Cyclin D3 Regulates Proliferation and Apoptosis of Leukemic T Cell Lines*

Ger J. J. C. Boonen, Brigitte A. van Oirschot, Angela van Diepen, Wendy J. M. Mackus, Leo F. Verdonck, Gert Rijksen, and René H. Medema†

From the Jordan Laboratory for Hemato-oncology, Department of Hematology, University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, The Netherlands

Activation of the T cell receptor in leukemic T cell lines or T cell hybridomas causes growth inhibition. A similar growth inhibition is seen when protein kinase C is activated through addition of phorbol myristate acetate. This inhibition is due to an arrest of cell cycle progression in G1 combined with an induction of apoptosis. Here we have investigated the mechanism by which these stimuli induce inhibition of proliferation in Jurkat and H9 leukemic T cell lines. We show that expression of cyclin D3 is reduced by each of these stimuli, resulting in a concomitant reduction in cyclin D-associated kinase activity. This reduction in cyclin D3-expression is crucial to the observed G1 arrest, since ectopic expression of cyclin D3 can abrogate the G1 arrest seen with each of these stimuli. Moreover, ectopic expression of cyclin D3 also prevents the induction of programmed cell death by phorbol myristate acetate and T-cell receptor activation, leading us to conclude that cyclin D3 not only plays a crucial role in progression through the G1 phase, but is also involved in regulating apoptosis of T cells.

Activation of resting T lymphocytes requires ligation of the T cell receptor (TCR) and a co-stimulatory signal, provided by IL-2 or co-ligation of CD28 (reviewed in Ref. 1). The combination of these stimuli enables the resting lymphocytes to exit G0 and proliferate. Remarkably, stimulation of the same TCR in T cells that are actively proliferating results in cessation of proliferation and subsequent apoptosis (2–5). This particular response is evident in thymocytes, but has also been described in leukemic T cells and T cell hybridomas (2, 6–8). This indicates that the signals from the activated TCR complex can affect cell proliferation very differently, depending on the proliferative state of the T cell.

Cell cycle progression from G0 to S phase in normal T cells requires the induction of the D-type cyclins (9). These cyclins are not expressed in resting T cells, but are induced upon activation of the TCR complex (10). Three D-type cyclins have been identified, of which cyclins D2 and D3 are predominantly expressed in T cells (11, 12). Cyclin D2 and D3 can form an active kinase complex with the cyclin-dependent kinase (cdk) 4 or cdk6, and the resulting kinase complexes are involved in phosphorylation of the retinoblastoma protein (pRb) (11, 13). Phosphorylation of pRb results in its functional inactivation and allows progression of the cell through the late G1 restriction point and subsequent entry into S phase (reviewed in Ref. 14). However, cyclin D-cdk complexes are able to phosphorylate pRb only partially and complete phosphorylation and functional inactivation of pRb requires additional phosphorylation by cyclin E-cdk2 complexes (15). Although cyclin D- and cyclin E-cdk complexes seem to phosphorylate overlapping sites in pRb, it has been demonstrated that some sites, such as Ser780, are only phosphorylated by cyclin D-cdk complexes (16).

Induction of cyclin D expression alone is not sufficient to drive resting T cells into S phase. This is due to the fact that resting T cells express abundant amounts of the cdk inhibitor p27kip1, that associates to the formed cyclin D-cdk complexes and inhibits their kinase activity (17). Expression of p27kip1 is down-regulated as T cells progress through G1, but this process requires the presence of a co-stimulatory signal supplied by IL-2 (17, 18). Thus, only the combination of TCR activation and costimulation with IL-2 allows for the formation of active cyclin D-cdk complexes and initiation of DNA replication. Down-regulation of p27kip1 in normal T cells can be prevented by immunosuppressive agents such as rapamycin and cyclosporin, but also by elevation of intracellular cAMP levels (18, 19). As such these agents can prevent the appearance of active cyclin D-cdk complexes and suppress the proliferation of immunoreactive T cells.

As mentioned above, the effect of TCR activation in actively proliferating T cells is dramatically different from that observed in resting T cells. In proliferating T cells, including leukemic T cells, engagement of the TCR complex results in a cell cycle arrest in G1 and TCR antigen-induced cell death (AID) (2–8). Such T cell activation can be mimicked by directly activating one of the downstream signaling pathways of the TCR complex. For example, activation of protein kinase C by adding PMA, either alone or in combination with calcium ionophores can cause growth inhibition and apoptosis in Jurkat T cells (2, 20, 21). AID was recently shown to occur from a late G1 checkpoint in a Rb-dependent fashion (22). Consistent with this notion is the finding that cyclin D3 is down-regulated by activation of the TCR in a T cell hybridoma (23). However, the mechanism by which leukemic T cell lines arrest in G1 has not been elucidated. Therefore, we set out to investigate the effect of TCR activation on the expression and activity of a variety of cell cycle regulatory proteins known to be involved in progression through G1. In this report we show that TCR activation in Jurkat or H9 leukemic T cells results in a G1 arrest, associated with hypophosphorylated pRb. We show that expression of

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: Jordan Laboratory, Dept. of Hematology, University Hospital Utrecht, Rm. G03.647, P. O. Box 85500, 3508 GA Utrecht, The Netherlands. Tel.: 31-30-2506515; Fax: 31-30-2511893; E-mail: R.H.Medema@lab.azu.nl.
‡ The abbreviations used are: TCR, T cell receptor; IL, interleukin; cdk, cyclin-dependent kinase; PMA, phorbol myristate acetate; AID, antigen-induced cell death; pRb, retinoblastoma protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; mAb, monoclonal antibody.
cyclin D3 is down-regulated in these cells, resulting in loss of phosphorylation of the Ser-780 residue on pRb. Very similar effects are seen upon stimulation with PMA. More importantly, ectopic expression of cyclin D3 cannot only overcome the growth inhibition induced by these stimuli, but also prevents the induction of apoptosis in response to these stimuli. This indicates that down-regulation of cyclin D3 is crucial to both the proliferative arrest as well as the induction of apoptosis seen with these stimulatory agents.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Antibodies, and Reagents—**Jurkat (E6.1, ATCC) and H9 (a kind gift of Dr. J.P. Medema, Leiden University) leukemic T cells were routinely cultured in RPMI 1640 medium (Life Technologies, Inc., Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah), L-glutamine (4 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 5 mM β-mercaptoethanol. Anti-CD3 mAb OKT3 and anti-CD4 mAb OKT4 (isotype control) were purified from hybridoma culture supernatant using protein A-Sepharose columns, anti-CD3 mAB UCHT-1 and anti-μR (G3-245) were obtained from Pharmingen (Hamburg, Germany), anti-p27<sup>kip1</sup> (clone 57) from Transduction Laboratories (Lexington, KY), anti-cyclin D3 (Ab-2) from Calbiochem (San Diego, CA), anti-cdk2 (M2) and anti-cyclin E (HE-12 for immunoblotting, HE-111 for immunoprecipitation and in vitro kinase activity) were all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Ser780 was a kind gift of Dr. M. Kitagawa (16) and anti-cyclin D2 (DCS-5) (24) was a kind gift from Dr. J. Lukas (Danish Cancer Society, Copenhagen, Denmark). Propidium iodide was purchased from Sigma and protein A/G-Sepharose beads from Santa Cruz Biotechnology.

**Cell Stimulation—**For treatment with anti-CD3 mAbs or anti-CD4 mAb, tissue culture plates (96- or 12-well) were coated overnight at room temperature with 10 μg/ml anti-CD3 (OKT3 or UCHT-1) or 10 μg/ml anti-CD4 (OKT4), respectively, in phosphate-buffered saline (PBS). Control plates were treated overnight with PBS without any antibodies. The coating solution was aspirated the next day and plates were washed three times with PBS. Plates were then immediately used for cell stimulation. For stimulation with PMA, cells were plated in the presence of 50 nM PMA. All cells were cultured in flasks in fresh medium at a density of 2 × 10<sup>5</sup> cells/ml at 24 h prior to cell stimulation, to ensure that they were exponentially growing at the time of stimulation. Cells were then counted the next day and replated in the presence or absence of the different stimuli at 5 × 10<sup>5</sup> cells/ml.

**Cell Cycle Analysis and Cell Viability—**For analysis of cell cycle distribution, anti-CD3- or PMA-stimulated cells were collected at the indicated time points and washed with ice-cold PBS, after which they were fixed overnight in 70% ethanol at 4 °C. Cells were then pelleted by centrifugation and cell pellets were washed once with ice-cold PBS. Cell pellets were then lysed in ELB (1 mg/ml G418. Clonal lines were obtained by limiting dilution resulting in the isolation of JD3-1 and JD5-11.

**Cell Cycle Analysis** was determined after 48 h of stimulation with the indicated agents. The percentage of cell death was determined by propidium iodide staining of whole cells and FACS analysis. Error bars represent the standard error of the mean.

**Immunoprecipitations and in Vitro Kinase Reactions—**After stimulation, cells were washed once with ice-cold PBS and cell pellets were resuspended and lysed in ELB lysis buffer (25). Protein concentrations were determined and equal amounts of protein were used for immunoprecipitation with anti-cyclin E or anti-cdk2 antibodies. The immunoprecipitates were then used for in vitro kinase assays as described previously (25).

**Immunoblotting—**After proper stimulation, cells were harvested and precipitates were then used for immunoblotting.

**RESULTS**

**Inhibition of Cell Proliferation in Leukemic T Cell Lines—**Activation of the TCR causes inhibition of proliferation of a variety of leukemic T cell lines, as well as different T cell hybridomas (2–8). We set out to study the mechanism by which this growth inhibition occurs in leukemic T cell lines. To this end, Jurkat and H9 leukemic T cell lines were stimulated by engaging the TCR complex with immobilized anti-CD3 mAbs. Treatment of both cell lines with the anti-CD3 mAb UCHT-1 for 24 h resulted in marked inhibition of proliferation, as measured by [3H]thymidine incorporation (Fig. 1A). Growth inhibi-
tion was also observed with immobilized OKT3, another anti-CD3 mAb or phytohemagglutinin both known to efficiently stimulate signaling by the TCR complex (Fig. 1A). Stimulation with an immobilized, isotype-matched anti-CD4 mAb or immobilized anti-CD28 mAb had no effect on cell proliferation (Fig. 1A). Activation of protein kinase C, by the addition PMA, also resulted in efficient inhibition of cell proliferation (Fig. 1A).

Induction of Apoptosis by TCR Activation in Leukemic T Cells—Engagement of the TCR complex in leukemic T cells can result in apoptosis. Induction of apoptosis is also observed when the downstream signaling molecules protein kinase C and Ca2+-dependent calcineurin are directly activated through engagement of the TCR complex (Fig. 1A). Stimulation with anti-CD3 or PMA are not the result of a resistance of these cells to Fas-induced cell death.

Fig. 3. Effect of anti-CD3 mAb or PMA on Rb phosphorylation and cdk2 kinase activity. A, phosphorylation state of pRb in Jurkat (left panel) or H9 cells (right panel) after stimulation with UCHT-1 or PMA. B, kinase activity of cyclin E immunoprecipitates prepared from Jurkat cells after stimulation with UCHT-1 or PMA. Cells were cultured in 12-well tissue culture plates coated with 10 μg/ml anti-CD3 antibody UCHT-1 or in the presence of 50 ng/ml PMA for 24 h. Cells were then harvested and lysed. Total lysates were separated on SDS-PAGE gels for the analysis of Rb phosphorylation on Western blots. Arrows indicate the hyper- and hypophosphorylated forms of pRb. To determine cdk2 kinase activity, cyclin E or cdk2 were immunoprecipitated (IP) from total cell lysates and immune complexes were then used for in vitro kinase assays using histone H1 as substrate. The arrow indicates the 1P-phosphorylated histone H1. Percentage of remaining kinase activity (relative to the untreated control) is indicated below each lane.

Effects on Cell Cycle Regulatory Proteins—As mentioned above, a critical event during passage through G1 is the phosphorylation of pRb. To investigate whether the G1 arrest occurred early, or late in G1, we examined the phosphorylation state of pRb after treatment of cells with immobilized anti-CD3 mAb or PMA for different time periods. In untreated asynchronous cultures, pRb is mostly present in its slower migrating hyperphosphorylated form (Fig. 3A). After 24 h of stimulation with the anti-CD3 mAb UCHT-1 or PMA we observed a clear
Down-regulation of Cyclin D3 in Leukemic T Cell Lines

Fig. 4. Expression of cell cycle regulatory proteins after treatment with anti-CD3 mAb or PMA. After stimulation with the indicated agents, cells were lysed and total lysates were separated on SDS-PAGE gels to determine the expression levels of p27\(^{kip1}\) (A), cyclin D3 (B), cyclin D2 (C), cdk2 (D), and cyclin E (E) by Western blotting. Equal amounts of protein was loaded in each lane. Total lysate of U2OS cells was used as a positive control for cyclin D2 expression in panel C.

We also measured the kinase activity of cyclin E-ckd2 complexes that are normally induced late in G1 and are involved in phosphorylation of pRb. After 24 h with anti-CD3 mAbs or PMA, cyclin E-associated kinase activity was inhibited by 22 or 38%, respectively (Fig. 3B), indicating that these cells still contain a considerable amount of cyclin E-associated kinase activity after stimulation. Thus, it seems unlikely that the moderate inhibition of cyclin E-associated kinase activity is responsible for the observed growth inhibition. Similar reductions in kinase activity were observed using immunoprecipitates prepared with anti-ckd2 in vitro kinase reactions (Fig. 3C).

TCR Activation Induces Down-regulation of Cyclin D3—Activation of G1 cyclin-ckd complexes in normal T cells involves down-regulation of the ckdk-inhibitor p27\(^{kip1}\) (17, 18). Stimulation of expression of p27\(^{kip1}\) by TCR activation in leukemic T cells could result in inhibition of the kinase activity of cyclin E-ckd2 complexes. Therefore, we examined if TCR activation can induce expression of p27\(^{kip1}\) in Jurkat T cells. Whole cell lysates were prepared from control cells and from cells stimulated for 24 h with immobilized anti-CD3 mAb UCHT-1 or PMA. As shown in Fig. 4A, expression of p27\(^{kip1}\) did not change significantly upon activation of the T cells with anti-CD3 mAb or PMA, indicating that another mechanism must be responsible for the observed inhibition of cyclin/ckd activity in these leukemic cells.

We next decided to analyze the effect of TCR activation on expression of the G1 cyclins. In T cells the major D-type cyclins are D2 and D3, whereas D1 is not expressed (11, 12), and therefore we examined expression of cyclins D2, D3, and cyclin E. Cells were stimulated with immobilized anti-CD3 mAb or PMA for 24 h, since pRb was efficiently dephosphorylated at this time point (Fig. 3A). Expression of cyclin D3 was dramatically reduced after 24 h in the presence of PMA or immobilized anti-CD3 mAb in Jurkat cells (Fig. 4B), suggesting that a reduction in cyclin D3 expression could be responsible for the observed G1 arrest. Cyclin D2 could not be detected in lysates from Jurkat (Fig. 4C) or H9 cells (not shown), in contrast to a control lysate from U2OS osteosarcoma cells shown previously to express cyclin D2 (24). This indicates that cyclin D3 is the major D-type cyclin expressed in these leukemic T cell lines. Expression of cyclin E or its kinase partner cdk2 was unaltered upon stimulation with anti-CD3 mAb or PMA (Fig. 4, D and E), demonstrating that regulation of cyclin E or cdk2 expression is not involved in the inhibition of cyclin E/ckd2 kinase activity.

In order to determine the timing of cyclin D3 down-regulation, we performed a time course experiment and total lysates were prepared for analysis of cyclin D3 expression levels. The timing of cyclin D3 down-regulation appears to be similar after stimulation with PMA or immobilized anti-CD3 mAbs. The reduction in cyclin D3 expression is first visible after 8 h of stimulation and continues to decline up to 24 h (Fig. 5A).

We next wanted to show that the reduction in cyclin D3 expression results in loss of cyclin D-associated kinase activity from the cell. Recently, it was shown that theSer\(^{780}\) residue in pRb is phosphorylated by cyclin D-ckd2 complexes, but not by cyclin E- or cyclin A-ckd2 complexes (16). Phosphorylation of Ser\(^{780}\) is therefore a good measure of cyclin D-ckd kinase activity without interference of the activity of other G1 cyclin-ckd complexes. Thus, we decided to study the phosphorylation state

FIG. 5. Kinetics of cyclin D3 down-regulation and phosphorylation of the serine 780 residue on pRb after stimulation with anti-CD3 mAb or PMA. A, Jurkat T cells were cultured in a 12-well tissue culture plate coated with 10 \(\mu\)g/ml anti-CD3 antibody UCHT-1 or in the presence of 50 ng/ml PMA for the indicated times. Cells were then harvested at the indicated time points and equal amounts of protein were used to determine the expression of cyclin D3. B, phosphorylation of serine 780 of pRb after stimulation with UCHT-1 or PMA for 24 h was determined by probing Western blots of total cell lysates with a phospho-specific antibody directed against Ser\(^{780}\)-phosphorylated pRb (16). C, kinetics of Ser\(^{780}\)-dephosphorylation of pRb in response to anti-CD3 stimulation. Cells were grown on plates coated with UCHT-1 for the indicated times and total cell lysates were probed on Western blot for Ser\(^{780}\)-phosphorylated pRb.
of the Ser<sup>780</sup> residue of pRb after stimulation with PMA or anti-CD3 mAbs. For this purpose we made use of an antibody recognizing only pRb phosphorylated on Ser<sup>780</sup> (anti-phospho-Ser<sup>780</sup>) (16). As shown in Fig. 5B, treatment of Jurkat cells with PMA or immobilized UCHT-1 for 24 h resulted in loss of phosphorylation of pRb-Ser<sup>780</sup>. This clearly demonstrates that the reduction in cyclin D3 expression results in loss of cyclin D3-associated kinase activity from the cell. Moreover, Ser<sup>780</sup> phosphorylation was reduced as early as 12 h after stimulation with UCHT-1, indicating that the reduction in cyclin D3-associated kinase activity closely follows the decrease in cyclin D3 expression (Fig. 5C).

**Ectopic Expression of Cyclin D3 Overcomes the Proliferative Arrest and Blocks Apoptosis**—To determine if down-regulation of cyclin D3 is crucial to the proliferative arrest seen in response to PMA and anti-CD3 mAbs, we analyzed the effect of ectopic expression of cyclin D3 in Jurkat T cells. For this purpose, Jurkat cells were transiently transfected with an expression vector for cyclin D3. To allow analysis of the transfected population we co-transfected a plasmid encoding a modified version of the green fluorescent protein (GFP) (26). Using bivariate flow cytometry we could then analyze the cell cycle distribution of the GFP-positive population (26). Transfection of an empty vector control did not affect the growth arrest induced by anti-CD3 mAbs or PMA, as can be seen from the increase in the percentage of cells in the G<sub>1</sub> phase after treatment with these agents (Fig. 6A). However, transfection of the cyclin D3 expression plasmid resulted in an efficient rescue of the cell cycle arrest that normally occurs in response to treatment with anti-CD3 mAbs or PMA (Fig. 6A), indicating that down-regulation of cyclin D3 is instrumental to the observed growth inhibition.

To further corroborate these data, we established cell lines of Jurkat T cells stably overexpressing cyclin D3. We isolated two independent clones, JD3.I and JD3.II, and these lines were tested for growth inhibition by anti-CD3 mAbs and PMA. As can be seen in Fig. 6B, growth inhibition of JD3.I cells by UCHT-1 was reduced approximately 2-fold, and the response to PMA was almost completely abolished. The effect of cyclin D3 overexpression was even more pronounced in the JD3.II clone which expressed slightly higher levels of cyclin D3 (not shown). JD3.II cells were hardly inhibited in their growth by TCR activation or PMA treatment. To confirm that cyclin D3 was indeed constitutively expressed in these cell lines, we analyzed cyclin D3 expression on Western blots. Whereas cyclin D3 is efficiently down-regulated in the parental Jurkat cell line, no effect on cyclin D3 expression by anti-CD3 mAbs or PMA is seen in JD3.I (data not shown) or JD3.II (Fig. 6C). Thus cyclin D3 can no longer be down-regulated in these cell lines by TCR activation and as a consequence no growth inhibition is observed. We then wanted to know if this rescue from growth inhibition had any influence on the apoptotic program induced by these stimuli. Interestingly, cell viability was no longer decreased in these cell lines after treatment with anti-CD3 mAbs or PMA (Fig. 6D). We confirmed this by analysis of DNA profiles of the JD3.I and JD3.II cell lines after stimulation with UCHT-1 or PMA. Only a very small increase (~6%) in the G<sub>1</sub> percentage was seen in these cells after stimulation with UCHT-1 (Fig. 7), compared with an increase of 21% seen in normal Jurkat (see Fig. 2). The effect of PMA appeared to be completely abolished in JD3.I as well as JD3.II (Fig. 7). Moreover, very little (1%) apoptotic cells were detected, confirming the data on cell viability obtained with these same cell lines. These data indicate that in addition to the growth arrest, AID also requires the down-regulation of cyclin D3.

**DISCUSSION**

In this report we have addressed the mechanism by which TCR activation induces a G<sub>1</sub> arrest in leukemic T cells. T cell activation was performed by stimulation with immobilized anti-CD3 mAbs. Under our experimental conditions, treatment with anti-CD3 mAbs resulted in a G<sub>1</sub> arrest, and a reduction in...
Down-regulation of Cyclin D3 in Leukemic T Cell Lines

To investigate the importance of cyclin D3 down-regulation to the observed growth inhibition, we studied the effects of ectopic expression of cyclin D3. We show that overexpression of cyclin D3, both transiently as well as in stable cell lines, is sufficient to override the cell cycle arrest induced by TCR activation, indicating that the growth inhibition requires down-regulation of cyclin D3. In addition, ectopic expression of cyclin D3 prevents the apoptosis induced by stimulation with anti-CD3 mAbs or PMA. These data implicate that the down-regulation of cyclin D3 is not only crucial to the growth arrest, but also for apoptosis induced by TCR activation. This would suggest that cyclin D3 integrates signals that regulate the balance between proliferation and apoptosis of T cells. Since D-type cyclins are required for inactivation of pRb and it is known that cells lacking functional pRb no longer require cyclin D-associated kinase activity, we would expect that T cells that lack functional pRb are not inhibited in their growth by TCR activation. Indeed, Lissy et al. (22) recently reported that Jurkat T cells can be rescued from AID when pRb is functionally inactivated through introduction of the HPV E7 protein. They showed that AID occurs from a late G1 phase cell cycle checkpoint. Our data provide further insight into the underlying mechanism and suggest that this checkpoint is activated through down-regulation of cyclin D3.

Expression of the D-type cyclins is low in cells in G0, such as resting T cells (10). Upon stimulation of G0 cells with the proper mitogens, expression of cyclin D is induced, and this remains high as long as the mitogens are present (9). In normal peripheral T cells, induction of cyclin D3 expression requires TCR activation and this is further stimulated after addition of IL-2 (31). However, leukemic cells, such as the Jurkat cells used in this study, no longer depend on TCR activation or IL-2 for their proliferation and express constitutively high levels of cyclin D3. Remarkably, our data show that TCR activation has the opposite effect on cyclin D3 expression from that observed in normal peripheral T cells. Future experiments are called for to elucidate the mechanism by which TCR activation can cause the reduction in cyclin D3 levels. It will be of significant interest to learn why this mechanism only exists in actively proliferating T cells and how the effect of TCR activation on cyclin D3 expression can be so radically different in resting T cells. This could help us understand how control of cellular proliferation in leukemic T cells is different from normal T cells and lead to more insight into potential treatments of such malignancies.

Acknowledgments—We thank Dr. Jiri Lukas (Danish Cancer Society, Copenhagen, Denmark) for the cyclin D2 antibodies, Dr. Masatoshi Kitagawa (Medical Institute of Bioregulation, Kyushu University, Japan) for the phosphospecific antibody recognizing Ser780-phosphorylated pRb, and Dr. Eric Lam (Ludwig Institute, London, UK) for helpful discussions. Also, we thank the other members of the Jordan Laboratory for helpful discussions.

REFERENCES

1. Lenschow, D. J., Walunas, T. L., and Bluestone, J. A. (1996) Annu. Rev. Immunol. 14, 293–258
2. Smith, C. A., Wilkins, G. I., Kingston, R., Jenkinson, E. J., and Owen, J. J. T. (1989) Nature 337, 181–183
3. Jones, L. A., Chiu, L. T., Lengy, D. L., and Kruisbeek, A. M. (1990) Science 250, 1726–1729
4. Lenardo, M. J. (1991) Nature 353, 858–861
5. Rocha, B., and von Boehmer, H. (1991) Science 251, 1225–1228
6. Ashwell, J. D., Cunningham, R. E., Noguchi, P. D., and Hernandez, D. (1987) J. Exp. Med. 165, 173–194
7. Meroep, M. J., Bluestone, J. A., Noguchi, P. D., and Ashwell, J. D. (1988) J. Immunol. 140, 324–330
8. Takahashi, S., Maeccker, H. T., and Levy, R. (1989) Eur. J. Immunol. 19, 1911–1919
9. Andj, K., Aichenaum-Cymbalista, F., and Griffin, J. D. (1993) Proc. Natl. Acad. Sci. USA 90, 6472–6476

FIG. 7. Cell cycle distribution and apoptosis of JD3.I and JD3.II cells after stimulation with anti-CD3 mAb or PMA. JD3.I cells (top panels) and JD3.II cells (lower panels) were cultured (2 × 10^5 cells/well) in 12-well tissue culture plates coated with 10 μg/ml anti-CD3 antibody UCHT-1 (middle panels) or in the presence of 50 ng/ml PMA (right panels), or left unstimulated (left panels). Stimulations were performed for 24 h. Cells were harvested, fixed in ethanol, and stained with propidium iodide. Cell cycle profiles were analyzed by flow cytometry and percentages of cells in the sub-G1 (apoptotic cells), G1, S, and G2/M phase of the cell cycle are indicated below each histogram. 

Cell viability. In parallel, cells were stimulated with the phorbol ester PMA, providing a somewhat more efficient stimulus. Activation of the same leukemic T cell lines with PMA induced a similar arrest in G1 and a similar reduction in cell viability.

The G1 arrest induced by anti-CD3 mAbs and PMA was associated with the appearance of dephosphorylated pRb, indicating an arrest at, or prior to, the G1 restriction point. In agreement with this notion was the finding that the kinase activity of cyclin E and cdk2 complexes was reduced after TCR activation. In order to address the mechanism by which the reduction in kinase activity of these complexes occurs, we analyzed expression of the various G1 cyclins. Expression of cyclin E is usually induced somewhere late in G1 as a function of cell cycle progression. No reduction in the expression level of cyclin E could be detected after treatment with anti-CD3 mAbs, indicating that the inhibition of cyclin E-associated kinase occurs through active repression of the cyclin-cdk complex. Further analysis of expression of G1 cyclins showed that cyclin D2 is not expressed in Jurkat or H9 cells and that the expression of cyclin D3 was reduced significantly after treatment with anti-CD3 mAbs or PMA. At present we do not know whether the reduction in cyclin D3 expression occurs through active transcriptional repression or by a post-translational mechanism affecting protein stability of cyclin D3. The decrease in cyclin D3 expression resulted in a drop in the kinase activity of cyclin D-cdk complexes, as evidenced by the reduction in Ser780 phosphorylation of pRb seen after stimulation with PMA or anti-CD3 mAbs. Since p27kip1 was associated with some of these complexes the reduction in cyclin D3 levels will cause a rise in the amount of p27kip1 available to associate with cyclin E-cdk complexes. Therefore, the decrease in cyclin D3 expression might be indirectly responsible for the inhibition of cyclin E-cdk2 complexes.
10. Ajchenbaum, F., Ando, K., DeCaprio, J. A., and Griffin, J. D. (1993) J. Biol. Chem. 268, 4113–4119
11. Meyerson, M., and Harlow, E. (1994) Mol. Cell. Biol. 14, 2977–2986
12. Tam, S. W., Theodoras, A. M., Shay, J. W., Draetta, G. F., and Pagano, M. (1994) Oncogene 9, 2663–2674
13. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J., and Kato, J-Y. (1994) Mol. Cell. Biol. 14, 2066–2076
14. Weinberg, R. A. (1995) Cell 81, 323–330
15. Lundberg, A. S., and Weinberg, R. A. (1998) Mol. Cell. Biol. 18, 753–761
16. Kitagawa, M., Higashi, H., Jung, H-K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J-Y., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. (1996) EMBO J. 15, 7060–7069
17. Firpo, E. J., Koff, A., Solomon, M. J., and Roberts, J. M. (1994) Mol. Cell. Biol. 14, 4889–4901
18. Nurse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M-H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994) Nature 372, 570–573
19. Kato, J. M., Matsuo, K., Polyak, K., Massague, J., and Sherr, C. J. (1994) Cell 79, 487–496
20. Wyllie, A. H., Morris, R. G., Smith, A. L., and Dunlop, D. (1984) J. Pathol. 142, 67–77
21. Shi, Y., Bironnette, R. P., Padrey, N., Szalay, M., Kubo, R. T., and Green, D. R. (1991) J. Immunol. 146, 3340–3346
22. Lissy, N. A., Van Dyk, L. F., Becker-Hapak, M., Vocera-Akbani, A., Mendler, J. H., and Dowdy S. F. (1998) Immunity 8, 57–65
23. Miyatake, S., Nakano, H., Park, S. Y., Yamazaki, T., Takase, K., Matsushima, H., Kato, A., and Saito, T. (1995) J. Exp. Med. 182, 401–408
24. Lukas, J., Bartkova, J., Welcker, M., Petersen, O. W., Peters, G., Strauss, M., and Bartek, J. (1995) Oncogene 16, 2125–2134
25. Medema, R. H., Klompmaier, E., Smits, V. A. J., and Rijksen, G. (1998) Oncogene 16, 431–441
26. Kaelja, R. F., Shenk, T., and Bearvis, A. J. (1997) Cytometry 29, 286–291
27. Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 91, 6289–6293
28. Zhu, L., and Anasetti, C. (1995) J. Immunol. 154, 192–200
29. Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G., and Bartek, J. (1995) Nature 375, 596–596
30. Koh, J., Enders, G. H., Dynlacht, B. D., and Harlow, E. (1995) Nature 375, 596–596
31. Boonen, G. J. C., van Dijk, A. M. C., Verdonck, L. F., van Lier, R. A. W., Rijksen, G., and Medema, R. H. (1999) Eur. J. Immunol. 29, 789–798
