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Overexpression of human cyclin D1 reduces the transforming growth factor β (TGF-β) type II receptor and growth inhibition by TGF-β1 in an immortalized human esophageal epithelial cell line

(carcinogenesis/G_1 cyclin/cell cycle/cyclin-dependent kinase)

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ABSTRACT **Cyclin D1 has been implicated in G_1 cell cycle progression and is frequently amplified, overtranscribed, and oversynthesized in human tumors, including esophageal carcinomas. To further address the role of cyclin D1 in cell cycle control and tumorigenesis, we have stably transfected the human cyclin D1 in the nontumorigenic esophageal epithelial cell line HET-1A. These transfected cells, which express increased amounts of cyclin D1, have enhanced colony-forming efficiency and saturation density and are resistant to growth inhibition by TGF-β1 compared with the parental cell line or a control vector cell clone. The clones which express increased amounts of cyclin D1 exhibited a decrease in the amount of TGF-β type II receptor, indicating a plausible mechanism for their diminished response to TGF-β1. Therefore, deregulated expression of the cyclin D1 gene can modulate the negative growth factor pathway of TGF-β1 and may disturb the control of epithelial cell proliferation in esophageal carcinogenesis.**

Since the major regulatory events leading to mammalian cell proliferation and differentiation occur in the G_0-to-G_1 and/or at the G_1-to-S phase transition of the cell cycle (1), the deregulated expression of G_1 or G_1/S phase cyclins or their related cyclin-dependent kinases (CDKs) might cause loss of cell cycle control and thus enhance carcinogenesis. The strongest connection between cyclins and carcinogenesis comes from studies on cyclin D1. The cyclin D1 gene was isolated as a gene that is rearranged and overexpressed in parathyroid adenomas (2, 3). In independent studies, cyclin D1 also rescued a G_1 cyclin-defective *Saccharomyces cerevisiae* strain, and its expression was induced in G_1 by growth factors (4, 5). Microinjection and electroporation of anti-cyclin D1 antibodies into mammalian cells revealed that cyclin D1 is essential for cell cycle progression in G_1 (5, 6). Amplification and overexpression of cyclin D1 were detected in several human carcinomas, including esophageal carcinomas (6).

Transforming growth factor βs (TGF-βs) are prototypic multifunctional negative growth factors that inhibit epithelial cell proliferation by delaying or arresting progression through the late portion of G_1 (7). It is believed that one of the mechanisms whereby cells undergo neoplastic transformation and escape from normal growth control involves an altered response to TGF-β1 (8). Previous studies have indicated links between TGF-β1-mediated G_1 growth arrest and the state of cyclins, CDKs, and the product of the retinoblastoma susceptibility gene, RB (9–13). Treatment of cells with TGF-β appears to prevent phosphorylation of RB and retains RB in the hypophosphorylated, growth-suppressive state. TGF-β1 also suppresses synthesis of CDK4, a major catalytic subunit of cyclin D1, in G_1 in mink lung epithelial cells, and constitutive CDK4 synthesis in these cells leads to TGF-β1 resistance (9). These results indicate that growth arrest by TGF-β1 might be mediated through the cyclins, CDKs, and RB-related pathways (9–13).

To further investigate the function of cyclin D1 and its role in tumorigenesis, we have stably transfected an expression vector containing the human cyclin D1 cDNA in HET-1A cells, a human normal esophageal epithelial cell line immortalized by simian virus 40 (SV40) large tumor antigen (T antigen) (14). Oversynthesis (>10 fold) of cyclin D1 protein in clones of HET-1A cells led to an increase in colony-forming efficiency and saturation density. In addition, the clones were less responsive to growth inhibition by TGF-β1 than the parental cells or control vector cell clones. Interestingly, these clones which express increased amounts of cyclin D1 exhibited a decrease in the amount of TGF-β type II receptor.

MATERIALS AND METHODS

Cell Culture. Human esophageal epithelial cell line HET-1A, which is immortalized by SV40 T antigen, has retained epithelial morphology and remained nontumorigenic in athymic *nude* mice for more than 12 months (14). The cell line was cultured in serum-free medium, KGM (Clonetics, San Diego). The human esophageal carcinoma cell line HCE-4 was cultured as previously described (6).

Overexpression of Human Cyclin D1 cDNA in HET-1A Cells. The viral supernatants containing either the human cyclin D1 cDNA or the retroviral expression vector pMV7pI alone were prepared as described (15). HET-1A cells (passage 27) were infected with the viral supernatant, and after 48 hr, they were selected with G418 (100 μg/ml).

Southern and Northern Blotting. DNA and RNA were prepared from the cell lines as described (6). Southern and Northern blot filters were hybridized with [^{32}P]dCTP-labeled probes for human cyclin D1, TGF-β1, TGF-β type I and II receptors, plasminogen activator inhibitor type I, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described (6).

Immunoprecipitation and Western Blot Analysis. Protein lysates were prepared as described (3). Samples (300 μg) of either ^{35}S-labeled or unlabeled protein were immunoprecipitated with the following antibodies: anti-cyclin D1 [kindly provided by Yue Xiong (University of North Carolina,

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Abbreviations: TGF-β, transforming growth factor β; CDK, cyclin-dependent kinase; RB, retinoblastoma protein; SV40, simian virus 40; T antigen, tumor antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen.

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Chapel Hill) and David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Upstate Biotechnology Inc. (UBI), Lake Placid, NY, and Santa Cruz Biotechnology], anti-cyclin D2 (Santa Cruz Biotechnology), anti-cyclin D3 (Santa Cruz Biotechnology), anti-cyclin E (UBI), anti-RB (Santa Cruz Biotechnology), anti-p53 (Oncogene Science), anti-PCNA (Oncogene Science), anti-CDK1 (Oncogene Science), anti-CDK2 (UBI), anti-CDK4 (UBI), anti-CDK5 (Santa Cruz Biotechnology), anti-cyclin A (UBI), and anti-TGF-β type II receptor (UBI). Immunoprecipitates were electrophoresed on SDS/polyacrylamide gels. For labeled experiments, gels were fixed and exposed to x-ray films at −70°C. For Western analysis, proteins were transferred to nitrocellulose filters, probed with antibodies, and detected by a chemiluminescence system (Amersham).

Colony-Forming Efficiency, Growth Rate, and Saturation Density. Cells were plated in triplicate at 1×10^3 cells per 60-mm dish and were fixed and stained at 10 days of incubation as described (14). The growth rate and saturation density of each cell line were determined as described before (14).

TGF-β1 Responsiveness. TGF-β1 responsiveness of each cell line was measured by both colony-forming efficiency as described above and [^{3}H]thymidine incorporation assay. Porcine TGF-β1 (R & D Systems) was added to the medium at a final concentration of 1–333 pg/ml for the colony-forming efficiency and 1–3300 pg/ml for [^{3}H]thymidine incorporation assay.

Tumorigenicity Assay. Either 5×10^6 or 1×10^7 cells were injected subcutaneously into multiple sites of athymic *nude* mice as previously described (15).

RESULTS

Overexpression of Human Cyclin D1 cDNA in HET-1A. Following G418 selection, six neomycin-resistant clones were randomly isolated from the cultures infected with the pMV7plCCND1 construct and a control clone (H1A-pMV7 CL13) was isolated from cultures infected with the pMV7pl vector. Northern blot analysis demonstrated that the mass culture of pMV7plCCND1-infected cells and all of the pMV7plCCND1-derived clones expressed high levels of the expected 4.8-kb 5′-long terminal repeat (LTR)-cyclin D1-tkneo-LTR-3′ transcript. Two of these clones, H1A-CCND1 CL5 and CL6, were selected for further studies. Fig. 1*A* displays the Northern blot analysis in which H1A-CCND1 CL5 and CL6 show high amounts of the 4.8-kb 5′-LTR-cyclin D1-tkneo-LTR-3′ transcript, whereas HET-1A and H1A-pMV7 CL13 show a low amount of 4.8-kb endogenous human cyclin D1 transcript. We confirmed the integration of exogenous cyclin D1 in these two cyclin D1-overexpressing clones by Southern blot analysis (Fig. 1*B*). Both H1A-CCND1 CL5 and CL6 showed a 5.5-kb band which corresponded to the *Eco*RI fragment of 5′-LTR-cyclin D1-tkneo-LTR-3′ in addition to the 4.0-, 2.2-, and 2.0-kb endogenous cyclin D1 bands. The HCE-4 cell line displayed amplification of the endogenous cyclin D1 sequences as previously described (6). We next examined whether the two overexpressing clones displayed increased synthesis of the related protein (Fig. 1*C*). An intense signal of a single 36-kDa band was seen in H1A-CCND1 CL5 and CL6. A faint band was observed in HET-1A and in H1A-pMV7 CL13. The intensity of the 36-kDa band in both overexpressing clones was more than 10-fold greater than that of H1A-pMV7 CL13 when measured by densitometric scanning, but it was still less than the very intense band seen in HCE-4. We also used mouse monoclonal cyclin D1 antibody, which did not cross-react with either cyclin D2 or D3, to confirm that cyclin D1 protein itself was overexpressed in the clones (data not shown).

Cyclin D1-Overexpressing Clones Show Abnormalities in Cell Growth Control. Both cyclin D1-overexpressing clones have a significant increase in colony-forming efficiency relative to control cultures (Fig. 2). H1A-CCND1 CL5 (248 ± 41 colonies per dish) expressed more cyclin D1 than H1A-CCND1 CL6 (170 ± 38 colonies per dish) ($P < 0.01$, by two-sample t test). Thus, there was a positive correlation between the concentration of cyclin D1 and colony-forming efficiency. Although both cyclin D1-overexpressing clones had population doubling times (32.4 and 33.3 hr) similar to those of HET-1A (33.4 hr) and H1A-pMV7 CL13 (35.3 hr), these overexpressing clones had 40–50% greater saturation densities than either HET-1A or H1A-pMV7 CL13 (data not shown). We also performed flow cytometric analysis on growing cell cultures, high-density cultures, and cultures that had been released from contact inhibition to examine the effect of constitutive overexpression of cyclin D1 on cell cycle progression. No significant differences in cell cycle distribution were found among these cultures (data not shown). We performed karyotypic analysis to examine whether overexpression of cyclin D1 increased chromosomal instability. No significant differences were observed in ploidy distribution, modal chromosomal number, marker chromosomal number, or chromosomal aberrations (data not shown). Neither H1A-CCND1 CL5 or CL6 displayed mor-

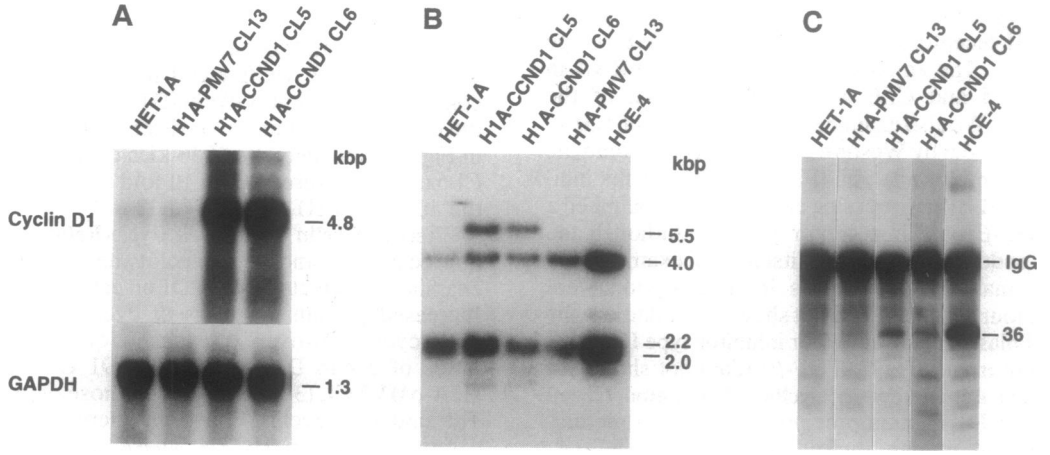

FIG. 1. Cyclin D1 expression and integration in SV40 T antigen-immortalized human esophageal cells (HET-1A) infected with PMV7 containing human cyclin D1 cDNA. (*A*) mRNA transcription of cyclin D1 was examined in HET-1A cells, a control clone infected with the pMV7pl vector alone (H1A-PMV7 CL13), and two clones infected with the pMV7plCCND1 construct (H1A-CCND1 CL5 and CL6). Right ordinate indicates the size of each hybridizing band in kilobase pairs (kbp). (*B*) Integration of exogenous cyclin D1 was confirmed in H1A-CCND1 CL5 and CL6 by Southern blot analysis using human cyclin D1 cDNA probe. (*C*) Western blot analysis of cyclin D1 protein was performed with anti-cyclin D antibody. The positions of the 36-kDa cyclin D1 and the IgG heavy chain are shown on the right.

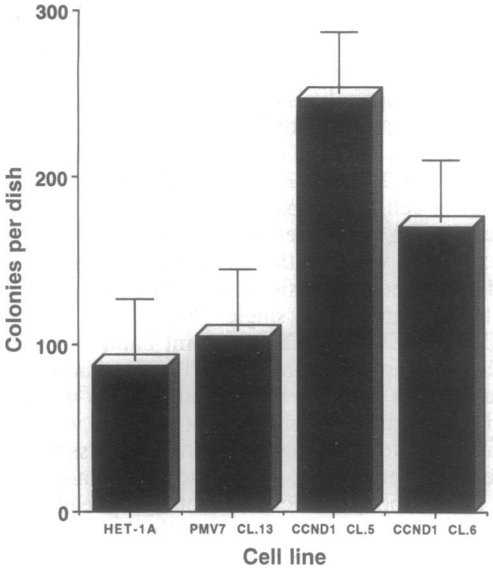

FIG. 2. Overexpression of cyclin D1 enhanced colony-forming efficiency. Colony-forming efficiency was examined in HET-1A, H1A-PMV7 CL13, H1A-CCND1 CL5, and H1A-CCND1 CL6. Values are represented as the mean ± SD of triplicates from six experiments.

phological evidence of neoplastic transformation. They did not grow in soft agar or form tumors in *nude* mice.

TGF-β1 Responsiveness of Cyclin D1-Overexpressing Clones. TGF-β1 responsiveness was examined by both colony-forming efficiency and [^{3}H]thymidine incorporation (Fig. 3). Compared with HET-1A and H1A-pMV7 CL13, the overexpressing clones were significantly more resistant to the inhibitory effects of TGF-β1. In the [^{3}H]thymidine incorporation assay, overexpressing clones were more resistant to TGF-β1 at a concentration of 3.3 ng/ml. H1A-CCND1 CL5, which displayed a higher level of expression of cyclin D1 than did CL6, showed more resistance to TGF-β1 than did CL6. Therefore, cells overexpressing cyclin D1 were more resistant to the growth-inhibitory effects of TGF-β1, and there was a positive correlation between the level of cyclin D1 expression and TGF-β1 resistance.

To further investigate the effect of cyclin D1 on TGF-β1 resistance, we examined the expression of TGF-β type I and II receptors by Northern blot analysis (Fig. 4*A*). Clones overexpressing cyclin D1 exhibited a decrease in the amount of TGF-β type II receptor mRNA but not of the TGF-β type I receptor mRNA. The amount of TGF-β type II mRNA in H1A-CCND1 CL5 and CL6 was 40–50% of that of the H1A-pMV7 CL13. We also examined the expression of TGF-β type II receptor protein by Western blot analysis to extend the data from Northern blot analysis (Fig. 4*B*). The results indicated that cyclin D1-overexpressing clones clearly exhibited a decrease in TGF-β type II receptor protein. Although the expression of endogenous TGF-β1 itself was examined by Northern blot analysis, no changes in transcription were observed in the four cell lines (data not shown). In addition, the transcription of plasminogen activator inhibitor type I in these cell lines was not influenced by TGF-β1 (data not shown).

Proteins Which Associate with Cyclin D1 in Immortalized Cells. Anti-cyclin D1 immunoprecipitations of [^{35}S]methionine-labeled WI-38 human diploid fibroblasts, HET-1A, H1A-pMV7 CL13, H1A-CCND1 CL5 and CL6, and HCE-4 were performed (Fig. 5*A*). WI-38 cells showed a 36-kDa band which corresponded to cyclin D1, 37-kDa PCNA, 33-kDa CDK2,4, and 21-kDa p21, which is consistent with previously published studies (16). HET-1A and its derivatives showed only the 36-kDa band, indicating that cyclin D1/PCNA/

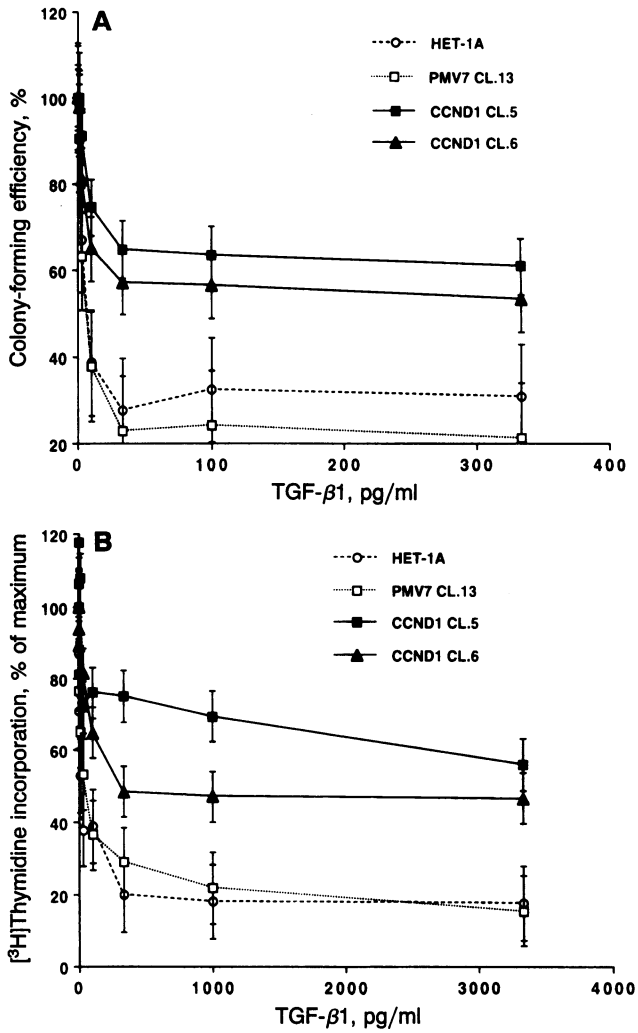

FIG. 3. TGF-β1 responsiveness in clones overexpressing cyclin D1. (*A*) Colony-forming efficiency was examined in the presence of TGF-β1 (1–333 pg/ml). Values are expressed as the percentage of the colony-forming efficiency in the absence of TGF-β1 and are represented as the mean ± SD of triplicates from three experiments. (*B*) [^{3}H]Thymidine incorporation was examined after 24 hr of incubation with TGF-β1 (1–3300 pg/ml). Values are expressed as the percentage of [^{3}H]thymidine incorporation in the absence of TGF-β1 and are represented as mean ± SD of triplicates from three experiments.

CDKs/p21 complexes are disrupted in immortalized epithelial cells. In the HCE-4 cell line, anti-cyclin D1 antibody coimmunoprecipitates cyclin D1 and CDKs 2 and 4, but not the p37 or p21 proteins. Consistent with the results presented in Fig. 1*C*, the amounts of 36-kDa cyclin D1 in H1A-CCND1 CL5 and CL6 were 28- and 10-fold higher, respectively, than the amount in H1A-pMV7 CL13.

Effect of Cyclin D1 on Cell Cycle-Related Proteins. Immunoprecipitation and Western blot analyses were performed to examine the effect of cyclin D1 on cell cycle-related proteins. Increased protein synthesis of cyclin D2 was observed in both cyclin D1-overexpressing clones, and the intensity ratios of cyclin D2 in H1A-CCND1 CL5 and CL6 versus H1A-pMV7 CL13 were similar to those of cyclin D1 (Fig. 5). The amount of cyclin D2 also was increased in the esophageal carcinoma cell line, HCE-4. These results suggest that cyclin D1 modulates the expression of cyclin D2 in these cells. Although protein synthesis of RB, p53, PCNA, CDKs 1, 2, 4, and 5, and cyclins A, D3, and E were examined in all cell lines, no significant alterations were observed in any of the cell lines (data not shown). TGF-β1 did not influence the degree of phosphorylation of RB or the degree of synthesis of

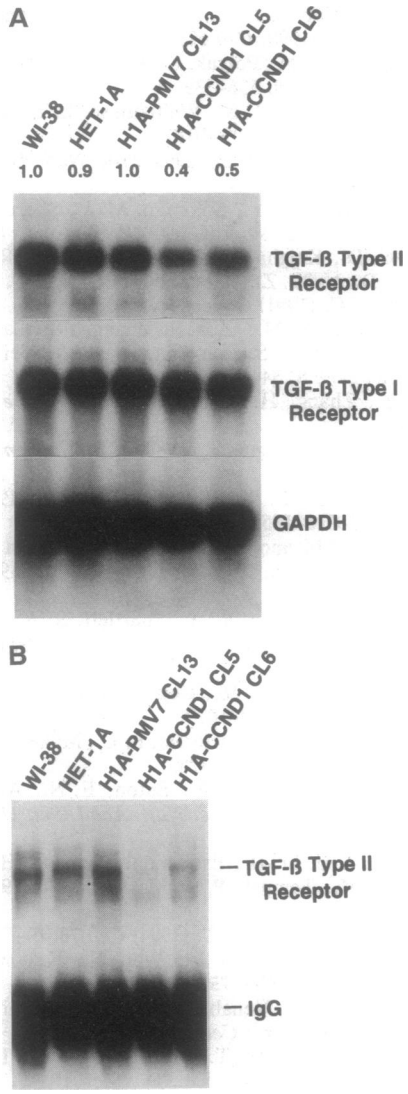

FIG. 4. Expression of TGF-β type I and II receptors in clones overexpressing cyclin D1. (*A*) Northern blot analysis was performed in HET-1A, H1A-PMV7 CL13, H1A-CCND1 CL5, and H1A-CCND1 CL6 with cDNA probes to TGF-β type I and II receptors. GAPDH is a control for RNA loading. The numeric ratios provide semiquantitative comparisons of TGF-β type II receptor expression in H1A-PMV7 CL13 cells. The densitometry value of TGF-β type II receptor for H1A-PMV7 CL13 was divided by the corresponding GAPDH value, and the ratio was normalized to 1.0 by a conversion factor. The same conversion factor was applied to the other cell lines to allow comparison to H1A-PMV7 CL13. Similar calculations for the type I receptor did not show significant variations. (*B*) Western blot analysis with anti-TGF-β type II receptor antibody was performed in the same cell lines as *A*.

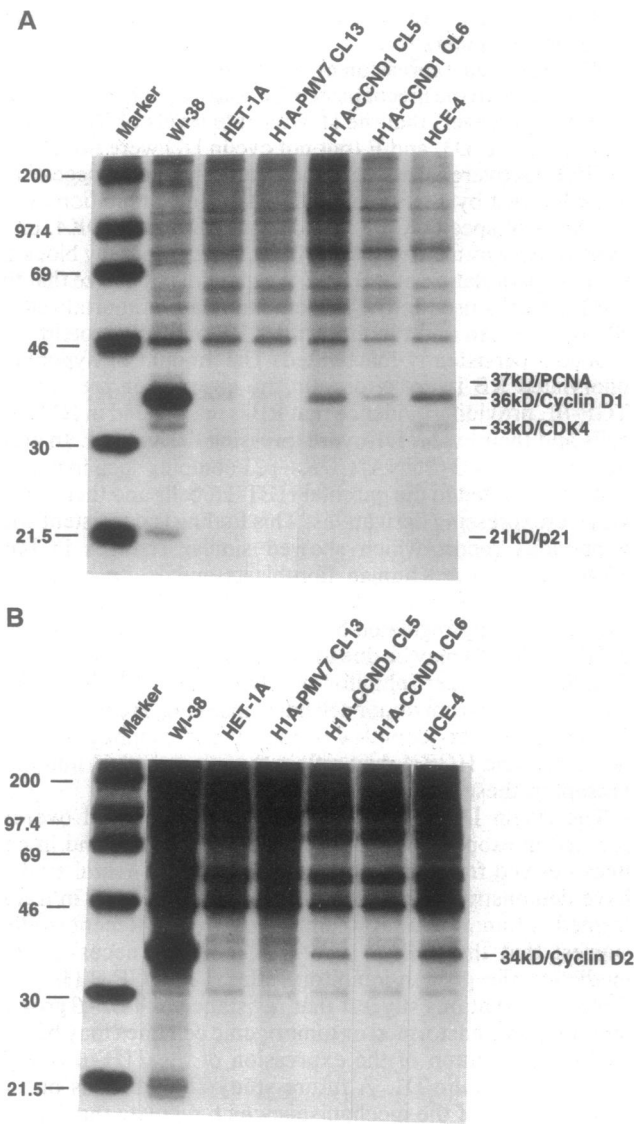

FIG. 5. Immunoprecipitation with anti-cyclin D1 and anti-cyclin D2. ^{35}S-labeled immunoprecipitates with cyclin D1 antibody (*A*) or cyclin D2 antibody (*B*) in WI-38, HET-1A, H1A-PMV7 CL13, H1A-CCND1 CL5, H1A-CCND1 CL6, and HCE-4 were examined. Positions of molecular mass markers (kDa) are shown on the left. PCNA, proliferating cell nuclear antigen.

CDK4 in either the control or cyclin D1-overexpressing H1A cell lines (data not shown).

DISCUSSION

Constitutive overexpression of cyclin D1 in HET-1A resulted in abnormalities in cellular growth control compared with control cells. Clones overexpressing cyclin D1 had higher colony-forming efficiency and saturation density, but their population doubling times were not reduced. In addition, overexpression of cyclin D1 renders cells resistant to the growth-inhibitory effects of TGF-β1. TGF-βs arrest cells in the G_1 phase of the cell cycle through ligand binding to specific cell surface receptors, and the type I and type II receptors are the most important for signal transduction (17–19). Our studies show that overexpression of cyclin D1 results in an inhibition of TGF-β type II but not type I receptor expression, suggesting a plausible mechanism for resistance of the cells to the negative growth effects of TGF-β1.

Previous studies on the effects of overexpression of cyclin D1 in rodent fibroblasts have also indicated that this causes abnormalities in growth control. Thus, utilizing the same cyclin D1 construct employed in the present study, we found that derivatives of Rat 6 fibroblasts that stably overexpressed cyclin D1 also had a normal doubling time but an increased saturation density (15). These cells formed small colonies in soft agar and were weakly tumorigenic. In addition, they had a shortened G_1 phase, were smaller, and displayed increased expression of cyclin A, c-*myc*, and c-*jun* compared with vector control cells. Other studies indicate that increased expression of cyclin D1 in rodent fibroblasts shortens the G_1 phase and also alters their responses to serum starvation (20, 21). Furthermore, in transfection studies cyclin D1 can enhance the transformation of rodent fibroblasts by an activated *ras* oncogene (22) or by a *ras* oncogene plus a mutant E1A gene (23). These findings, taken together with the present studies, provide strong evidence for amplification

and increased expression of the cyclin D1 gene in human esophageal cancers (6).

We examined expression of cell cycle-related proteins for a possible clue to the mechanism of action of cyclin D1. Our data indicate, however, that the steady-state levels of RB, CDKs, and cyclins A, D3, and E (but not cyclin D2) were not altered in HET-1A overexpressing clones. Recent studies suggest that growth arrest by TGF-β is mediated through inhibition of the synthesis of specific CDKs, such as CDK2 and CDK4, or of their activity by a p27 protein (10–13, 24, 25), thereby blocking the phosphorylation of RB (11). We should emphasize that the HET-1A cells used in the present study are immortalized by SV40 T antigen and therefore their RB and p53 proteins are probably persistently inactivated. The amount of hypophosphorylated RB is not altered in the absence or presence of TGF-β1, providing evidence that RB is inactivated in HET-1A cells and their cyclin D1-overexpressing derivatives. In addition, the cyclin D/PCNA/CDKs/p21 complex seen in normal cells is disrupted in the parental HET-1A cells and their cyclin D1-overexpressing derivatives. This finding is consistent with a previous report which showed similar changes in both SV40-immortalized human fibroblasts and HeLa cells (16). Since p21, also named WAF1, Cip1, or Sdi1 (26–29), is transcriptionally regulated by p53, disruption of this complex in HET-1A cells may be due to inactivation of p53 by the SV40 T antigen. Another inhibitor of CDK4, $p16^{INK4}$ (30), is frequently absent from tumor cell lines, including HCE-4 (31–33). Disruption of this complex in the human esophageal carcinoma cell line HCE-4 may reflect the missense p53 mutation present in these cells (34).

The cyclin D1 gene is commonly amplified and overexpressed in esophageal, breast, and liver tumors, and in cell lines derived from these tumors (6, 35, 36). Several studies have demonstrated the loss of sensitivity to TGF-β in transformed or tumorigenic epithelial cells (37, 38). Recent studies suggest that the TGF-β type II receptor is necessary for mediating the growth-inhibitory effects of TGF-β (39, 40). These observations suggest that resistance to TGF-β growth inhibition in transformed or tumorigenic cell lines may be due to down-regulation of the expression of the TGF-β type II receptor by cyclin D1. A future study should focus on the determination of the mechanisms which regulate the expression of TGF-β type II receptor by cyclin D1 and examination of the TGF-β type II receptor expression in cancers with or without the cyclin D1 amplification and/or overexpression.

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