that the cyclin D1-Cdk2 fusion protein is transforming in that it confers anchorage-independent cell proliferation and resistance to the growth inhibitory effects of TGFβ and TGFβ+ rapamycin. Cyclin D1-Cdk2 complexes were previously identified in multiple human mammary carcinoma cell lines (20). Here we have shown that cyclin D1-Cdk2 complexes are present at relatively high levels in NMuMG cells and human colorectal carcinoma HCT116 cells, and at somewhat lower levels in T98G and SW480 carcinoma cells. In fact, we have detected the presence of cyclin D1-Cdk2 complexes in all epithelial cell lines examined to date (data not shown). Together, our results indicate for

FIG. 8. A model for the role of cyclin D1-Cdk2 complexes in the cell cycle. Our results suggest that cyclin D1-Cdk2 complexes promote cell cycle progression through two mechanisms. First, cyclin D1-Cdk2 complexes phosphorylate key cell cycle targets such as Rb. Since Cdk2 does not bind to the Ink family of inhibitors, cyclin D1-Cdk2-dependent phosphorylation events would occur even in the presence of p15, p16, p18, and p19. Second, cyclin D1-Cdk2 complexes sequester p21 and p27, relieving p21- and p27-mediated inhibition of cyclin E-Cdk2 and cyclin A-Cdk2 complexes.
the first time that cyclin D1-Cdk2 complexes may play an important role in driving cell proliferation and tumorigenesis.

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Construction of a Cyclin D1-Cdk2 Fusion Protein to Model the Biological Functions
of Cyclin D1-Cdk2 Complexes

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