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.

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 × 10⁵ 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 × 10⁶ 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 × 10⁶ 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), 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 pSP65Ⅲ-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 graciously received from Dr. E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA). The mouse cyclin D3 cDNA was cloned from a XZAP 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′-CAAAAGCTTGCNT-AAYTGGATGYTY and 5′-TCGAATTCARKTAKTTCATNGC3'. Mouse cyclin E cDNA was cloned from the same library with human cyclin E cDNA as a probe. The mouse cyclin E cloned 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 × 10⁶ 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 × 10⁶–1 × 10⁷ 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 dithiobitol [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 antimouse, 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 × 10⁶ 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 MgCl₂, 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, 10 mM PMSF, 1 mM NaF, 0.1 mM sodium orthovanadate, 20 μM ATP, 2 mM reduced glutathione, and 10 μCi γ[32P]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 EcoRI fragment of the mouse cyclin D3 into the EcoRI site of the pMKItneo vector containing SRα promoter and neo 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, 10⁶ DO11.10 cells were washed three 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 Na₂HPO₄, and 1.46 mM KH₂PO₄). 30 μg of linearized plasmid DNA was added to the cell suspension in a cuvette (Gene Pulser...
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 DOI1.10 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 DOI1.10 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 DOI1.10 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.

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. DOI1.10 cells were synchronized and stimulated 2 h later when most cells were in

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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.
S phase decreased to <30% within 8 h after stimulation in revealed significant recovery from the arrest. At 8 h after stimulation was analyzed by flow cytometry (Fig. 5 E). As described parental DO1.10 cells. In contrast, cyclin D3 transfectants inhibition detected by [3H]thymidine incorporation after above, G1 arrest was induced, and the percentage of cells in above stimulation was significantly suppressed in the transfectants (Fig. 5 D). These data strongly suggest that activation-induced 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 [3H]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.

We thank Drs. Y. Ishida, H. Ohno, and T. Aoe for helpful discussions, Ms. H. Ohno for preparing the manuscript, and Ms. C. Ogata for technical assistance. We are grateful to Drs. S. I. Reed for plasmids containing G1 cyclins, C. J. Sherr for plasmids carrying G1 cyclins and anti-cyclin D3 mAb, E. Harlow for plasmids containing cdk2 and cdk2, S. Sakifyama for the β-actin probe, and K. Maruyama for the pMKTneo vector.

This work was supported by grants-in-aid for scientific research and for cancer research from the Japanese Ministry of Education, Science, and Culture, and in part by the Uehara Memorial Foundation.

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Received for publication 16 November 1994 and in revised form 27 February 1995.

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