Induction of G1 Arrest by Down-regulation of Cyclin D3 in T Cell Hybridomas

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Summary

The relationship between activation-induced growth inhibition and regulation of the cell cycle progression was investigated in T cell hybridomas by studying the function of the cell cycle–regulating genes such as G1 cyclins and their associated kinases. Activation of T cell hybridomas by anti-T cell receptor antibody induces growth arrest at G1 phase of the cell cycle and subsequently results in activation-driven cell death. Rapid reduction of both messenger RNA and protein level of the cyclin D3 is accompanied by growth arrest upon activation. Although the residual cyclin D3 protein forms a complex with cdk4 protein, cyclin D3–dependent kinase activity is severely impaired. Stable transfectants engineered to express cyclin D3 override the growth arrest upon activation. These results imply that the activation signal through T cell receptor induces the down-regulation of cyclin D3 expression and cyclin D3–dependent kinase activity, leading to growth arrest in G1 phase of the cell cycle in T cells.

Mature T lymphocytes in peripheral tissues start to proliferate in response to antigen stimulation. However, the same stimulation through the TCR complex induces growth arrest and activation-driven cell death (ADCD)[1] in T cell hybridomas or tumor cells (1). Moreover, growth inhibition and ADCD have been found to be induced even in normal T cells and T cell clones if they are reactivated through TCR when cells are proliferating in the presence of IL-2 (2–4). These observations raise the hypothesis that the activation signal through the TCR complex modifies cell cycle regulation and results in cell cycle arrest and ADCD in proliferating T cells.

D-type cyclins, cyclin E, and cdk's are important regulators of cell progression from G1 to S phase and of initiation of DNA synthesis (5–9). When quiescent cells are stimulated to initiate proliferation by the addition of growth factor, both D-type cyclins and cyclin E are induced (9–14). Generally, D-type cyclins are induced earlier than cyclin E in G1 phase (15–18). It has been shown that cyclin E associates mainly with cdk2, whereas the major partners of D-type cyclins are cdk4 and the recently identified kinase, cdk6 (15–21). The constitutive expression of cyclin E as well as of D-type cyclins suppresses the growth-inhibitory activity of retinoblastoma protein (pRb) in a pRb-deficient osteosarcoma cell line (22–24). In addition, the enforced expression of cyclin E and D-type cyclins has been shown to shorten the duration of G1 phase, and microinjection of cyclin D1 antisense DNA or of antibodies specific for cyclin D1 during G1 phase inhibits cells from exiting to S phase (25, 26).

The role of cyclin E and D-type cyclins and their associated cdk kinases in inhibiting the G1 phase progression was also studied. TGF-β induces G1 arrest by down-regulation of cdk4 kinase, and it activates p27 and p15, the inhibitors for cdk's (27–33). Growth arrest at G1 phase by radiation-induced DNA damage is due to another inhibitor of cyclin E–cdk complex, p21 (34–37). Cell cycle arrest at G1 phase appears to be regulated mainly by these inhibitors in these systems. Whether this mechanism is important in lymphocytes is yet unknown.

We took advantage of the unique situation of T cell hybridomas, namely, that the activation signal through the TCR complex induces cell cycle arrest, to understand the function of G1 cyclins and cdk's in cell cycle regulation. Here we report that down-regulation of cyclin D3–associated kinase activity, mainly due to the reduction of cyclin D3 expression, is critical for the induction of G1 arrest in T cell hybridomas.

[1] *Abbreviations used in this paper:* ADCD, activation-driven cell death; DTT, dithiothreitol; GST, glutathione *S*-transferase; mRNA, messenger RNA; PI, propidium iodide; pRb, retinoblastoma protein; TBST, Tris-buffered saline with 0.2% Tween 20.

Materials and Methods

Cells and mAbs. T cell hybridoma DO11.10 (38) is a chicken ovalbumin–specific T cell hybridoma expressing Vβ8 that can be

stimulated with anti-Vβ8 mAb F23.1 (39). This cell line was kindly provided by Dr. P. Marrack (National Jewish Center, Denver, CO). Hybridoma cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 4 mM glutamine, 100 μg/ml kanamycin, and 50 μM 2-ME (complete medium) at a density of 1–6 × 10^5 cells per ml. F23.1 mAb was purified from ascites by ammonium sulfate precipitation and protein A-Sepharose column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Anti-mouse cyclin D2 and D3 mAbs were kindly provided by Dr. C. J. Sherr (St. Jude Children's Hospital, Memphis, TN). Anti-cdk4 mAb was raised against a synthetic COOH-terminal peptide of mouse cdk4 and will be described elsewhere (Kato, A., and H. Matsushime, manuscript in preparation). Anti-human cdk2 antiserum was purchased from Pharmingen (San Diego, CA).

T Cell Hybridoma Synchronization. Cells were synchronized at the G1/S phase boundary by the double-thymidine block protocol (40). Briefly, DO11.10 T hybridoma cells were incubated in complete medium containing 12 mM thymidine for 5 h, washed three times, and incubated for another 5 h without thymidine. Thymidine was then added again at the same concentration, and the cells were incubated for another 5 h. The cell cycle analysis confirmed that >75% of the cells were in G1 phase.

Cell Cycle Analysis. 3–5 × 10^6 cells were pelleted, resuspended in 0.5 ml of 4-mM sodium citrate containing 0.05% NP-40, 0.45 mg/ml RNase, and 50 μg/ml propidium iodide (PI), and incubated for 10 min on ice. Then 50 μl of 1.5-M NaCl was added to the suspension. The proportions of cells in G1, S, and G2 phases of the cell cycle were analyzed by flow cytometry on a FACScan® with Cellfit software (Becton Dickinson Advanced Cellular Biology, San Jose, CA).

Activation of T Cell Hybridoma. 12-well flat-bottomed plates were coated with 1 ml of 20 μg/ml of mAb F23.1 for 12 h at 4°C and then washed three times with PBS. 3 ml of 1 × 10^5 cells per ml hybridoma cells was placed on the F23.1-coated plates for activation.

RNA Blotting. Total RNA was extracted from hybridoma cells as previously described (41), fractionated on 1% agarose-formaldehyde gel, and blotted to membrane (Hybond-N^+; Amersham International, Little Chalfont, UK) by conventional capillary blotting. Membranes were hybridized with cDNA probes labeled with α-[^{32}P]dCTP by a DNA labeling kit (Megaprime; Amersham International), washed at a final stringency of 0.5 × SSC, 0.1% SDS at 65°C, and analyzed with an image analyzer (BAS2000; Fuji Photo Film Corporation, Tokyo, Japan). The signals were quantitated and normalized to the β-actin signals. The following probes for various G1 cyclins, cdk2, and actin were used: human cyclin C, 2.1-kb BglII-HindIII fragment (6); mouse cyclin D1, 1.1-kb BamHI EcoRI fragment excised from plasmid pGEX-3X-CYL1 (9); mouse cyclin D2, 1.2-kb EcoRI fragment excised from plasmid pcN9cyl2 (9); mouse cyclin D3, 2.2 kb EcoRI fragment excised from plasmid pmCyD3; human cyclin E cDNA, 1.5-kb EcoRI-HindIII fragment (6); human cdk2 cDNA, 1 kb HindIII-BamHI fragment (42); mouse β actin cDNA of 3′ untranslated region, 1-kb EcoRI-BamHI fragment excised from plasmid pSPMβ-′3UT (43). The plasmids containing the human cyclin C and E were kindly provided by Dr. S. I. Reed (Scripps Research Institute, La Jolla, CA). The plasmids pGEX-3X-CYL1 and pcN9cyl2 were generously donated by Dr. C. J. Sherr (St. Jude Children's Hospital). The plasmids containing cdk2 cDNA were gratefully received from Dr. E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA). The mouse cyclin D3 cDNA was cloned from a λZAP cDNA library prepared from mouse thymus cDNA (Stratagene, Inc., La Jolla, CA) and screened with mouse cyclin D3 cDNA obtained by PCR using the synthesized primers from the published amino acid sequences (9). The primers used were 5′CAAAGCTTGCNTAYTGGATGYT3′ and 5′TCGAATTCARRTARTTCATNGC3′. Mouse cyclin E cDNA was cloned from the same library with human cyclin E cDNA as a probe. The mouse cyclin E clone was sequenced and the homology to the human cyclin E cDNA sequence was confirmed.

Cell Viability. For determining the viability of cells, 0.5–1 × 10^6 cells were pelleted and resuspended in PBS containing 5% FCS and 10 μg/ml PI. The numbers of cells stained with PI (dead cells) and not stained with PI (live cells) were determined by flow cytometry.

Immunoprecipitation and Western Blotting. 5 × 10^6–1 × 10^7 cells were washed twice with PBS and lysed on ice in 1 ml of lysis buffer (250 mM NaCl, 0.1% NP-40, 50 mM Hepes, pH 7.0, 5 mM EDTA, 50 mM NaF, 0.1 mM sodium orthovanadate, 50 μg/ml of PMSF, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, and 1 mM dithiothreitol [DTT]). 1 μl of anti-mouse cyclin D2 or D3 mAb was used for immunoprecipitation as described by Harlow and Lane (44). The proteins were resolved on 12% SDS-PAGE and transferred to polyvinyl difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). Blots were blocked in Blockace (Yukijirushi, Sapporo, Japan), washed four times with Tris-buffered saline with 0.2% Tween 20 (TBST), and incubated with first antibodies in TBST for 1 h at room temperature. Rabbit anti-human cdk2 and affinity-purified mAbs against cdk4, cyclin D2, and cyclin D3 were all diluted 1,000-fold. The blots were then washed four times with TBST, incubated with secondary antibodies (affinity-purified sheep anti-mouse, sheep anti-rat antibody, or donkey anti-rabbit antibody conjugated with horseradish peroxidase [Amersham International]) at a 1:10,000 dilution, washed four times with TBST, and developed with enhanced chemiluminescence reagent (Amersham International).

Immune Complex Kinase Assay. Immune complex kinase assay was performed as described previously (45). 5 × 10^6 cells were suspended in IP buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, and 0.1% Tween-20) containing 10% glycerol, 0.1 mM PMSF, 10 μg/ml of leupeptin, 20 U/ml of aprotinin, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate, and they were sonicated at 4°C. The clarified lysates were precipitated with mAbs against mouse cyclin D2 or cyclin D3 and rabbit antisera against human cdk2 or mouse cdk4. The immune complexes were precipitated for 2 h at 4°C with protein G–Sepharose beads. Immunoprecipitated proteins were washed with IP buffer and suspended in a kinase buffer (50 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT) containing 0.2 μg of soluble glutathione *S*-transferase (GST)-Rb fusion protein and 2.5 mM EGTA, 10 mM β-glycerophosphate, 20 U/ml of aprotinin, 0.1 mM PMSF, 1 mM NaF, 0.1 mM sodium orthovanadate, 20 μM ATP, 2 mM reduced glutathione, and 10 μCi γ-[^{32}P]ATP. After incubation for 20 min at 30°C with occasional mixing, the samples were boiled for 5 min and resolved on 10% SDS-PAGE. GST-Rb was prepared as described previously (45).

DNA Transfection. The expressible construct for mouse cyclin D3 was prepared by subcloning of the *Eco*RI fragment of the mouse cyclin D3 into the EcoRI site of the pMKITneo vector containing SRα promoter and neo^r gene (kindly provided by Dr. K. Maruyama, Tokyo Medical and Dental University, Tokyo, Japan). The linearized plasmid was transfected into cells by electroporation as described previously (46). Briefly, 10^7 DO11.10 cells were washed thee times with ice-cold PBS and resuspended in 0.8 ml ice-cold K-PBS buffer (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.46 mM KH_2PO_4). 30 μg of linearized plasmid DNA was added to the cell suspension in a cuvette (Gene Pulser

Cuvette; Bio-Rad Laboratories, Richmond, CA), and an electric pulse (310 V, 960 μF) was applied with a Gene Pulser (Bio-Rad Laboratories). Cells were cultured at 10^5 cells per well in 96-well plates in the complete medium and selected after 24 h in the presence of 1 mg/ml Geneticin (GIBCO BRL, Gaithersburg, MD).

Results

T Cell Activation Induced Rapid Changes of the Expression of G1 Cyclins and Their Associated Kinases before Cell Cycle Arrest and Cell Death. T cell hybridoma DO11.10 was stimulated by engaging the TCR complex with immobilized anti-TCR-β antibody F23.1. Flow cytometric analysis of the cell cycle indicated that the activation of T cells induced the cell cycle arrest at G1 phase (Fig. 1 *A*). The number of cells in early S phase started to decrease 4 h after stimulation, whereas cells in G1 phase started to increase. The reduction of the cell number in S phase continued until 8 h after stimulation, with the percentage changing from 60 to 30% (Fig. 1 *A*).

Cell viability was also measured by staining dead cells with PI and was analyzed by flow cytometry. Dead cells were first observed at 6 h after stimulation, and about half of the population died after 12 h (Fig. 1 *B*).

To study the role of G1 cyclins and cdk2 kinase in the induction of the G1 arrest, the expression of these genes after activation of T cells through TCR was analyzed. Total RNA was isolated from DO11.10 cells at each time point after stimulation and analyzed on RNA blots using probes of G1 cyclins and cdk2 as well as β-actin as a control (Fig. 2). Messenger RNAs (mRNA) of cyclin D2 and C increased gradually and reached a maximum level two- to threefold higher than the basal level. Their expression returned to the basal level at 12 h (Fig. 2). Importantly, the mRNA level of cyclins D3, E, and cdk2 sharply decreased and reached their lowest level within 4 h after stimulation. Thereafter, while cyclin E mRNA recovered rapidly, cyclin D3 recovered partially and cdk2 remained at a low level (Fig. 2). Cyclin D1 expression was not detected in this T cell hybridoma (Miyatake, S., and T. Saito, unpublished observation).

Cyclin D3 Protein Decreased after Stimulation through TCR. To determine whether the protein levels of these genes correlate with the amounts of mRNAs, cyclin D2, D3, cdk2, and cdk4 proteins were immunoprecipitated from the cell lysates of synchronized and stimulated DO11.10 cells and detected by immunoblot analysis. The cell cycle was synchronized by double-thymidine block procedure to obtain clear results for these proteins (Fig. 3 *A*). At 2 h after release from the G1 block (time 0 h), ~90% of the cells were in mid-S phase, and half of the cells were stimulated with anti-TCR mAb and harvested at various times after stimulation. After 3 h (time 3 h), ~35% of nonstimulated cells were in S phase, whereas 65% of stimulated cells were still in S phase. After 6 h (time 6 h), nonstimulated cells were not left in G2/M phase, whereas ~10% of stimulated cells were in G2/M phase. Overall, the cell cycle became slower after stimulation but, nonetheless, was not arrested until the cells entered G1 phase (Fig. 3 *A*).

The protein level of cyclin D3, which was found to remain constant throughout the cell cycle, was reduced to approximately one-sixth at both 3 and 6 h after TCR stimulation (Fig. 3 *B*). The level of cyclin D2 protein, which does not fluctuate throughout the cell cycle, gradually increased after stimulation.

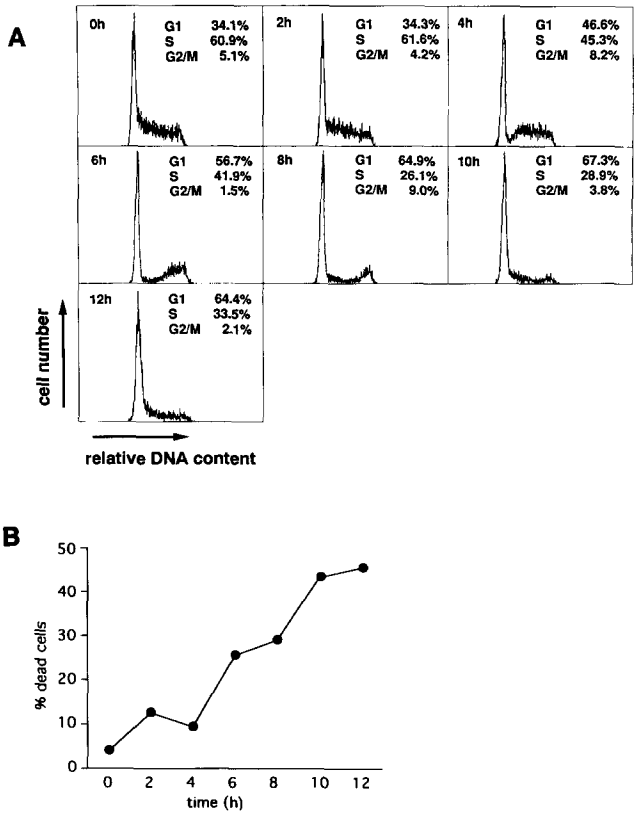

Figure 1. Cell cycle arrest and cell death induced upon T cell activation. (*A*) Cell cycle arrest of DO11.10 hybridomas upon activation. DO11.10 cells were stimulated by immobilized F23.1 mAb, stained with PI at the indicated times after stimulation, and subjected to cell cycle analysis by FACScan®. (*B*) Kinetics of cell death of DO11.10 cells upon stimulation. The number of PI-stained cells, i.e., dead cells, was determined by FACS® analysis. All results represent the analysis of 10^4 cells.

Fig. 3 *B* also shows that the changes of the protein level of both cdk2 and cdk4 were minimal compared with those of cyclin D2 and D3 proteins. The cyclin D3–cdk4 complex was then analyzed by immunoprecipitation with anti-cyclin D3 mAb followed by immunoblot with anti-cdk4 antiserum. As shown in Fig. 3 *C*, the amount of cdk4 protein within the complex decreased to the same extent as that of cyclin D3 upon stimulation, indicating that the complex formation of cyclin D3 and cdk4 was not altered by TCR stimulation, and the amount of the cyclin D3–cdk4 complex was determined by the level of cyclin D3.

Stimulation through TCR Inhibits Cyclin D3–associated Kinase Activity. GST-Rb protein has been reported to be a specific substrate for detecting D-type cyclin-associated kinases such as cdk4 and cdk6 (15–17, 21). We used this system to analyze cyclin D3–associated kinase activity. DO11.10 cells were synchronized and stimulated 2 h later when most cells were in

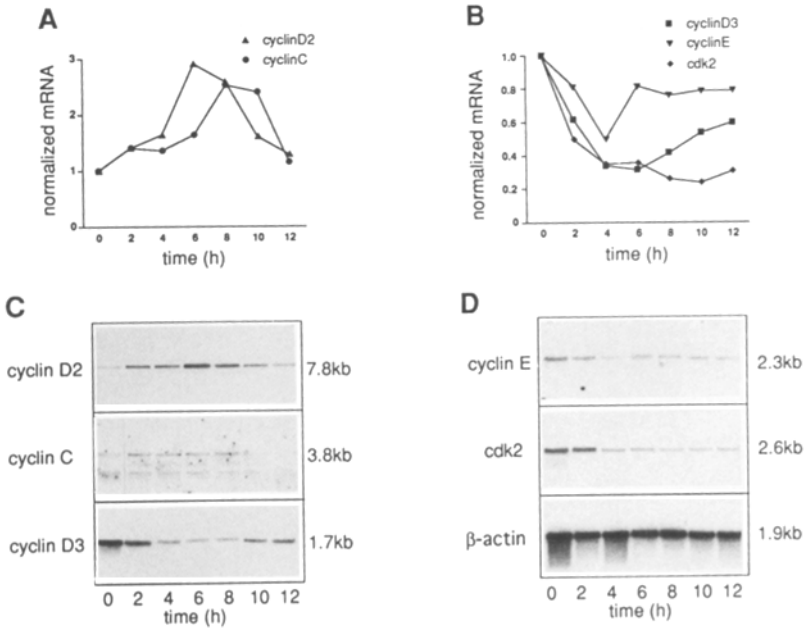

Figure 2. Expression of transcripts of G1 cyclins and cdk2 in DO11.10 hybridoma cells upon TCR stimulation. Total RNAs from DO11.10 cells at the indicated periods after stimulation with immobilized F23.1 mAb were analyzed with probes corresponding to cyclin C, cyclin D2, cyclin D3 (*C*), cyclin E, cdk2, and β-actin (*D*). The radioactivity of each band was quantitated and normalized to that of β-actin (*A*, *B*).

S phase (time 0 h). Cell lysates were prepared from unstimulated cells (time 0 h) and stimulated cells 6 h later (time 6 h). A significant level of cyclin D3–associated kinase activity was detected in nonstimulated cells, which was reduced to <10% after TCR stimulation (Fig. 4). Since cyclin D3 decreased to one-sixth of the original level, inhibition of cyclin D3–associated kinase activity is mostly attributable to the reduction of cyclin D3 expression. However, the kinase activity was impaired more severely in comparison with the reduction of the cyclin D3 level.

Although cyclin D2 protein was detected in the DO11.10 cell line, there was very little cyclin D2–associated kinase activity. cdk2 and cdk4 kinase activities were also measured. cdk2 had a significant level of kinase activity, while cdk4 kinase activity was lower than that of the cyclin D3 complex in unstimulated cells. Both kinase activities were inhibited to ~40% upon stimulation (Fig. 4).

Constitutive Expression of Cyclin D3 Altered T Cells to Be Resistant to Growth Arrest. To prove the hypothesis that the level of cyclin D3 and D3-associated kinase activity controls

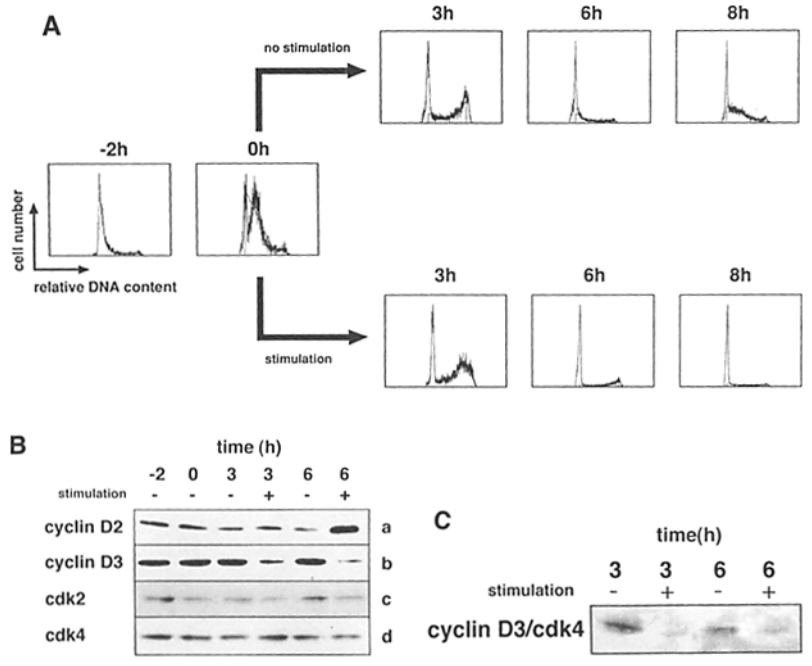

Figure 3. Analysis of protein expression of cyclin D2, cyclin D3, cdk2, and cdk4 protein as well as cyclin D3–cdk4 complex in synchronized DO11.10 cells upon TCR stimulation. (*A*) Cell cycle analysis of synchronized DO11.10 hybridomas. DO11.10 cells were synchronized as described in Materials and Methods. Cells were stimulated (or not) with immobilized F23.1 mAb 2 h after synchronization. At the indicated times, cell cycle analysis as well as protein analysis were performed. (*B*) Protein level of cyclin D2 (*a*), D3 (*b*), cdk2 (*c*), and cdk4 (*d*) in DO11.10 cells upon TCR stimulation. The lysates were prepared from the synchronized and stimulated cells at indicated time points as in *A*. For cyclin D2 and D3, mAbs were used for both precipitation and immunoblots. For cdk2 and cdk4, the lysates were blotted with rabbit anti–human cdk2 antiserum and mouse anti-cdk4 mAb. (*C*) Protein analysis of the cyclin D3–cdk4 complex. The same blot for cyclin D3 (lane *b* in *B*) was reblotted with anti–mouse cdk4 antiserum.

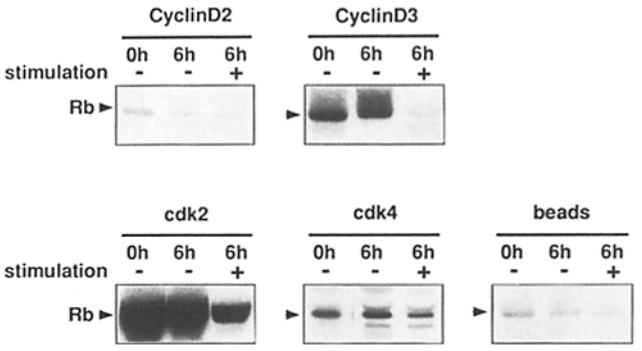

Figure 4. Activity of cyclin D2- and cyclin D3–associated kinases as well as cdk2 and cdk4. Cell lysates of DO11.10 cells stimulated for 8 h with F23.1 were immunoprecipitated with mAb (cyclin D2 and D3), antiserum (cdk2 and cdk4), or without Ab (beads), and the kinase activity of the immune complex was analyzed using GST-Rb as a substrate. In all experiments, lysates equivalent to 1×10^6 cells were loaded per lane. The radioactivity incorporated in the Rb band of SDS-PAGE was quantitated by an image analyzer.

the cell cycle progression in T cells, stable transfectants constitutively expressing cyclin D3 were established.

Expressible construct of mouse cyclin D3 was prepared in a plasmid vector pMKITneo containing SRα promoter and introduced into DO11.10 cells. More than 10 G418-resistant clones were isolated, and the expression of the introduced genes was analyzed by Northern blot, immunoblot, and kinase assay (Fig. 5, *A–C*). Two representative clones, DOCyD3-3 (clone 3) and DOCyD3-18 (clone 18), were described here. We could not obtain any transfectants expressing higher levels of mRNA and cyclin D3 protein than the endogenous one (Fig. 5, *A* and *B*). While the protein level of cyclin D3 decreased upon stimulation in parental DO11.10 cells, that in the two clones was rather enhanced upon stimulation. Consequently, these stimulated transfectants expressed a higher level of cyclin D3 protein than the stimulated parental cell line, and this resulted in the enhancement of cyclin D3–associated kinase activity in the transfectants (Fig. 5, *B* and *C*). cdk2 kinase activity was also increased in these transfectants (Fig. 5 *D*).

The cell cycle of the cyclin D3 transfectants upon stimulation was analyzed by flow cytometry (Fig. 5 *E*). As described above, G1 arrest was induced, and the percentage of cells in S phase decreased to <30% within 8 h after stimulation in parental DO11.10 cells. In contrast, cyclin D3 transfectants revealed significant recovery from the arrest. At 8 h after stimulation, the percentage of cells in S phase only decreased to ~50% in the two clones (Fig. 5 *E*). Furthermore, growth inhibition detected by [^{3}H]thymidine incorporation after stimulation was significantly suppressed in the transfectants (Fig. 5 *F*). These data strongly suggest that activation-induced growth arrest at G1 to S transition was suppressed by the ectopic expression of cyclin D3. Doubling time and cell size were not affected by the enforced expression of cyclin D3 in T cell hybridoma cells.

Discussion

The activation signal upon TCR stimulation induces cell cycle arrest at G1 phase but not in other stages of the cell cycle of T cell hybridomas, as reported before (1). This was clearly demonstrated when the cell cycle of T cell hybridomas was synchronized and the cells were stimulated during S phase. T cells continued the cell cycle until entering G1 phase, where they stopped its progression. Furthermore, induction of ADCD was observed after the cell cycle was arrested at G1 phase, suggesting that ADCD occurs at G1 phase.

The induction of growth arrest is accompanied by the rapid reduction of the mRNA level of three genes: cyclin E, cyclin D3, and cdk2. The reduction of cyclin E mRNA was small and recovered rapidly. The expression of cdk2 protein was minimally changed upon stimulation in spite of the reduction of the transcript. By contrast, cyclin D3 protein decreased in parallel with mRNA after stimulation. Furthermore, cyclin D3–associated kinase activity also decreased. In cyclin D3–expressing transfectants, the expression of cyclin D3 protein increased upon stimulation, resulting in the elevated level of cyclin D3–associated kinase activity. Subsequently, the transfectants gained resistance to the induction of G1 arrest upon TCR stimulation. Together, these results indicate that the cyclin D3–associated kinase activity is controlled by the expression level of cyclin D3, that this regulation seems to be critical for T cells to proceed from G1 to S phase, and that TCR stimulation induces growth inhibition by down-regulating the expression of cyclin D3 protein.

One of the mechanisms of TGF-β to induce G1 arrest in epithelial cells is the down-regulation of cdk4 rather than D-type cyclins (28). Therefore, the target of the antiproliferative signal induced through TCR is different from that of TGF-β. The other mechanism of TGF-β–induced G1 arrest is the activation of inhibitors for the kinase activity of cdk's (27–33). The inhibitor activity was demonstrated when the cell lysate of TGF-β–treated cells was mixed with that of proliferating cells. In our system, the cyclin D3–associated kinase activity was more severely impaired upon stimulation as compared with the reduction of the cyclin D3 protein, suggesting that additional mechanisms may operate to suppress the kinase activity. In cyclin D3 transfectants, the cyclin D3 protein level was increased upon stimulation, but the kinase activity in these transfectants was still slightly reduced, and weak inhibition of the cell cycle progression was observed. Taken together, these results indicate that cyclin D3–associated kinase activity is inhibited not only by the reduction of cyclin D3 protein but also by additional mechanisms such as the suppression by inhibitors. We analyzed the expression of mouse p21 (Cip1/WAF1/Sdi1) and p27 (Kip1) on RNA blots. A low level of p21 mRNA was detected, and it was slightly decreased upon stimulation. On the other hand, the p27 mRNA was increased by threefold upon stimulation through TCR (Miyatake, S., and T. Saito, unpublished observation), suggesting that p27 may contribute to the inhibition of cyclin D3–associated kinase activity in T cell hybridoma DO11.10.

The kinase activity of cdk4, a major partner of D-type

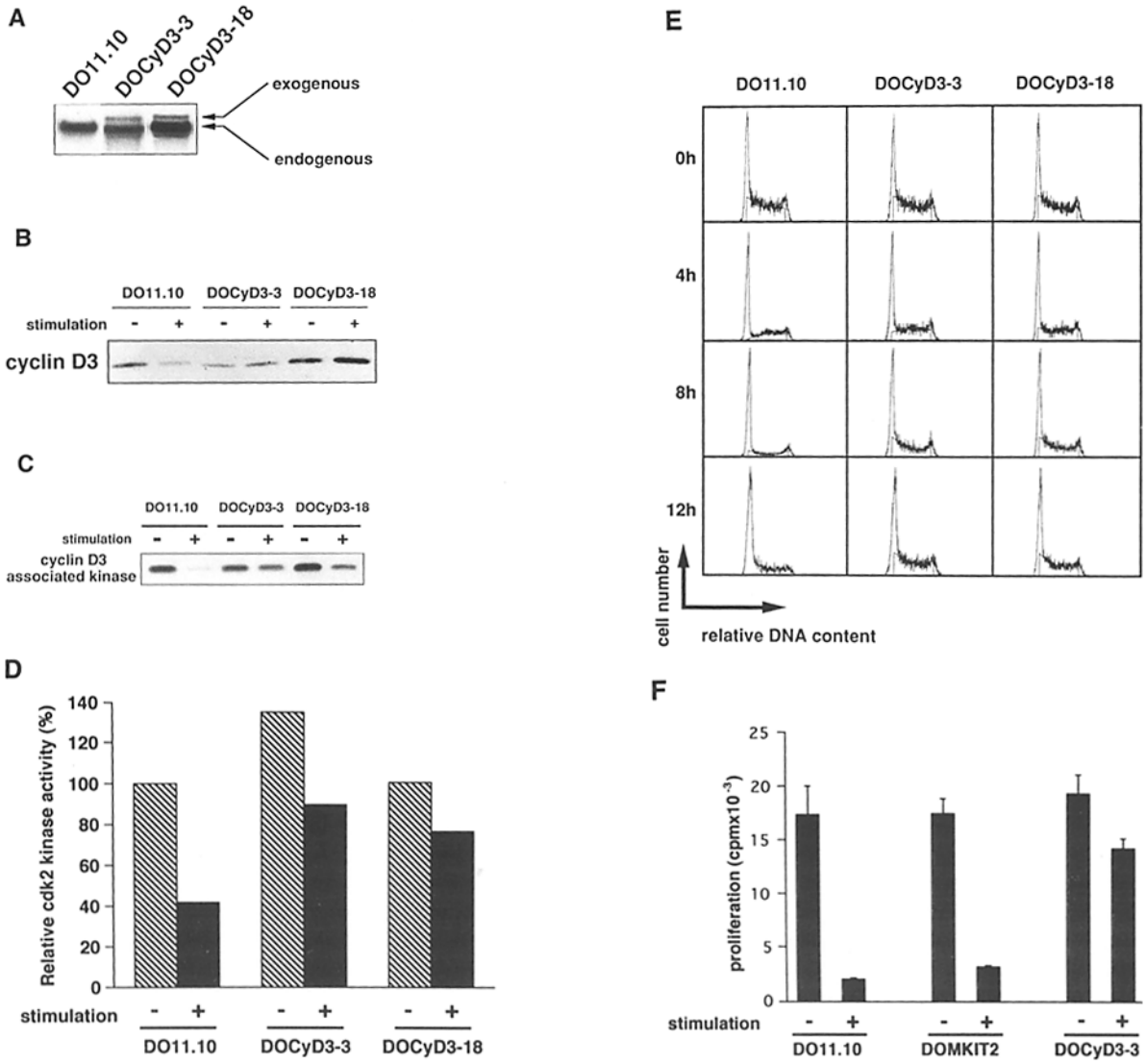

Figure 5. Effects of constitutive expression of cyclin D3 on the activation-induced cell cycle arrest in T cell hybridomas. (*A*) Expression of cyclin D3 mRNA in DO11.10 and cyclin D3 transfectants (DOCyD3-3 and -18). (*B*) Protein level of cyclin D3 in stimulated or unstimulated DO11.10 cells and cyclin D3 transfectants. (*C*) Activity of cyclin D3–associated kinase in stimulated or unstimulated DO11.10 cells and cyclin D3 transfectants. (*D*) Activity of cdk2 in stimulated or unstimulated DO11.10 cells and cyclin D3–transfectants. Results of cdk2 kinase activity were quantitated with an image analyzer and plotted relative to the amount of kinase activity recovered from immunoprecipitates from unstimulated parental DO11.10 cells. (*E*) Cell cycle analysis of DO11.10 and two cyclin D3 transfectants upon TCR stimulation. (*F*) Proliferation of DO11.10, a pMKITneo vector transfectant (DOKIT-2), and a cyclin D3 transfectant (DOCyD3-3). T cells were stimulated for 10 h and pulsed with [^{3}H]thymidine for 1 h. The results were expressed as the mean ± SD of the triplicate culture.

cyclin in various cell types, was not as strong as the cyclin D3–associated kinase activity in unstimulated cells. After stimulation, cdk4 kinase activity and cyclin D3–associated kinase activity were suppressed to 40 and <10%, respectively. cdk4 protein in the complex with cyclin D3 changed in parallel with cyclin D3 protein, indicating that the complex formation of cdk4 and cyclin D3 was not affected by the signal through TCR. These results suggest that cyclin D3 in T cell hybridomas is associated not only with cdk4 but also with other kinase(s), presumably including cdk6, since cdk6 has recently been reported as an important partner of D-type cyclins in T cells (17). It is possible that the signal through TCR may affect the association of cyclin D3 and these kinases.

cdk2 kinase activity was very strong in comparison with that of cyclin D3–associated kinase or cdk4. cdk2 kinase activity decreased to ~40% of that of nonstimulated cells. In cyclin D3 transfectants, cdk2 kinase activity was elevated in stimulated cells, suggesting that cyclin D3–associated kinase activity controls cdk2 kinase activity.

Cyclin D2 has been shown to possess activities similar to those of cyclin D3, such as the activation of cdk2 or cdk4 in insect cells (21, 24), inhibition of pRb function to induce growth arrest in an osteosarcoma cell line (22–24), and inhibition of granulocyte differentiation (47). In contrast with cyclin D3, cyclin D2 mRNA and protein increased, although the kinase activity of the cyclin D2 complex was very low

and was not affected by TCR stimulation. Therefore, cyclin D3 but not D2 plays a crucial role in governing the transition from G1 to S phase in T cell hybridomas.

Activation-induced growth arrest followed by ADCD has been found to be induced even in normal T cells when they are proliferating in the presence of IL-2 (3, 4). The question of whether the cell cycle arrest in normal T cells and cloned T cell lines upon Ag stimulation is induced by the same mechanism as described here in T cell hybridomas is important for understanding the physiological regulation of T cell growth. Preliminary experiments indicated that the expression patterns of G1 cyclins and cdk2 genes in a cloned T cell line proliferating in the presence of IL-2 were almost the same as those in T cell hybridomas when the G1 arrest was induced upon activation through TCR. Furthermore, the reduction of cyclin D3 protein in the cloned T cell line after stimulation was more significant than in hybridomas (Miyatake, S., and T. Saito, unpublished observation). Therefore, we assume that, in normal T cells, the activation signal through TCR blocks the growth-stimulatory signal through the IL-2 receptor mainly by suppressing the cyclin D3 expression and its associated kinase activity.

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