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Cyclin D1 Is Dispensable for G_1 Control in Retinoblastoma Gene-Deficient Cells Independently of cdk4 Activity

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To elucidate the regulator-versus-target relationship in the cyclin D1/cdk4/retinoblastoma protein (pRB) pathway, we examined fibroblasts from *RB-1* gene-deficient and *RB-1* wild-type littermate mouse embryos (ME) and in human tumor cell lines that differed in the status of the *RB-1* gene. The $RB^{+/+}$ and $RB^{-/-}$ ME fibroblasts expressed similar protein levels of D-type cyclins, cdk4, and cdk6, showed analogous spectra and abundance of cellular proteins complexed with cdk4 and/or cyclins D1 and D2, and exhibited comparable associated kinase activities. Of the two human cell lines established from the same sarcoma biopsy, the RB-positive SKUT1B cells contained cdk4 that was mainly associated with D-type cyclins, contrary to a predominant cdk4-$p16^{INK4}$ complex in the RB-deficient SKUT1A cells. Antibody-mediated neutralization of cyclin D1 arrested the RB-positive ME and SKUT1B cells in G_1, whereas this cyclin appeared dispensable in the RB-deficient ME and SKUT1A cells. Lack of requirement for cyclin D1 therefore correlated with absence of functional pRB, regardless of whether active cyclin D1/cdk4 holoenzyme was present in the cells under study. Consistent with a potential role of cyclin D/cdk4 in phosphorylation of pRB, monoclonal anti-cyclin D1 antibodies supporting the associated kinase activity failed to significantly affect proliferation of RB-positive cells, whereas the antibody DCS-6, unable to coprecipitate cdk4, efficiently inhibited G_1 progression and prevented pRB phosphorylation in vivo. These data provide evidence for an upstream control function of cyclin D1/cdk4, and a downstream role for pRB, in the order of events regulating transition through late G_1 phase of the mammalian cell division cycle.

Progression through G_1 phase and transition from G_1 to S phase of the cell division cycle is controlled, at least in part, by orderly activation of a distinct series of serine/threonine kinase complexes which comprise a cyclin regulatory subunit and a catalytic subunit, termed cyclin-dependent kinase (cdk) (reviewed in references 43, 44, 56, and 60). The active cyclin-cdk complexes are assumed to modify critical target proteins by phosphorylation, thereby promoting cell cycle progression toward DNA replication. At least five candidate G_1-phase cyclins, termed cyclins C, D1, D2, D3, and E, have been identified in mammalian cells (19, 25, 26, 29, 38, 39, 45, 46, 67). Each of these cyclins can associate with one or more of the cdk family members (40), of which at least cdk2, cdk4, and cdk6 have been shown to operate in G_1 (5, 8, 27, 35, 36, 37, 41, 52, 57, 65, 68). While a partner cdk(s) for cyclin C has not yet been identified, cyclin D1- and D2-associated cdk4 and/or cdk6 kinase activities have been detected in mid-G_1, prior to the activation of any other known cdk, and they culminate in late G_1 phase (37, 41, 65). The cyclin D3-associated cdk4 and/or cdk6 and cyclin E-bound cdk2 exhibit kinase activities at the G_1/S transition (27, 37, 41, 57; reviewed in references 60 and 61), followed by cyclin A-cdk2, cyclin A-cdc2, and cyclin B-cdc2 kinases at the later stages of the cell division cycle (43, 44, 51, 58, 60). The timely activation and inactivation of cyclin-cdk complexes appear to be regulated at multiple levels, including periodic oscillation of the cyclin regulatory subunits, posttranslational modifications of cdks, dynamic subcellular compartmentalization, and subunit composition of the complexes (reviewed in references 35, 43, 44, 51, 56, 60, and 61). As for the subunit composition, several negative cell cycle-regulatory polypeptides ranging from 15 to 28 kDa in size have recently been identified and shown to function as cdk inhibitors. Some of these inhibitors appear to influence a wide range of cdks (reviewed in references 18, 49, and 54), whereas another subset represented by $p16^{INK4}$ (59) and $p15^{INK4B}$ (14) appear to specifically block activities of the cdk4 and cdk6 partner kinases of D-type cyclins.

A large body of experimental evidence indicates that the major regulatory decisions controlling cell cycle progression of mammalian cells take place during G_1 (reviewed in references 43, 53, 56, and 60). The critical G_1-phase regulators are also frequently targeted by tumorigenic aberrations, either through deregulation by DNA tumor virus oncogenes or by oncogenic mutations of the respective genes (reviewed in references 13, 16, 21, 43, 44, 50, 53, and 61). Dominant among the physiological control events is a checkpoint in late G_1 phase, often called the R (restriction) point (53), defined as the time in G_1 after which cells are no longer sensitive to metabolic inhibitors and no longer require growth factors in order to enter S phase. Consistent with an essential role in normal cell proliferation, the R-point control appears to be frequently lost in cancer (53, 56, 60). Although the molecular mechanism underlying the R-point control remains to be elucidated, accumulating evidence suggests that pRB, the product of the retinoblastoma gene, fits the criteria expected for a candidate critical substrate of such a checkpoint (13, 15, 33, 42, 61). pRB functions as a negative regulator of the cell cycle in its un(der)phosphorylated form during G_1, and phosphorylation of pRB in late G_1 appears to down-regulate its growth-restraining activities. The active, underphosphorylated pRB can bind to and temporally govern the functions of a series of transcription factors, including some of the E2F family members (13, 16, 23, 50, 61). Phosphorylation of pRB in mid- to late G_1 releases the seques-

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tered transcription factors, at least some of which are important regulators of the genes controlling the G_1/S transition and subsequent DNA synthesis (reviewed in references 13, 16, 50, and 61). Inactivating phosphorylation in pRB occurs at sequences typically targeted by cdk/cyclin kinases, and recent data strongly implicate at least the D-type cyclins and possibly also cyclins E and A in this process (8, 9, 10, 15, 17, 24, 41, 61). Consistent with the candidacy of pRB for the R-point substrate, the G_1 R point appears to be under the control of the accumulation of unstable proteins whose properties match those of G_1 cyclins (53). While others have suggested cyclin A and/or cyclin E as potential executors of the R-point switch (8, 9), we have recently proposed that a D-type cyclin-pRB interaction operating in late G_1 may perform this key checkpoint control (33, 47). The latter model has been based on the data from Sherr's laboratory demonstrating specific pRB kinase activities of D-type cyclin–cdk complexes (24, 36, 37), on the evidence for cyclin D1 requirement in cells harboring functional pRB as opposed to the lack of cyclin D1 function in human RB-deficient cells (4, 32, 33, 34, 64), the timing of the cyclin D1 execution point in late G_1 (1, 2, 33, 55, 57), and the lower dependence on exogenous growth factors of cells lacking functional pRB or overexpressing D-type cyclins (21, 48, 55).

Regardless of whether D-type cyclins and pRB indeed account for the R-point control, the close functional interplay between these crucial cell cycle regulators has been well documented (reviewed in reference 61). D-type cyclin proteins can also directly bind to the pocket domain of underphosphorylated form of pRB, through their LXCXE sequences shared with pRB-neutralizing oncoproteins of several DNA tumor viruses but absent from other cyclins (9, 10). Two alternative scenarios have been proposed to explain the functional significance of these interactions. Dowdy et al. (9) have suggested that D-type cyclins might be negatively regulated via physical sequestration by pRB, which could be relieved through phosphorylation of pRB by cyclin E/cdk in late G_1, thereby releasing active cyclin D/cdks to allow for phosphorylation of downstream targets at the G_1/S transition (9). In contrast, Ewen et al. (10) have proposed that the complex formation between pRB and D-type cyclins could transiently target the former for phosphorylation by the relevant kinase(s), most likely cdk4/cyclin D (24, 37) and cdk6/cyclin D (41). Our data on the tight correlation between the positive cell cycle-regulatory role of cyclin D1 and the expression of functionally active pRB (4, 33, 34) were consistent with the latter proposal by Ewen et al. (10) and indicated that cyclin D1 performs its function upstream of pRB along the same pathway in G_1. In addition, the lack of any effect of cyclin D1 neutralization in cells with mutant or virally sequestered pRB implied that this tumor suppressor protein represents the major target of the cell cycle control function exerted by cyclin D1 (33). On the other hand, the fact that our experiments were performed with virally transformed and/or cancer-derived human cell lines which have suffered other mutations besides the RB defects somewhat undermined the conclusions made, especially since rigorously matched control cells from the same donors were mainly not available for those studies. To clarify the order of events along the cyclin D/pRB pathway, and to avoid the above-mentioned limitations of the previously used model systems, we have now examined the requirements for cyclin D1 function in cells from embryos of RB knockout mice and those from their normal littermates, as well as in two human cell lines established from the same tumor sample but different with respect to RB gene status (3, 12, 62, 64). Our present results confirm and extend the previous analyses, being consistent with the upstream function of cyclin D1, and a downstream role for pRB, in the key pathway controlling the G_1 phase progression in mammalian cells.

MATERIALS AND METHODS

Cell culture and synchronization. Cells from mouse embryos (ME) at day 13 of gestation were prepared by culture of small tissue explants in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco) and antibiotics. The outgrowing primary cell population was passaged by trypsinization at a ratio of 1:3 to 1:4 upon confluency and continuously cultured in DMEM with 10% serum to favor growth of fibroblastic cells. Exponential cultures at passage 2 were used to determine the RB gene and protein status (see below), and cells from selected $RB^{+/+}$ and $RB^{-/-}$ embryos between passage 6 and 15 were used for subsequent analyses. Cells were synchronized either in G_0 by serum starvation for 3 days (in DMEM with 0.1% serum), followed by stimulation in the complete medium with 20% FCS, or blocked in mitosis by 18 h of incubation in the presence of 40 ng of nocodazole (Sigma) per ml, followed by shake-off, washing, and replating into nocodazole-free medium. Progression through the cell cycle was monitored by flow cytometry (see below) or through immunodetection of bromodeoxyuridine (BrdU) incorporation (2, 32, 34). Mouse NIH 3T3 cells, human cell lines U-2-OS (osteosarcoma), UMSCC-2 (squamous cell carcinoma), and MCF7 (breast cancer), and two human cell lines derived from the same uterine mesodermal mixed tumor (12) (SKUT1B [ATCC HTB 115] and SKUT1 [ATCC HTB 114]; referred to as SKUT1A in this study to distinguish it clearly from SKUT1B) were all grown in DMEM with 10% FCS and antibiotics.

PCR analysis of RB gene status. The presence in mouse genomic DNA of the *RB-1* gene disrupted via insertion of the hygromycin resistance gene into RB exon 19 (7) was detected by PCR analysis, using 10 ng of genomic DNA isolated from individual ME cell cultures as a template and one of the following upstream and downstream primers: CGATCTTAGCCAGACGAGCG (within the hygromycin resistance gene insert) and TGAGGCTGCTTGTGTCTCTG (within RB exon 19). Alternatively, the latter primer was used in combination with another upstream primer, GACTAGGTGAAGGAATGCAGAG (within intron 18 of the *RB-1* gene), to demonstrate the presence of wild-type RB gene sequences. Thirty cycles were performed, each cycle consisting of denaturation (94°C, 1 min), annealing (65°C, 1 min), and extension (72°C, 1 min), and the PCR products were resolved on 1.5% agarose gels.

Immunoreagents. Preparation and characterization of mouse monoclonal antibodies (MAbs) DCS-6 and DCS-11, specific to cyclin D1, have been reported previously (34). Mouse MAbs DCS-1 (immunoglobulin G1 [IgG1]), recognizing both cyclins D1 and D2, DCS-5 (IgG2b), specific to cyclin D2, and DCS-22 (IgG1), specific to cyclin D3, were produced by immunization with the respective human recombinant full-length cyclin proteins (31); MAbs 5D4, against cyclins D1 and D2, and 245, against pRB, were kindly provided by M. Seto and W.-H. Lee, respectively. All mouse MAbs were used as either neat tissue culture supernatant or protein A-Sepharose-purified immunoglobulin. Other immunoreagents included rabbit polyclonal antisera against cdk4 (three different antisera, one provided by C. Sherr and the other two purchased from Pharmingen and Santa Cruz Biotechnology, were used with similar results), cdk6 (provided by G. Peters), $p16^{INK4}$ (Pharmingen), cyclins D1 and D2 (2), MAb to BrdU (Becton Dickinson), and control nonimmune mouse and rabbit immunoglobulins (Sigma).

Immunochemical analyses and pRB kinase assay. Total cell extracts were prepared by direct lysis with hot Laemmli sample buffer. Extracted proteins were electrophoretically separated on 8, 10, 12.5, or 15% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS). Gels were blotted onto nitrocellulose (enhanced chemiluminescence grade; Amersham) by a semidry method, and immunodetection was performed with an enhanced chemiluminiscence system (Amersham) according to the manufacturer's instructions. The biotin-streptavidin-based immunofluorescence, combined cyclin D immunostaining with immunodetection of BrdU incorporation, and immunoprecipitation protocols were described previously (34). Two milligrams of total extracted protein was used per assay in coimmunoprecipitations followed by immunoblotting, whereas 500 μg of protein per assay was used to assess the associated kinase activity. In the immunoprecipitations of metabolically labeled extracts, cells were labeled with [^{35}S]methionine and then subjected to immunoprecipitation, SDS-polyacrylamide gel electrophoresis (PAGE), and autoradiography (2). Reimmunoprecipitations of ^{35}S-labeled proteins were performed upon dissociation of the immunoprecipitated complexes in 100 μl of elution buffer (25 mM Tris [pH 7.5], 100 mM NaCl, 2 mM EDTA, 0.4% SDS, 2 mM 2-β-mercaptoethanol) at 95°C for 2 min; this procedure was followed by centrifugation, incubation of the supernatants with 10 mM iodoacetamide at 30°C for 15 min, dilution to 1 ml with lysis buffer containing 2% Triton X-100 (2), and a second round of immunoprecipitation with the same antibody. For kinase assays, standard immunoprecipitation was followed by washing the protein G beads four times in extraction buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.1% Tween 20, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 2 μg of aprotinin per ml) and twice in kinase assay buffer (50 mM HEPES, 10

mM $MgCl_2$, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate). The final pellet was resuspended in 30 μl of kinase buffer supplemented with 1 μg of the substrate (soluble glutathione *S*-transferase [GST]-pRb C-terminus [pRB amino acids 773 to 928] fusion protein produced in bacteria and purified exactly as described by Meyerson and Harlow [41], 20 μM ATP, 10 μCi of [γ-^{32}P]ATP [6,000 Ci/mmol; NEN Dupont]) and incubated for 30 min at 30°C with occasional mixing. The reaction was stopped by addition of 30 μl of 2×-concentrated Laemmli sample buffer and separated on an SDS–10% polyacrylamide gel, and phosphorylated pRB C terminus was visualized and quantified with a PhosphorImager (Molecular Dynamics).

Microinjection, electroporation, and flow cytometry techniques. Cells synchronized either by mitotic arrest-release or by serum starvation-restimulation were microinjected with either the affinity-purified anti-cyclin D1 MAb DCS-6 or DCS-11 or control mouse immunoglobulin (Sigma) at a final concentration of 4 to 5 mg/ml, using an automatic AIS system (Zeiss) connected with an Eppendorf injector (2). The procedure used for subsequent monitoring of cell cycle progression by double immunofluorescence combining detection of microinjected immunoglobulin and BrdU incorporation in situ was described earlier (33, 34). On average, around 70 cells were successfully microinjected with each antibody in every experiment in which the four ME cell types, synchronized by either procedure, were compared (see Results). Alternatively, cells arrested in mitosis and washed free from nocodazole were electroporated in suspension with DCS-6 or control IgG, using a Gene Pulser apparatus (Bio-Rad) as described by Lukas et al. (30). The settings used to electroporate NIH 3T3 cells and the human cancer cell lines were 270 V, 125 μF, and infinite resistance. After the pulse, cells were grown in complete medium with 100 μM BrdU for a period of 24 h, and the effect of electroporated antibody on the progression of the cells into S phase was analyzed either by in situ double immunofluorescence as described above for the microinjection experiments or by multiparameter flow cytometry as described by Lukas et al. (32). Each experiment was performed two to three times, and the data are expressed as percentage of specific S-phase inhibition, calculated as $(A - B)/A \times 100$, where A is the percentage of nonspecific immunoglobulin-containing cells incorporating BrdU and B is the percentage of DCS-6-containing cells incorporating BrdU at the time of evaluation.

RESULTS

Expression of pRB, D-type cyclins, and cdks in cell cultures from normal and RB-knockout mouse embryos. To establish cellular model systems with selective RB gene defects and genetically matched normal counterparts, mice heterozygous for the *RB-1* gene disruption as described by Clarke et al. (7) (provided by H. te Riele and A. Berns) were first mated with wild-type FVB animals. Heterozygous $RB^{+/-}$ mice were selected among offspring based on PCR identification of the disrupted *RB-1* gene in tail biopsy material and mated to generate embryos homozygous (−/−) at the RB-1 locus. Primary fibroblast cell cultures designated the ME series were initiated from eight littermate embryos at 13 days of gestation and propagated in DMEM with 10% serum. To identify the cells from embryos carrying a disrupted *RB-1* gene, genomic DNAs from ME cultures at passage 2 were analyzed by PCR amplification using an upstream primer within the hygromycin resistance gene used for insertion knockout and a downstream primer flanking the expected disruption in exon 19 (Fig. 1A). While two of the eight cultures (ME1 and ME2) showed no detectable PCR product, the remaining six embryonic cultures (ME3 to ME8) clearly contained the hybrid *RB-1*–hygromycin resistance gene sequences, indicating RB gene disruption in at least one of the alleles (Fig. 1A). Additional PCR analysis with two primers corresponding to the wild-type RB gene sequences flanking the targeted region of exon 19 revealed the presence of at least one normal *RB-1* allele in six of eight cultures (ME1, ME2, ME4, ME5, ME6, and ME7; data not shown), indicating that ME1 and ME2 cells are genotypically homozygous ($RB^{+/+}$), ME3 and ME8 are homozygous ($RB^{-/-}$), and the remaining four cultures of the ME series are heterozygous for the introduced mutation.

To compare the genotypic status with RB expression at the protein level, whole-cell lysates from the ME cultures were examined by immunoblotting with MAb 245, specific for pRB (Fig. 1B). Consistent with the PCR genotyping data, pRB of the expected full-length size was found in six of the ME cultures, while it was not detectable in the ME3 and ME8 cells (Fig. 1B). On the basis of the combined PCR and pRB immunoblotting data, the ME1, ME2 (both homozygous for wild-type RB), ME3, and ME8 (both homozygous for the mutant RB) cells were chosen for subsequent comparative analyses. To further characterize the RB protein expressed in ME1 and ME2 cells, cell lysates of exponential cultures at passage 10 were resolved on 8% denaturing gels, and migrations of the pRB species detected on immunoblots were compared with results for control mouse NIH 3T3 cells. In both NIH 3T3 and ME1 cells, pRB migrated as a series of bands, consistent with its normal processing through phosphorylation (Fig. 1C). Immunofluorescence staining with an anti-pRB antibody gave no

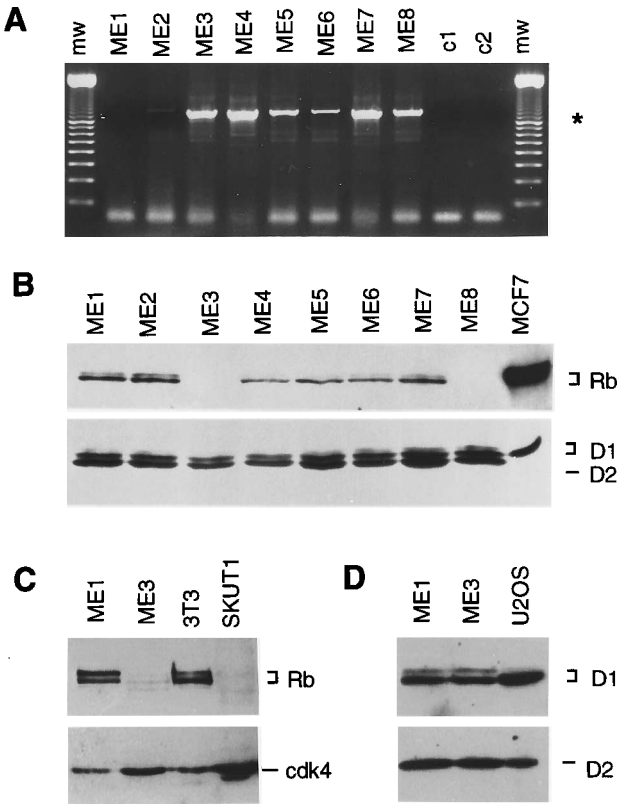

FIG. 1. Characterization of RB gene status and expression of pRB, cyclins D1 and D2, and cdk4 in cells cultured from ME obtained upon mating between $RB^{+/-}$ heterozygotes. (A) Screening of genomic DNA from cell cultures established from eight embryos, ME1 to ME8, by PCR analysis of gene disruption through hygromycin resistance gene insertion into exon 19 of the RB gene. The position of the 1,008-bp amplification product indicating successful RB gene disruption in at least one allele is marked by an asterisk. mw, marker DNA ladder; c1 and c2, negative controls with no DNA and normal mouse DNA, respectively. (B) Immunoblot analysis of pRB and cyclins D1 and D2 in total cell lysates of ME cells ME1 to ME8 at passage 2. Upper and lower parts of the blot were probed with MAb 245 against pRB and MAb DCS-1 to cyclins D1 and D2 as indicated; human breast cancer cell line MCF7 was used as a positive control for positions of pRB and cyclin D1. Note the total lack of pRB expression in ME3 and ME8 lysates and approximately equal levels of cyclins D1 and D2 in all ME cultures. (C) Immunoblot analysis of total cell lysates separated on an 8% gel reveals a normal pattern of pRB processing through phosphorylation, as reflected by differentially migrating pRB bands in the $RB^{+/+}$ ME1 cells in contrast to no pRB in $RB^{-/-}$ ME3 cells, both at passage 10. Mouse NIH 3T3 cells served as a positive control for pRB, and human RB-deficient SKUT1A sarcoma cells served as a negative control for pRB. The lower part of the blot was probed with rabbit antiserum to rodent cdk4. (D) Immunoblot analysis of total cell lysates (50 μg per lane) from ME1 ($RB^{+/+}$) and ME3 ($RB^{-/-}$) cells at passage 10 shows comparable levels of cyclins D1 and D2, as detected by monospecific MAbs DCS-6 and DCS-5, respectively. Human U-2-OS sarcoma cells were used as a positive control.

detectable signal in ME3 and ME8 cells, in contrast to granular positivity localized to nuclei of both ME1 and ME2 cells (data not shown), again supporting the conclusion that the pRB expressed in the homozygous (+/+) embryo fibroblasts represents the wild-type protein.

Another series of immunoblotting and immunofluorescence experiments was designed to compare the patterns of expression of the D-type cyclin proteins and their partner kinases in the ME series of embryo fibroblasts. The immunoblots showed easily detectable levels of cyclins D1 and D2 (Fig. 1B) and a low abundance of cyclin D3 (data not shown) in cell lysates from all eight ME cultures. Refined analysis on higher-resolution gels and immunoblotting with monospecific reagents to cyclins D1, D2, and D3 and to cdk4 and cdk6 confirmed the comparable abundances of the D-type cyclins in the $RB^{+/+}$ and $RB^{-/-}$ cultures (Fig. 1D) and detected significant amounts of cdk4 in both the RB-positive and RB-negative ME cells (Fig. 1C), in contrast to very low expression of cdk6 in either cell type (data not shown). Reminiscent of the immunostaining patterns previously observed in human cells (2, 4, 22, 33, 34), the cyclin D1 immunofluorescence signal was highly variable from cell to cell in terms of intensity, and the protein was predominantly localized to nuclei (Fig. 2A). Concomitant visualization of S-phase cells and cyclin D1 expression by double immunofluorescence demonstrated that the cell cycle period of DNA synthesis was associated with a low to undetectable cyclin D1 signal (Fig. 2). We found no significant difference between the RB-positive and RB-defective ME cells in any of the parameters examined, indicating that neither the abundance nor the subcellular localization of the D-type cyclins is dramatically influenced by the RB genotype in these cells.

Antibody-mediated neutralization of cyclin D1 function prevents phosphorylation of pRB in vivo. Several studies demonstrated that entry into S phase can be specifically inhibited by microinjection or electroporation of neutralizing antibodies to cyclin D1 in normal rodent (33, 55) and human (2, 64) fibroblasts and in RB-positive human tumor cell lines (4, 32, 33, 34). However, none of these studies established whether the antibody treatment had any influence on the state of phosphorylation of pRB, as manifested by the appearance of an electrophoretically slower-migrating form(s) of pRB in advanced G_1 (13, 17, 42). To examine the timing of the cyclin D1 function that is targeted by the antibodies, relative to the phosphorylation-associated shift in pRB's electrophoretic mobility, we electroporated several antibodies against cyclin D1 into mitotic human UMSCC-2 cells and evaluated the relative abundances of the two major electrophoretic bands of pRB in cell extracts prepared 24 h after electroporation. The UMSCC-2 carcinoma cell line was selected as a model for this experiment since it responds well to nocodazole synchronization, harbors wild-type RB, expresses a high level of cyclin D1 but no detectable cyclin D2, is known to be arrested in late G_1 by neutralizing antibodies to cyclin D1, and proliferates rapidly such that the vast majority of the mitotic cell population mock electroporated and released from the nocodazole block progresses into S, G_2, and M phases by 24 h (34). As can be seen in Fig. 3A, UMSCC-2 cells electroporated with a control mouse immunoglobulin and extracted after a 24-h culture period contained pRB almost exclusively in the slower-migrating, hyperphosphorylated form. By contrast, introduction of equal amounts of the DCS-6 antibody, previously shown to interfere with cyclin D1 function (32, 33, 34), resulted in significantly higher accumulation of the un(der)phosphorylated pRB species (Fig. 3). No significant effect in this assay was observed when either DCS-11 or 5D4, both of which are mouse MAbs to cyclin D1, were electroporated into UMSCC-2 cells in parallel experiments (Fig. 3). When the data were quantitated by densitometry, it was apparent that the introduction of DCS-6 led to an approximately twofold increase in abundance of the faster-migrating pRB form compared with the effects of control IgG or the other antibodies used (Fig. 3B). Considering that the efficiency of electroporation was about 60% in this experiment and that the evaluation was performed on extracts of total, nongated cell populations, the differences in accumulation of un(der)phosphorylated pRB in the subpopulations of UM-

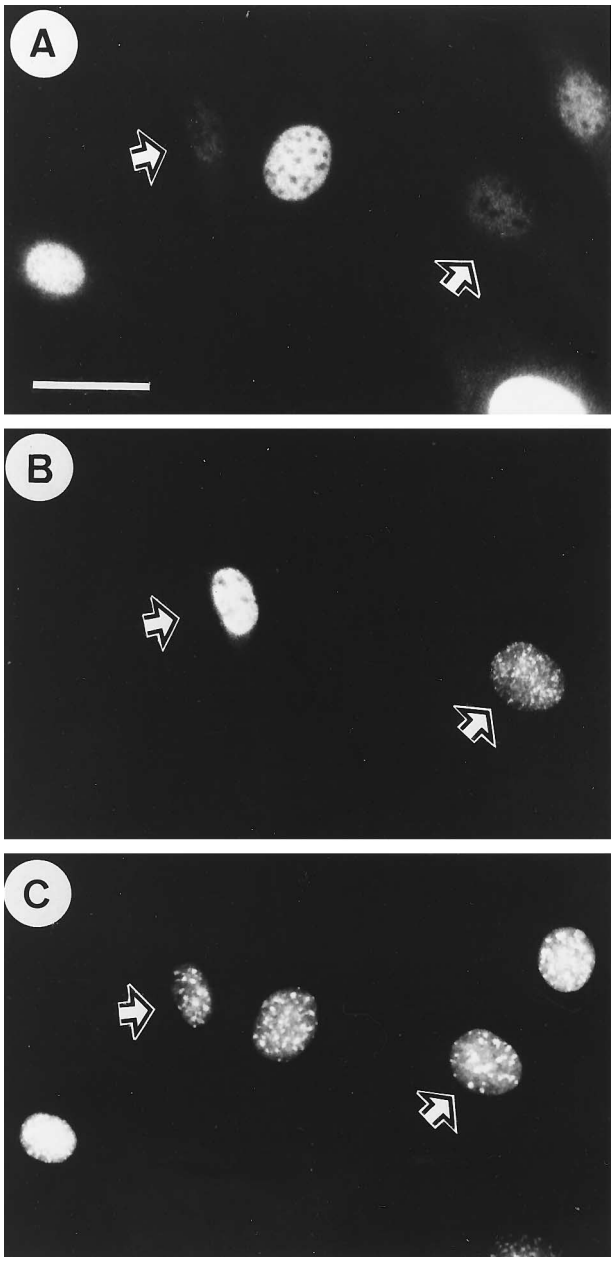

FIG. 2. Three-parameter immunofluorescence staining demonstrating variable expression of cyclin D1 in exponentially proliferating ME2 ($RB^{+/+}$) cells, with very low to undetectable levels of cyclin D1 in S-phase cells. An exponentially proliferating culture was labeled by BrdU for 1 h, fixed, and stained for cyclin D1 by MAb DCS-6 and a Texas red detection system (A); this procedure was followed by anti-BrdU antibody and fluorescein isothiocyanate detection (B) and DNA counterstaining with Hoechst stain (C). Arrowheads point to the BrdU-positive S-phase nuclei in all three photographs. Scale bar = 20 μm.

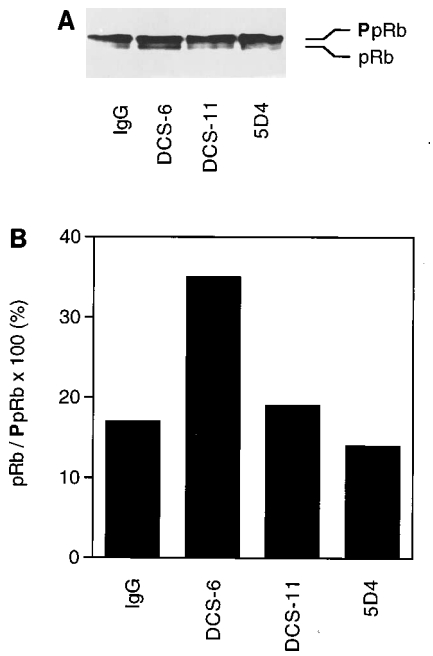

FIG. 3. Effects of antibodies to cyclin D1 on phosphorylation of pRB. (A) Immunoblot analysis of equal amounts of SDS-PAGE-separated total extracts of human UMSCC-2 cells electroporated with affinity-purified antibodies (as indicated on the top; final concentration, 2 mg/ml) at mitosis and harvested 24 h later. pRB was detected with MAb 245; the positions of hypophosphorylated and hyperphosphorylated forms of pRB are marked on the right as pRB and PpRB, respectively. IgG is a control nonimmune mouse immunoglobulin (Sigma). (B) Densitometry quantitation of the ratio between the intensities of the two bands in each lane.

SCC-2 cells successfully electroporated with DCS-6 versus those electroporated with the other antibodies must be even significantly more pronounced. Comparison of several properties of the individual antibodies to cyclin D1 (Table 1) revealed an inverse correlation between the ability to inhibit pRB phosphorylation and G_1 progression and the ability to coprecipitate cellular cyclin D1 with cdk4 and/or cdk6 in a complex which is active in a pRB kinase assay. Thus, DCS-6 is clearly the most potent reagent to interfere with cyclin D1-associated function(s), and MAbs DCS-11 and 5D4 represent the opposite end of the spectrum in that they immunoprecipitate cyclin D1-cdk complexes, support their kinase activity, and do not inhibit G_1 progression and/or phosphorylation of pRB, whereas the rabbit antiserum showed intermediate performance in these assays (Table 1; see also Fig. 5, 6, and 8 for examples). These data suggest that the ability of cyclin D1 to positively regulate cell cycle progression correlates with its ability to activate the cdk4 and/or cdk6 kinases and that this function preceeds or coincides with phosphorylation of pRB in advanced G_1. In addition, the distinct properties of the individual antibodies to cyclin D1 allowed us to select the most appropriate reagents for examination of various aspects of the function of cyclin D1 in the established ME fibroblasts.

TABLE 1. Characteristics of various antibodies to cyclin D1

Antibody	Specificity	Performance in assay[a]			
		Coprecipitation of cdk4	Coprecipitation of pRB kinase activity	Inhibition of pRB phosphorylation	Inhibition of G_1 progression
DCS-11[b]	D1	++	++	+/−	+/−
5D4[b]	D1, D2	++	++	−	ND
RabD1 and D2[c]	D1, D2	++	+	ND	+
DCS-6[b]	D1	−	−	++	++

[a] ++, strong; +, moderate; +/−, weak; −, undetectable signal or effect; ND, not determined. Results from this study and references 2, 31, and 34 are summarized.
[b] Mouse MAb.
[c] Rabbit antiserum raised against a full-length human cyclin D1 (2).

Inhibition of S-phase entry by antibodies to cyclin D1 in ME cells is RB dependent. To investigate whether mouse fibroblasts carrying a wild-type or disrupted *RB-1* gene differ in their requirements for cyclin D1 function in G1, we performed microinjection experiments on ME1, ME2, ME3, and ME8 cells, using two independent methods of cell cycle synchronization: serum starvation-restimulation and mitotic block-release. Preliminary experiments designed to compare responsiveness of RB^+ and RB^- ME cells to such synchronization treatments demonstrated a reduced requirement for serum growth factors in the $RB^{-/-}$ ME cells compared with the $RB^{+/+}$ counterpart cells. Thus, the proportion of BrdU-incorporating cells in cultures grown in low serum for 3 days (and pulsed with BrdU for the last 12 h of the serum starvation period) was reproducibly two- to threefold higher in the RB-deficient fibroblasts, ranging from 2.5 to 6 and 8 to 15% in the RB^+ and RB^- ME cells, respectively (Fig. 4A and data not shown). In addition, the RB-negative ME cells enter S phase before the RB-positive controls upon either serum restimulation (Fig. 4A and B) or replating of mitotically arrested cells (Fig. 4C). Under exponential growth conditions, the $RB^{-/-}$ ME cells proliferate with a shorter doubling time (27 to 28 h, versus 34 to 36 h in the $RB^{+/+}$ cells) and a higher fraction of cells in S-phase (32 and 21.5% upon a 30-min BrdU pulse in $RB^{-/-}$ and $RB^{+/+}$ ME cells, respectively), and they are 20 to 25% smaller. All of these properties are consistent with the shortening of G_1 phase in the $RB^{-/-}$ ME cells as observed in the synchronization experiments described above. These data showed that the lack of the RB gene in the ME3 and ME8 fibroblasts is associated with significant alterations of their growth properties, a fact that is essential to consider when designing the microinjection experiments. In the first series of experiments, the serum-restimulated cells were microinjected in early G_1 (3 to 5 h after serum addition) with either the DCS-6 or DCS-11 antibody to cyclin D1, and their effects were evaluated at the time when the majority of cells uninhibited in their cell cycle progression were expected to traverse S phase. Microinjection of affinity-purified DCS-6, but not DCS-11, resulted in pronounced delay in S-phase entry of the control mouse NIH 3T3 cells and the RB-positive ME fibroblasts, in contrast to the RB-defective ME cells, whose progress toward DNA replication was unaffected or even marginally accelerated (Fig. 5A). Microinjection performed several hours after restimulation with serum is optimal for detection of G_1 inhibition in RB-positive cells, but it cannot exclude the possibility that some cyclin D1-dependent event occurred considerably earlier during the G_0-to-G_1-to-S phase transition in the RB-deficient cells and consequently was completed before the antibody injections in early G_1. This possibility was ruled out in the second series of experiments, in which the cells were microinjected shortly before serum stimulation (Fig. 5B). The inhibition of S-phase entry was moderately less pronounced in the $RB^{+/+}$ and the control 3T3 cells under these conditions because of the longer interval between the injections and the time of evaluation, which may result in some metabolic degradation of the antibody, but the RB-deficient ME fibroblasts remained unaffected in their cell cycle progression (Fig. 5B).

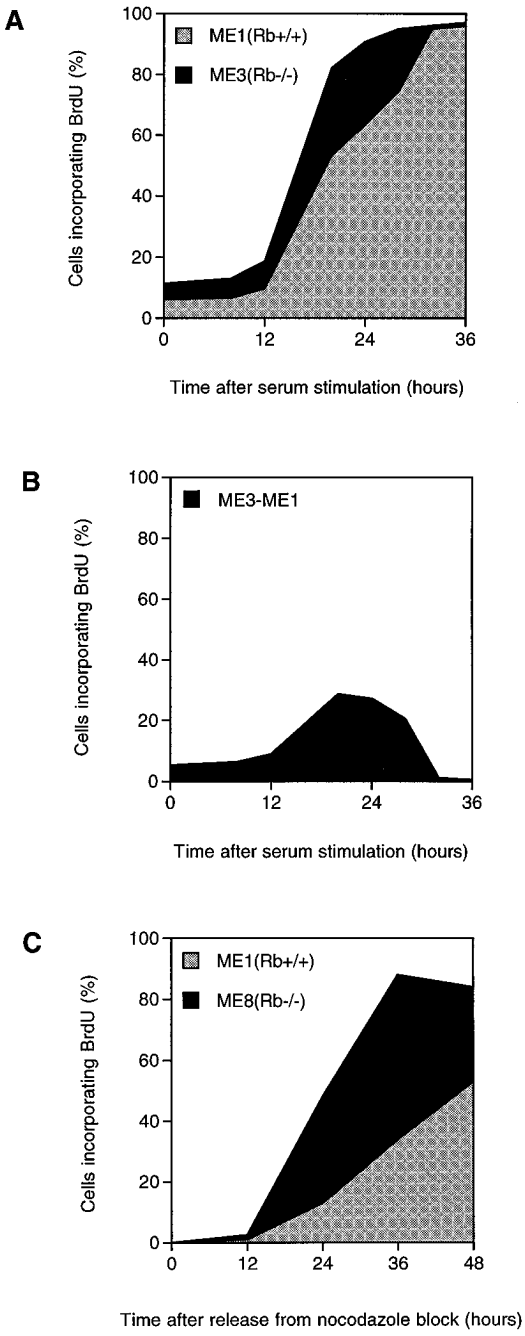

FIG. 4. Differential behaviors of RB-positive and RB-negative ME fibroblasts in growth synchronization experiments. (A) Response of ME cells with distinct RB genotypes to serum starvation and restimulation. ME cells were grown in medium with 0.1% serum for 72 h and then restimulated with fresh medium containing 20% FCS. S-phase entry was monitored at 4-h intervals by incorporation of BrdU (0.1 mM), added at the time of restimulation. Note the lower degree of quiescence and the entry into S phase several hours earlier in the case of the RB-negative cells. (B) Subtraction of the two curves shown in panel A, demonstrating a shorter duration of G_1 in the RB-deficient ME cells. Analogous data, not shown here, were obtained also for ME2 ($RB^{+/+}$) and ME8 ($RB^{-/-}$) fibroblasts. (C) Cell cycle progression of ME cells with distinct RB genotypes after release from mitotic block. Cells were grown in the presence of 40 ng of nocodazole per ml for 18 h, and mitotic cells were collected by gentle shaking, repeatedly washed in phosphate-buffered saline, and replated in nocodazole-free medium with 10% FCS and BrdU (0.1 mM) to monitor entry into S phase. Note the significantly earlier onset of DNA synthesis in $RB^{-/-}$ ME8 cells than in $RB^{+/+}$ ME1 cells. At least 200 cells were counted at each of the time points in all panels.

Analogous results were obtained with both early-passage and late-passage ME cells, suggesting that the observed correlation between the requirement for cyclin D1 and RB gene status is a relatively stable feature of the ME cells (Fig. 5B). Finally, the microinjection experiments were performed with cells released from a nocodazole block, again resulting in a significant G_1 arrest of cells expressing wild-type RB but not in the RB-deficient fibroblasts (Fig. 5C). Additional data obtained in repeated experiments using ME1, ME2, ME3, and ME8 cells were very similar to those shown in Fig. 5 as examples. We conclude from these microinjection experiments that regardless of the synchronization method used, cyclin D1 function is required in NIH 3T3 cells and ME cells expressing wild-type RB, whereas it is dispensable for G_1 phase progression/S-phase entry in the ME cells lacking functional pRB.

Cyclin D-cdk4 complexes and their pRB kinase activities are comparable in ME cells regardless their RB genotype. To clarify whether the lack of requirement for cyclin D1 function in RB-deficient ME cells is accompanied by down-regulation of the kinase activity toward pRB, we next examined the composition of the protein-protein complexes of cyclins D1 and D2 and their respective kinase activities. Exponential cultures of ME1, ME2, ME3, and ME8 cells were extracted, and equal amounts of cellular protein were immunoprecipitated with MAbs DCS-11 and DCS-5, specific for cyclins D1 and D2, respectively. The immunoprecipitated complexes were resolved on denaturing gels, blotted, and probed for the presence of cyclin D1, cyclin D2, and cdk4. As can be seen from examples of the resulting immunoblots shown in Fig. 6, comparable amounts of cyclin D1 and D2 proteins were precipitated from lysates of ME1 ($RB^{+/+}$) and ME3 ($RB^{-/-}$) cells (Fig. 6A). Both DCS-11 and DCS-5 antibodies specifically coprecipitated the cdk4 protein, whose abundances were again very similar in complexes from either cell type (Fig. 6B). Consistent with the existence of independent complexes between individual D-type cyclins and cdk4, significantly higher amounts of the latter protein were found to be coprecipitated by an antiserum recognizing both cyclins D1 and D2 compared with complexes containing either of these cyclins alone (Fig. 6B). Results virtually identical with those for ME1 and ME3 cells were obtained upon immunoprecipitation analysis of ME2 ($RB^{+/+}$) and ME8 ($RB^{-/-}$) cell extracts (data not shown), indicating that in ME fibroblasts, the D-type cyclins efficiently associate with cdk4 regardless of the presence or absence of functional pRB. This result was unexpected since RB-deficient human cells examined so far showed very little if any evidence of cyclin D-cdk4 association, partly because of preferential complexing of cdk4 with the p16 kinase inhibitor (14, 54, 59). Since none of the available antibodies to human p16 recognized any putative mouse homolog (data not shown), we approached this issue by immunoprecipitations of cdk4-bound and cyclin D-bound proteins from extracts of [^{35}S]methionine-labeled RB-positive and RB-deficient ME cells (Fig. 7). No significant differences were seen between the autoradiography patterns of such immunoprecipitates from $RB^{+/+}$ and $RB^{-/-}$ fibroblasts, including the spectrum and relative abundance of cdk4- and/or cyclin D-associated polypeptides in the 15- to 28-kDa range, potentially corresponding to mouse homologs of the human cdk inhibitors (Fig. 7).

The existence in ME cells of cyclin D-cdk4 complexes prompted us to assess their kinase activity toward pRB (24, 37, 41). When GST-pRB was used as a substrate, immunoprecipitations obtained with antibodies to cyclin D1, cyclin D2, or cdk4 from extracts of the ME cells exhibited significant specific kinase activity (Fig. 8A). The observed differences among the antibodies with respect to the ability to support the coprecipi-

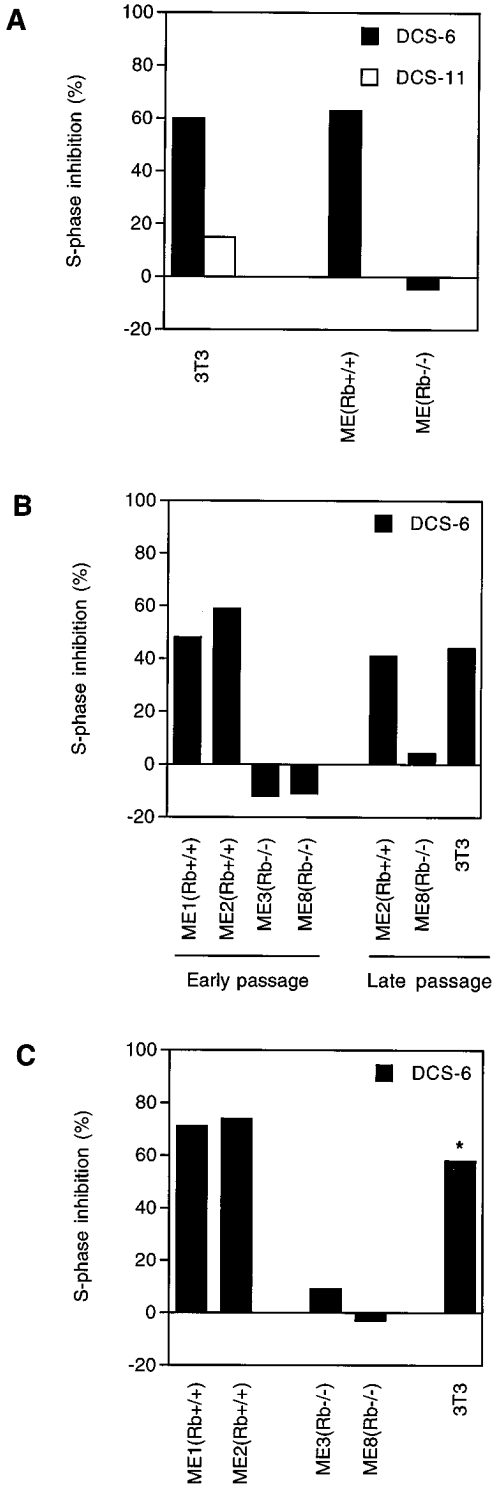

FIG. 5. Microinjection of antibodies to cyclin D1 inhibits S-phase entry in RB-positive but not in RB-deficient ME cells. (A) Extent of specific inhibition of S-phase entry by MAb DCS-6 or DCS-11 (as indicated) in mouse cells after serum starvation and restimulation. Reflecting distinct rates of cell cycle progression, ME1 and ME2 (indicated as average for ME $RB^{+/+}$) were microinjected at 6 to 8 h, and the effect was evaluated between 30 and 36 h after serum readdition; ME8 and ME3 (indicated as average for ME $RB^{-/-}$) were injected at 4 to 6 h and evaluated at 24 to 36 h; and the control (fast-growing) NIH 3T3 cells were microinjected immediately after serum restimulation and evaluated 24 h later. (B) Effects of antibody-mediated neutralization of cyclin D1 by MAb DCS-6 in ME cells microinjected before serum restimulation and evaluated 24 h later. The individual cell types are indicated at the bottom; the early-passage ME cells were at passages 5 to 7, and the late-passage ME cells were at passages 15 to 18. (C) Effects of antibody-mediated neutralization of cyclin D1 by MAb DCS-6 in ME cells released from mitotic block. The $RB^{+/+}$ ME cell types were microinjected at 5 to 8 h and analyzed 30 to 36 h after replating; the $RB^{-/-}$ ME cells were injected at 3 to 4 h and analyzed 24 h after replating. NIH 3T3 cells were electroporated (*) rather than microinjected with the antibody immediately after nocodazole removal, replated, and evaluated 24 h later. All graphs show representative results from one of the repeatedly performed experiments.

tated kinase activities (Fig. 8A) are consistent with the data of Matsushime et al. (37), and this phenomenon is likely to reflect location of the target epitope(s) and other characteristics of the reagents used. Reminiscent of the relative abundance of the cyclin D-cdk4 complexes in RB-positive and RB-deficient ME cells, no obvious difference in kinase activity was found when lysates of ME1 ($RB^{+/+}$) cells (top panel in Fig. 8A) and ME3 ($RB^{-/-}$) cells (bottom panel in Fig. 8A) were compared. Quantitative PhosphorImager analysis of the kinase assay data from two independent experiments (Fig. 8B) confirmed the conclusions made upon visual comparison of the gels. In summary, these results show that both the abundances of cyclin D-cdk 4 complexes and their kinase activities toward pRB are similar in all ME cells and therefore independent of whether a wild-type or mutant RB gene is present in the ME cell cultures under study.

The requirement for cyclin D1 and the composition of cdk4-cyclin D complexes correlate with RB status in human tumor cell lines. In search for a matched pair of human cell types from the same donor which would differ in terms of RB status but express comparable and easily detectable amounts of D-type cyclins and cdk4, we have found two cell lines, designated SKUT1A and SKUT1B, which were originally established from a single primary uterine tumor (12). While SKUT1A cells have been reported to lack detectable *RB-1* transcripts (62) and pRB (3), SKUT1B cells express pRB which exhibits properties expected for the wild-type protein, as judged from its abundance and electrophoresis mobility pattern (Fig. 9A), nuclear localization, and ability to form a complex with simian virus 40 T antigen (31). We have now characterized this pair of cell lines by immunoblotting and immunoprecipitation and found that both SKUT1A and SKUT1B express approximately equal levels of all three D-type cyclins (Fig. 9A) and comparable levels of cdk4 (Fig. 9B). In contrast, the $p16^{INK4}$ inhibitor of cdk4 is expressed at a high level in the RB-deficient SKUT1A cells but is barely detectable in the RB-positive SKUT1B cell line (Fig. 9A). The latter difference appears to have a profound influence on the composition of the protein complexes immunoprecipitated by antibodies to cyclins D1 and D2 and cdk4 (Fig. 9B). In the SKUT1B cell lysates, both cyclins D1 and D2 form easily detectable complexes with cdk4, and these associations are also obvious upon analysis of cdk4 immunoprecipitates (Fig. 9B). On the other hand, the D-type cyclins in the RB-deficient SKUT1A cell lysates appear to be free from cdk4, which instead forms an abundant complex with $p16^{INK4}$ (Fig. 9B). The observed preferential association of cdk4 with $p16^{INK4}$ in RB-deficient cells despite the presence of relatively high levels of D-type cyclins is interesting and suggests a high-affinity interaction between these partner proteins.

Using the two sarcoma cell lines as a model, we next examined whether antibody-mediated neutralization of cyclin D1 protein has similar or distinct effects upon cell cycle progression of SKUT1A and SKUT1B cells. Since the SKUT1 cell lines easily detach from the tissue culture substrate when microinjected, we chose electroporation to introduce antibodies into cell populations synchronized in mitosis. In two indepen-

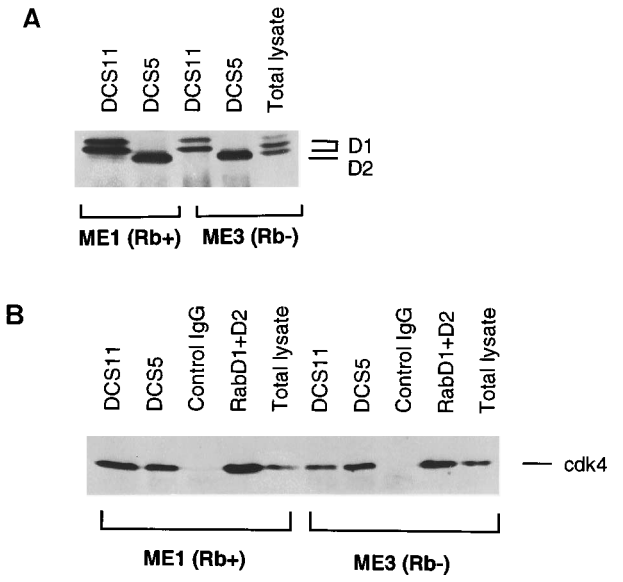

FIG. 6. Immunoprecipitation of cyclins D1 and D2 from lysates of $RB^{+/+}$ and $RB^{-/-}$ ME cells and coprecipitation of cdk4. (A) Relative amounts of cyclins D1 (MAb DCS11) and D2 (MAb DCS5) immunoprecipitated from ME1 and ME3 cells, as visualized by immunoblotting of the separated precipitates with MAb DCS1, recognizing both cyclins D1 and D2. The positive control lane (Total lysate) shows a direct blot (with DCS-1) of SDS-PAGE-separated whole-cell lysate. (B) cdk4 (visualized by blotting with rabbit antiserum) coprecipitated with cyclins D1 (DCS11), D2 (DCS5), or D1 and D2 (rabbit antiserum); control lanes (Total lysates) show direct blots with rabbit antibodies to cdk4.

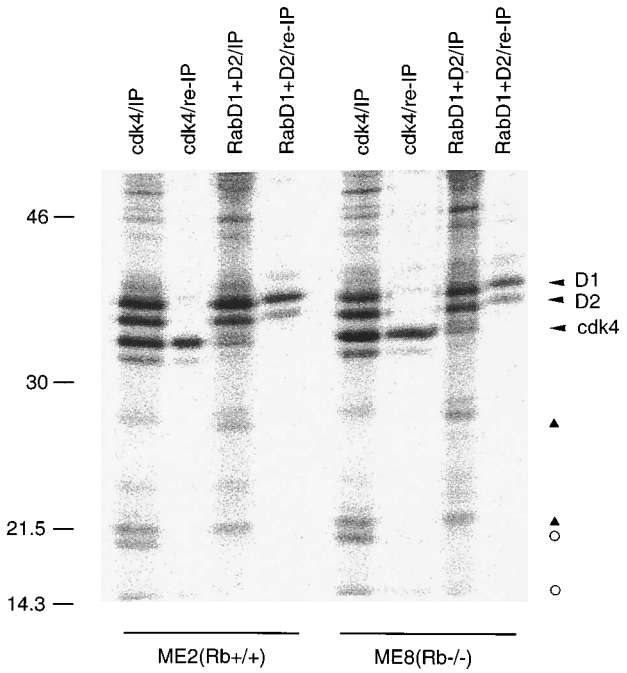

FIG. 7. Autoradiographic analysis of [^{35}S]methionine-labeled immunoprecipitates from lysates of exponentially growing RB-positive and RB-deficient ME fibroblasts. Shown is the spectrum of cellular proteins coprecipitated (lanes marked IP) with rabbit antibodies to cdk4 or cyclins D1 and D2, as indicated at the top, and separated on an SDS–12% polyacrylamide gel. Specific protein-protein associations were verified by dissociating the complexes and reprecipitation with the same antibody (lanes marked re-IP). Positions of the molecular weight markers are given on the left in kilodaltons; positions of cyclin D1, cyclin D2, and cdk4 are indicated on the right, along with positions of lower-molecular-weight proteins present in both cdk4 and cyclin D complexes (the 27- and 21-kDa bands marked by triangles) or selectively coprecipitated with cdk4 only (18- and 16-kDa bands marked by circles). The composition of the complexes was verified by analyses on 15% gels with essentially the same results, except for an additional cdk4-bound protein present in similar amounts in lysates from all ME cultures that migrated at 14 kDa and that is not visible on this gel.

dent experiments, electroporation of the DCS-6 MAb caused a specific delay in cell cycle progression toward DNA synthesis in the RB-positive SKUT1B cells (Fig. 10), comparable to the effect observed in a control human sarcoma cell line U-2-OS (Fig. 10) expressing wild-type RB and all three D-type cyclins. In contrast, electroporation of the same antibody preparation resulted in a marginal stimulation rather than inhibition of S-phase entry in the RB-defective SKUT1A cells (Fig. 10), whereas analogous treatment had no detectable effect on cell cycle progression of control human Bristol-8 cells, which express no detectable cyclin D1 (5, 31). Thus, consistent with the effects seen with the ME cells, cyclin D1 was required for G_1 progression/S-phase entry in the RB-positive cell line but not in the RB-deficient cell line derived from the same biopsy specimen.

DISCUSSION

Transgenic mice lacking the RB gene die in utero, usually before day 14 of embryonic development. Analyses of developmental abnormalities in the RB-knockout ME pointed to the essential role of pRB in linking cell proliferation with control of differentiation (7, 22, 28). We show that cells isolated from the RB-deficient ME at day 13 of gestation are viable, can be propagated in culture for a number of generations, and therefore may provide an attractive cellular model system with which to study various aspects of RB function. It is apparent from the growth properties in vitro that the lack of functional pRB has profound effects on the cell cycle parameters of the ME fibroblasts. First, the $RB^{-/-}$ ME cells proliferate faster under exponential growth conditions, with a significantly shorter G_1 phase, and the cells are smaller. Second, they arrest less effectively in a G_0 state after removal of serum compared with the $RB^{+/+}$ homozygous counterpart cells derived from littermate embryos. Consequently, the altered behavior of the RB-deficient ME cells must be taken into consideration when any cell cycle experiments based on this model system are designed. The acceleration of cell proliferation is reminiscent of the behavior of human fibroblasts treated with RB antisense constructs (63) and is consistent with the evidence for pRB's role in negative regulation of cell proliferation (reviewed in references 13 and 61). Although the principal mechanisms regulating cell cycle progression and key differentiation programs appear to be analogous in human and rodent cells, some interesting differences related to function of the pRB pathway in mice and humans are also obvious. For instance, the heterozygous RB-knockout mice do not develop retinoblastomas but suffer from frequent pituitary tumors instead (7, 20, 28). Another example is the unexpected finding in this study of the different behaviors of cyclin D-cdk4 complexes in RB-deficient mouse and human cells. Thus, both the abundances of the complexes and their kinase activities appeared very similar in RB-positive and RB-deficient ME cells. In human cells, by contrast, complexes of cyclins D1 and D2 with cdk4 were abundant in the RB-positive SKUT1B cells, whereas they were virtually undetectable in their RB-defective SKUT1A counterparts, despite the comparable levels of the D-type cyclins and cdk4 in the two cell lines. This observation is consistent with a previous report of Bates at al. (6), who noted that cyclin D-cdk complexes were lacking in human cells

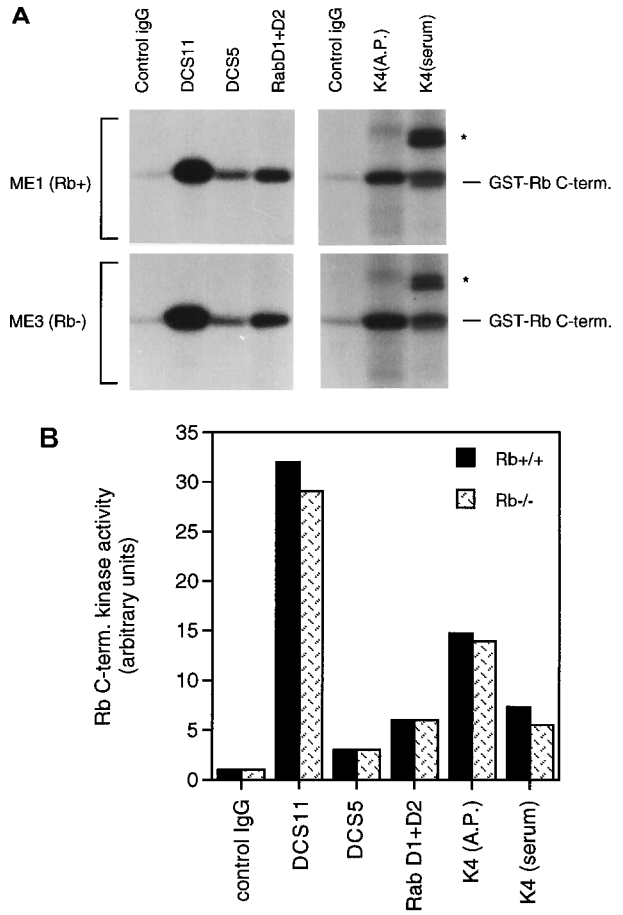

FIG. 8. Cyclin D- and cdk4-associated kinase activity immunoprecipitated from ME cells. (A) Antibodies used for immunoprecipitations are indicated at the top. GST-pRb C terminus served as a substrate, and normal immunoglobulin immunoprecipitate served as a background control; asterisks mark an extra phosphorylated band present only in the precipitates with nonpurified serum to cdk4 (K4); K4(A.P.) indicates an affinity-purified preparation from the rabbit anti-cdk4 serum. (B) Graphs summarizing mean values from densitometric scanning quantification of pRB C terminus kinase activities, immunoprecipitated from ME1 and ME2 ($RB^{+/+}$) and ME3 and ME8 ($RB^{-/-}$) extracts. The antibodies used for immunoprecipitations are indicated at the bottom.

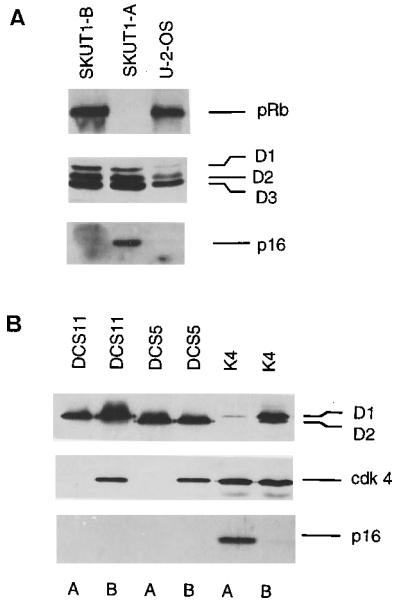

FIG. 9. Immunoblotting and immunoprecipitation analysis of cyclin D-cdk4 complexes in human SKUT1A and SKUT1B cell lysates. (A) Direct blots with MAb 245 (top), a mixture of DCS1 and DCS22 (middle), and rabbit antiserum to p16 (bottom panel). U-2-OS sarcoma cells served as a positive control for pRB and cyclins D1, D2, and D3. (B) Immunoblots of immunoprecipitates from SKUT1A (RB^{-}) and SKUT1B (RB^{+}), marked as A and B at the bottom, by antibodies to cyclin D1 (DCS11), D2 (DCS5), and cdk4 (K4), were probed with reagents indicated on the right; note abundant D1-cdk4 and D2-cdk4 complexes in SKUT1B cells, in contrast to predominant cdk4-p16 complex in the RB-negative SKUT1A cells.

transformed by simian virus 40 T antigen, adenovirus E1A and E1B, or human papillomavirus type 16 E7 and E6, while they seemed to be preserved in mouse cells transformed by the same DNA tumor virus oncogenes. Our data extend this information and demonstrate that also the spectra and relative abundances of metabolically labeled cellular proteins that can be coprecipitated with either cdk4 or cyclins D1 and D2 are virtually identical in both RB^{+} and RB^{-} ME fibroblasts. The series of cdk4-associated mouse proteins included several polypeptides between 15 and 28 kDa in size, reminiscent of the presence of cdk inhibitors such as p16, p21, and p27 in analogous complexes isolated from human cells (69). Since the human $p16^{INK4}$ inhibitor plays an important role in determining the subunit composition of the cyclin D-cdk complexes in human cells (6, 54, 59, 69), it will be interesting to identify the gene encoding a mouse homolog of $p16^{INK4}$ and test the biological and biochemical consequences of its knockout in transgenic mice. Despite some intriguing mechanistic differences between rodent and human cells, the spectra of cyclins and cyclin-dependent kinases and their orderly cell cycle appearance and functions, including evidence that the D-type cyclin proteins are required and rate limiting for G_1 progression in both human and rodent cells, strongly argue for the evolutionary conservation of the cell cycle control principles from mice to humans (reviewed in references 43, 44, 49, 56, and 60). This notion is fully supported also by our present results demonstrating that cyclin D1 becomes dispensable for G_1 phase regulation in the absence of functional pRB in cells from either species.

The primary aim of this study was to elucidate the order of events along the pRB pathway, in particular the issue of whether D-type cyclins perform their cell cycle-regulatory function upstream or downstream of pRB (9, 10, 33, 61). Our previous experiments with a wide range of human normal, cancer-derived, and virally transformed cell types showed a clear correlation between the absence of functional pRB and the lack of requirement for cyclin D1 function, a result interpreted by us as evidence for an upstream role of cyclin D1 and a target-substrate role for pRB (4, 32–34). On the other hand, limitations such as the occurrence of multiple genetic defects in the tumor cell lines, exceptionally low levels of cyclin D1 in the RB-defective human cells, and the paucity of rigorously matched pairs of RB-positive versus RB-deficient cells led us to address some functional aspects of the cyclin D/pRB pathway in the genetically defined model system of RB-knockout mice (7). Also the matched human cell lines SKUT1A (RB^{-}) and SKUT1B (RB^{+}) represent a more convenient model than the previously used cell lines (33, 34), being established from a single uterine sarcoma biopsy (12) and expressing comparable, relatively high levels of all three D-type cyclin proteins (this study). While the relatively high abundance of cyclins D1, D2, and D3 in the human SKUT1 cell lines was of advantage for

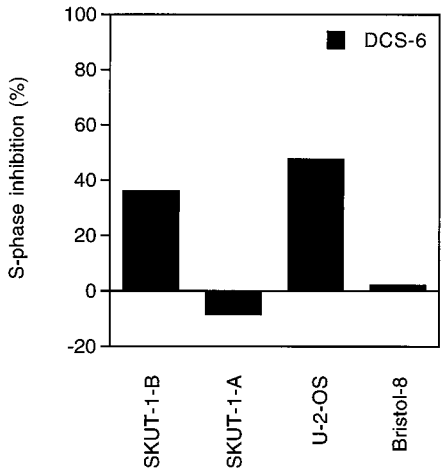

FIG. 10. Effects of anti-cyclin D1 antibody electroporation into mitotic SKUT1A and SKUT1B cells upon S-phase entry. Cells were electroporated with DCS6 and control normal mouse immunoglobulin immediately upon release from nocodazole block, cultured for 24 h in the continuous presence of BrdU, fixed, and evaluated for percentages of DNA-synthesizing cells among successfully electroporated cells identified by anti-immunoglobulin immunofluorescence. The graphs show representative values of percentage of S-phase inhibition obtained in repeated experiments; human Bristol-8 cells lacking cyclin D1 served as refractory cells, and U-2-OS sarcoma cells served as responsive control cells.

this study and in favor of the conclusions made, it clearly represents an exception in the sense that the cyclin D protein levels exceed those in the majority of other human cell types, especially the RB-deficient ones (33, 64). This appears to be due to enhanced stability of the D-type cyclin proteins whose half-lives are four- to fivefold longer in the SKUT1 cell lines (31) than in, for example, U-2-OS, another human sarcoma cell line expressing all three D-type cyclins (10).

The essential new findings from our present analysis that can shed some light on the order of events in the G_1 control pathway can be summarized as follows. First, antibody-mediated neutralization experiments showed that cyclin D1 function was required in the RB-positive ME and SKUT1B cells, whereas it was dispensable in the RB-deficient ME and SKUT1A cells. Second, the lack of requirement for cyclin D1 correlated with absence of functional pRB, regardless of whether active cyclin D1/cdk4 kinase was present (as in ME3 and ME8) or absent (as in SKUT1A) in the RB-deficient cells. Third, cyclin D1 antibodies that coprecipitated cdk4 and supported the cyclin D1-dependent kinase activity failed to inhibit phosphorylation of pRB and G_1/S transition of RB-positive cells. Fourth, antibody DCS-6, which immunoprecipitated only a cdk4-free cyclin D1 and did not coprecipitate any detectable pRB kinase activity, effectively prevented G_1 progression and phosphorylation of pRB in vivo. These findings, when considered together with other reports relevant to this topic (1, 2, 9, 10, 17, 18, 33, 37, 41, 47, 55, 57, 61), can significantly reduce the number of plausible scenarios of a physiological mechanism underlying the cyclin D-pRB interplay. The fact that neutralization of cyclin D1 had no effect upon S-phase entry in the RB^- ME cells injected either in early G_1 or in G_0 effectively eliminates the possibility that a cyclin D1-dependent regulatory event was only shifted toward G_0/G1 transition rather than becoming dispensable because of a lack of functional pRB. Since the RB gene disruption is likely to be the only genetic change, at least at the population level, in the ME3 and ME8 cells compared with the RB^+ ME1/ME2 cells, the observed loss of cyclin D1 function appears to be attributable to lack of pRB. Although other D-type cyclins were expressed in the cells under study, in particular the closely related cyclin D2 (19, 39, 44), it is unlikely that they selectively substituted for the antibody-neutralized cyclin D1 in the RB-deficient cells, thereby performing the cyclin D checkpoint function and only mimicking its loss as detected by our cyclin D1 assay. In the human system, this was ruled out by the high levels of the p16 inhibitor of cdk4/cdk6 that prevented formation of active complexes between cdk4/cdk6 and any of the D-type cyclins (6, 14, 18, 54, 59). In the mouse model, cyclin D2-cdk4 complexes and kinase activity were indistinguishable in the RB-positive and RB-deficient cells and therefore showed no sign of any compensatory elevation of the cyclin D2-dependent kinase in the RB^- ME cells. The mechanism through which antibody DCS-6 neutralized the essential G_1-regulatory function of cyclin D1 is not entirely clear at present. However, since this antibody recognizes the form of cyclin D1 that is inactive in supporting the cdk4 kinase, it is likely that it inhibits G_1 progression, at least in part, by preventing assembly of an active cyclin D1/cdk holoenzyme. Another plausible mechanism that could operate in parallel might be a reduction by antibody DCS-6 of a capacity of cyclin D1-cdk complexes to titrate out some cdk inhibitors, such as p21 or p27 (18, 49, 54), thereby indirectly preventing activity of other cellular cdks. One important observation in this study was that the introduction of DCS-6 into RB-positive cells resulted in accumulation of the un(der)phosphorylated form of pRB, suggesting that the progression through G_1 was inhibited at the time preceding and/or coinciding with phosphorylation of pRB in advanced G_1 (13, 42, 61). A mechanism that appears consistent with all of the data is that cyclin D1-cdk4 complex may perform a checkpoint function by phosphorylating pRB itself (10, 24, 37, 41, 61), one or more of the pRB-binding cellular proteins such as E2F (11, 16, 23, 61), or both. In any case, the expected outcome of such a phosphorylation event(s) would be dissociation of the multiprotein pRB complexes, followed by pleiotropic downstream events eventually leading to G_1/S transition (50, 61). Our data therefore strongly suggest that cyclin D1 plays an essential G1-regulatory role upstream of pRB and that it may contribute to inactivation of the growth-restraining complexes of pRB with transcription factors and/or other proteins.

Finally, the existence of a second, downstream role of cyclin D1 along the G_1 control pathway (9) seems unlikely in the light of our present data and those obtained previously in time course experiments inhibiting cyclin D1 function at different time points in G_1 (2, 31, 55). Such a regulatory role, if required for G_1/S transition, would have to be performed after phosphorylation of pRB yet before the onset of DNA synthesis and therefore would be expected to be inhibited by microinjections of cyclin D1 antibodies and antisense constructs performed in late G_1, i.e., contrary to what was found in the aforementioned time course studies (2, 31, 55). The accumulating evidence therefore indicates that the cyclin D-pRB interplay is unlikely to regulate the activity of D-type cyclins. Phosphorylation of pRB in mid- to late G_1 does not appear to be a means to liberate active cyclin D-cdk complexes poised to perform their putative downstream cell cycle regulatory function. Rather, the data available are consistent with a major role of cyclin D/cdks in abrogating the proliferation-constraining activity of pRB under conditions favorable for commitment to cell division.

With respect to a broader perspective of cell proliferation control, our results appear to be compatible with the proposed role of the D-type cyclins as potential sensors that integrate extracellular signals with the cell cycle clock (reviewed in reference 60). The mitogenic stimuli, transmitted through signal transduction pathways, may converge to activate the cyclin

D-cdk complexes by mid-G_1 (41, 48, 57, 66), followed by inactivation of the growth-constraining role of pRB in mid- to late G_1 and subsequent liberation of a series of transcription factors that activate expression of a range of genes whose products are required to initiate DNA replication (16, 50, 61). The key element of this pathway is the commitment decision in mid- to late G_1, at the time when pRB becomes phosphorylated, potentially by cyclin D/cdk kinases (reviewed in references 13 and 61). At this checkpoint, the growth factor-dependent phase culminating in assembly and activation of cyclin D-cdk complexes would become translated into inactivation of pRB, thereby switching the cell cycle control to the growth factor-independent phase governed by the autonomous cell cycle machinery (51, 56, 60). This concept is remarkably reminiscent of the R-point definition and again points to a candidacy of the D cyclins and pRB as plausible executor and substrate, respectively, of the R-point switch in late G_1 phase (33, 53).

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