Protein Kinase A Regulates Expression of p27kip1 and Cyclin D3 to Suppress Proliferation of Leukemic T Cell Lines*

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Brigit A. van Oirschot†, Marie Stahl§, Susanne M. A. Lens§, and René H. Medema§†

From the †Jordan Laboratory for Hemato-Oncology, G03-647, Utrecht Medical Center, Heidelberglaan 100, 3584 CX Utrecht and the ‡Department of Molecular Biology H8, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Peripheral homeostasis and tolerance requires the suppression or removal of excessive or harmful T lymphocytes. This can occur either by apoptosis through active antigen-induced death or cytokine withdrawal. Alternatively, T cell activation can be suppressed by agents that activate the cAMP-dependent protein kinase (PKA) signaling pathway, such as prostaglandin E2. Stimulation of PKA inhibits lymphocyte proliferation and immune effector functions. Here we have investigated the mechanism by which activation of PKA induces inhibition of proliferation in human leukemic T cell lines. Using a variety of agents that stimulate PKA, we can arrest Jurkat and H9 leukemic T cells in the G1 phase of the cell cycle, whereas cell viability is hardly affected. This G1 arrest is associated with an inhibition of cyclin D/Cdk and cyclin E/Cdk kinase activity. Interestingly, expression of cyclin D3 is rapidly reduced by PKA activation, whereas expression of the Cdk inhibitor p27kip1 is induced. Ectopic expression of cyclin D3 can override the growth suppression induced by PKA activation to some extent, indicating that growth inhibition of leukemic T cells by PKA activation is partially dependent on down-regulation of cyclin D3 expression. Taken together our data suggest that immunosuppression by protein kinase A involves regulation of both cyclin D3 and p27kip1 expression.

Exposure to an antigen elicits a dramatic expansion of resting peripheral T lymphocytes that specifically recognize and target this antigen. However, the proliferative capacity and effector functions of the evolving T cell population need to be kept in check in order to ensure a restricted reactivity and protect the host from an uncontrolled immune response. To this end, several mechanisms have evolved that can cause deletion or suppression of reactive mature T cells. One mechanism involves apoptosis of the activated T cell population that can occur either via restimulation of the T cell receptor (TCR) with the appropriate antigen or through cytokine deprivation (1). Another mechanism involves suppression of T cell proliferation and effector functions by glucocorticoid hormones and prostaglandins of the E series (PGE) (2). These agents are thought to regulate the intensity of the immune response at the site of inflammation. For example, PGE2 is secreted by many cell types, such as IL-1-stimulated macrophages, and is a potent activator of the cAMP-dependent protein kinase (PKA) pathway in lymphocytes (3). Stimulation of PKA results in the inhibition of T cell proliferation and dampens T cell effector functions (2). As such, PGE2 conveys an inhibitory signal to produce a properly tuned immunoreactivity.

At the onset of the immune response, resting peripheral T lymphocytes enter the cell cycle from quiescence (G0). At this time D-type cyclins and cyclin E are sequentially synthesized during the G1 interval (4–6), both being rate limiting for S phase entry (7). Three D-type cyclins have been identified, of which cyclins D2 and D3 are predominantly expressed in T cells (5, 8). Cyclins 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) (8, 9). Phosphorylation of pRb results in its functional inactivation and allows progression of the cell cycle through the late G1 restriction point and subsequent entry into S phase (10). However, 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 assembled cyclin D/Cdk complexes and inhibits their kinase activity (11, 12). Expression of p27kip1 is down-regulated as cells progress through G1, but this process requires the presence of a co-stimulatory signal supplied by IL-2 or B7–1 and B7–2, the ligands for CD28 (6, 11, 12). Thus, only the combination of T cell receptor (TCR) complex activation and co-stimulation with IL-2 or B7 molecules allows for the formation of active cyclin D/Cdk complexes and initiation of T cell proliferation.

Leukemic T cell lines, such as Jurkat or H9 cells, are not dependent on TCR activation or co-stimulatory signals for their proliferation, and these cells express low levels of p27kip1 (6). Nevertheless, cell proliferation can be blocked in these cell lines upon restimulation of the TCR complex, and this is associated with activation-induced cell death (13–15). As such, these cells have been used as a model system to study the signaling events triggered by TCR activation that induce apoptosis in cycling T cells. Using these cell lines, we have previously shown that down-regulation of cyclin D3 is crucial to TCR-induced apoptosis (13). Here we have used these cell lines as a model to study PKA-mediated immunosuppression. We show that these cells are inhibited in their growth by agents that activate PKA and show that expression of cyclin D3 is down-regulated in leukemic T cell lines in response to activation of PKA. This down-regulation of cyclin D3 plays an important role in the PKA-mediated growth inhibition, since ectopic expression of cyclin D3 could overcome the suppressive effects.
of PKA to some extent. However, ectopic expression of cyclin D3 was unable to fully prevent the growth suppression that occurred in response to activation of PKA. Indeed, we could show that PKA activation also results in the induction of p27kip1 expression, independent of its effect on cyclin D3, indicating that PKA can cause growth suppression in T cells via multiple independent pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Antibodies, and Reagents**—Human Jurkat and H9 leukemic T cell lines were routinely cultured as described (13). Jurkat T cells stably expressing cyclin D3 (clones JD-I and JD-II) have been described previously (13). Anti-pRb (G5-245) was obtained from PharMingen (Hamburg, Germany), anti-p27kip1 (clone 57) from Transduction Laboratories (Lexington, KY), anti-cyclin D3 (Ab-2) from Calbiochem (San Diego, CA), anti-Cdk2 (M2), anti-cyclin E (HE-12 for immunoblotting and HE-111 for immunoprecipitation and *in vitro* kinase activity) were all from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D2 (DCS-5) was a kind gift from Dr. Jiri Lukas (Danish Cancer Society, Copenhagen, Denmark). Propidium iodide, N-6,2'-O-dibutyryl-cAMP (dibutyryl cAMP) and 8-bromo-cAMP were purchased from Sigma, and protein A/G-Sepharose beads from Santa Cruz Biotechnology.

**Cell Stimulation**—All cells were plated in fresh medium at a density of 2 × 10⁵ cells/ml at 24 h prior to cell stimulation to ensure that they were growing exponentially at the time of stimulation. Cells were counted the next day and replated in the presence or absence of forskolin, dibutyryl cAMP, and 8-bromo-cAMP at the indicated concentrations or on plates coated with an antibody (UCHT-1) as previously described (13). Anti-cyclin D3 monoclonal antibody (UCHT-1) (10 µg/ml) or dibutyryl cAMP (1 mM) (gray bars) or dibutyryl cAMP (1 mM) (white bars). C, Jurkat cells were treated with increasing amounts of forskolin for 24 h. D, Jurkat T cells were incubated for 24 h on tissue culture plates coated with an antibody (UCHT-1) at the indicated concentrations. E, Jurkat cells were incubated on tissue culture plates coated with anti-CD3 monoclonal antibody (UCHT-1) (10 µg/ml) for 8 or 24 h. In all cases cell proliferation was determined by [3H]thymidine incorporation and expressed as a percentage of the untreated control population. Error bars represent S.E. and are derived from at least three independent experiments.

**FIG. 1. Activation of PKA causes growth inhibition in Jurkat leukemic T cells.** A, Jurkat T cells were stimulated with forskolin (50 µM), dibutyryl cAMP (1 mM), or 8-bromo-cAMP (1 mM) for 24 h or left untreated. B, Jurkat cells were treated for 8 or 24 h with forskolin (50 µM) (gray bars) or dibutyryl cAMP (1 mM) (white bars). C, Jurkat cells were treated with increasing amounts of forskolin for 24 h. D, Jurkat T cells were incubated for 24 h on tissue culture plates coated with an antibody (UCHT-1) at the indicated concentrations. E, Jurkat cells were incubated on tissue culture plates coated with anti-CD3 monoclonal antibody (UCHT-1) (10 µg/ml) for 8 or 24 h. In all cases cell proliferation was determined by [3H]thymidine incorporation and expressed as a percentage of the untreated control population. Error bars represent S.E. and are derived from at least three independent experiments.

**Cell Cycle Analysis and Viability**—Cells were plated at a density of 2 × 10⁵ cells/ml in 96-well plates and grown with or without forskolin, dibutyryl cAMP, and 8-bromo-cAMP for the indicated times. Alternatively, cells were plated on anti-CD3-coated 96-well plates and grown for the indicated times. During the last 2 h, 0.5 µCi [3H]thymidine (Amersham Pharmacia Biotech) was added to each well, after which cells were harvested and [3H]thymidine incorporation was determined in a scintillation counter. To determine which phase of the cell cycle was affected by activation of protein kinase A or TCR activation, cells were grown at a density of 2 × 10⁵ cells/ml on 12-well tissue culture plates (Costar, Cambridge, MA) and treated with forskolin or anti-CD3 monoclonal antibodies or left untreated. At different time points after the stimulation, cells were collected and washed with ice-cold phosphate-buffered saline, after which they were fixed overnight in 70% ethanol at 4 °C. Cells were then pelleted by centrifugation, and cell pellets were stained with propidium iodide as described (13). Cell viability after stimulation for different time periods with forskolin or anti-CD3 monoclonal antibodies was determined by staining whole cells with propidium iodide as described (13). Samples were analyzed on a FACScan and cell viability was subsequently determined using Cell Quest software (BD PharMingen).

**Immunoprecipitation**—Cells were stimulated with forskolin or anti-CD3 monoclonal antibodies or left unstimulated and harvested at the indicated time points. Cells were washed once with ice-cold phosphate-buffered saline, and cell pellets were resuspended and lysed in E1A lysis buffer as described previously (6). Protein concentrations were determined, and equal amounts of protein were used for immunoprecipitation, using 100 µg of whole cell lysate, 20 µl of protein A/G-Sepharose beads with 4 µl of anti-cyclin E antibody, or 2 µl of anti-Cdk2 antibody. The resulting immunocomplexes were washed three times with ELB lysis buffer and used in *in vitro* kinase assays using histone H1 as substrate as described previously (16). Samples were analyzed on SDS-PAGE gels and exposed to PhosphorImager plates (Molecular Dynamics).

**Immunoblotting**—After proper stimulation, cells were harvested and washed once with ice-cold phosphate-buffered saline. Cell pellets were then lysed in 2× Laemmli buffer without β-mercaptoethanol and bromphenol blue. Samples were then incubated at 95 °C for 5 min. Protein concentrations were determined using a standard Lowry protein deter-
Inhibition of Cell Proliferation of Human Leukemic T Cell Lines by Activation of PKA—Activation of PKA can suppress T cell activation and causes growth inhibition in a large variety of lymphocytes, including leukemic T cell lines. We set out to study the mechanism of PKA-mediated growth inhibition in more detail. To this end, we stimulated Jurkat leukemic T cells with a variety of agents known to activate PKA. Treatment of Jurkat T cells with forskolin, which directly activates the catalytic subunit of adenylate cyclase, resulted in marked inhibition of proliferation, as measured by [3H]thymidine incorporation (Fig. 1A). A similar inhibition was seen after addition of dibutyryl cAMP or 8-bromo-cAMP (Fig. 1A), both membrane-permeable cAMP analogs known to efficiently stimulate PKA and thereby increase cAMP levels in the cells. Inhibition of cell proliferation in response to forskolin or dibutyryl cAMP was apparent as early as 8 h after stimulation and reached >75% inhibition after 24 h (Fig. 1B). Inhibition of [3H]thymidine incorporation was dependent on the concentration of forskolin added to the cells (Fig. 1C). Because 50 μM forskolin caused an efficient growth inhibition, we chose to use this concentration in our further experiments.

In addition to immunosuppression by agents that modulate the activity of PKA, tolerance of mature T lymphocytes can be induced through antigen-driven cell death (1). This latter process has been studied extensively in leukemic T cell lines that undergo apoptosis upon direct activation of the TCR complex (13–15). Therefore, we compared growth inhibition mediated by PKA with that observed in response to TCR activation. To this end we incubated cells with a cross-linked anti-CD3 mAb in increasing concentrations (13) and could observe an efficient inhibition of [3H]thymidine incorporation after a 24 h stimulation, similar to the effects of forskolin (Fig. 1D). Similar to PKA-induced inhibition, the suppression of thymidine incorporation was visible as early as 8 h after stimulation with UCHT-1 and reached ~75% after 24 h (Fig. 1E).

PKA Stimulation Causes a G1 Arrest but Does Not Affect Cell Viability—Cross-linking of the CD3 complex on leukemic T cell lines can result in programmed cell death (13–15), and we previously showed that the TCR-induced growth inhibition of Jurkat cells is in part due to a reduction in cell viability (13). Therefore, we wanted to determine the contribution of cell death to the growth inhibition seen upon activation of PKA. To this end, we cultured the cells in the presence of forskolin for 24 or 48 h and measured cell viability by propidium iodide uptake. Under these experimental conditions, forskolin induced only minor increases in the percentage of dead cells (from 4 to 6% after 48 h) (Fig. 2A), indicating that the effect of PKA cannot be explained by programmed cell death in these cells. In the same experiments, we observed a more significant increase in the number of dead cells in response to TCR activation, from 4% in the control population to 27% after 48 h (Fig. 2A).

Because activation of PKA induced a significant growth inhibition in the absence of apoptosis, we decided to study the effects of PKA activation on the cell cycle progression in more detail. We, therefore, stimulated Jurkat cells for 24 or 48 h with forskolin and analyzed the DNA profiles using propidium iodide staining and flow cytometry. As shown in Fig. 2B, the percentage of cells in the G0/G1 phase rises from 39 to 52% within 24 h of forskolin treatment and reaches ~63% at 24 h after treatment. As can be seen in Fig. 2B, the fraction of cells with a sub-G1 DNA content (apoptotic cells) remains unchanged after treatment with forskolin (Fig. 2B), consistent with our data on cell viability (Fig. 2A). Stimulation with anti-CD3 mAbs resulted in a similar increase in the percentage of cells in G1 (Fig. 2B). In contrast with forskolin, anti-CD3 mAbs induced a clear increase in the fraction of apoptotic cells (~2n), from 6% in the unstimulated cells to 19% in the cells stimulated for 48 h, similar to the percentages obtained by measuring cell viability.

PKA-mediated Inhibition of Cyclin/Cdk Activity—To investigate at which point in G1 cells arrest in response to PKA activation, we examined the phosphorylation state of pRb after treatment of cells with forskolin. In untreated, asynchronous cultures of Jurkat cells, pRb is mostly present in its slower migrating hyperphosphorylated form (Fig. 3A, lane 1). After 24 h of stimulation with forskolin we observed a clear mobility shift in the pRb protein from the hyperphosphorylated to the hypophosphorylated form (Fig. 3A, lane 2). In order to rule out that these effects of PKA activation were restricted to Jurkat leukemic cells, we analyzed the effects of PKA activation on pRb phosphorylation in another human leukemic cell line, H9. Indeed, proliferation of H9 leukemic cells was also efficiently

**RESULTS**

**Inhibition of Cell Proliferation of Human Leukemic T Cell Lines by Activation of PKA**—Activation of PKA can suppress T cell activation and causes growth inhibition in a large variety of lymphocytes, including leukemic T cell lines. We set out to study the mechanism of PKA-mediated growth inhibition in more detail. To this end, we stimulated Jurkat leukemic T cells with a variety of agents known to activate PKA. Treatment of Jurkat T cells with forskolin, which directly activates the catalytic subunit of adenylate cyclase, resulted in marked inhibition of proliferation, as measured by [3H]thymidine incorporation (Fig. 1A). A similar inhibition was seen after addition of dibutyryl cAMP or 8-bromo-cAMP (Fig. 1A), both membrane-permeable cAMP analogs known to efficiently stimulate PKA activity when added to cells. Inhibition of cell proliferation in response to forskolin or dibutyryl cAMP was apparent as early as 8 h after stimulation and reached >75% inhibition after 24 h (Fig. 1B). Inhibition of [3H]thymidine incorporation was dependent on the concentration of forskolin added to the cells (Fig. 1C). Because 50 μM forskolin caused an efficient growth inhibition, we chose to use this concentration in our further experiments.

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inhibited by forskolin (data not shown), and a similar inhibition of pRb phosphorylation was observed in response to forskolin (Fig. 3A, lanes 3 and 4).

Phosphorylation of pRb is mediated by cyclin/Cdk complexes (10), and therefore, we measured the kinase activity of cyclin E-Cdk2 complexes, normally induced late in G1. For this purpose, cyclin E or Cdk2 were immunoprecipitated from whole cell lysates prepared from control and forskolin- or anti-CD3-stimulated Jurkat or H9 cells. The obtained immunocomplexes were then subjected to in vitro kinase reactions using histone H1 as a substrate. After a 24 h stimulation with forskolin or anti-CD3 mAbs, both cyclin E as well as Cdk2-associated kinase activity were inhibited (Fig. 3B). Expression of cyclin E and Cdk2 were unaltered upon stimulation with forskolin or anti-CD3 mAbs (Fig. 3C), demonstrating that regulation of cyclin E expression is not involved in the inhibition of cyclin E-Cdk2 kinase activity.

In addition to phosphorylation by cyclin E-Cdk2, Rb phosphorylation also requires the activity of cyclin D-Cdk4 or -Cdk6 complexes (17). Cyclin D/Cdk complexes phosphorylate residues on pRb that are distinct from the residues phosphorylated by cyclin E- or cyclin A-Cdk2 complexes (18). For example, Ser^780 is uniquely phosphorylated by cyclin D/Cdk complexes, and its phosphorylation state is, therefore, a good measure of cyclin D/Cdk kinase activity. Using an antibody recognizing Ser^780-phosphorylated pRb (anti-phospho-Ser^780), we noted that treatment of Jurkat cells with forskolin for 24 h resulted in the loss of phosphorylated pRbSer^780, similar to what was observed after stimulation with anti-CD3 mAbs (Fig. 3D). These experiments clearly demonstrate that PKA stimulation results in the loss of cyclin D- and cyclin E-associated kinase activities from the cell.

**PKA Activation Induces Down-regulation of Cyclin D3 and Up-regulation of p27kip1**—We have previously shown that Jurkat and H9 cells express abundant amounts of cyclin D3 but no cyclin D2 and that its expression is down-regulated upon activation of the TCR (13). Therefore, we next decided to analyze the effect of PKA activation on expression of the cyclin D3. Expression of cyclin D3 was dramatically reduced after 24 h in the presence of forskolin or anti-CD3 mAbs in Jurkat and H9 cells (Fig. 4A). The timing of cyclin D3 down-regulation appears to be similar after stimulation with anti-CD3 mAbs or forskolin. In both cases, a reduction in cyclin D3 expression is first visible after 8 h of stimulation and expression continues to decline up to 24 h (Fig. 4B). This timing slightly precedes the decrease in Ser^780 dephosphorylation on pRb (Fig. 4C), suggesting that the reduction in cyclin D3 expression is responsible for the observed pRb dephosphorylation.

To examine if changes in the levels of cyclin-dependent kinase inhibitors (Cdki) contribute to the observed inhibition of cyclin/Cdk activity, we examined if PKA activation could induce expression of p27kip1 or p21cip1 in leukemic T cells. Whole cell lysates were prepared from control cells and from cells stimulated for 24 h with anti-CD3 mAbs or forskolin. As shown in Fig. 4D, expression of p27kip1 did not change significantly upon activation of the T cells with anti-CD3 mAbs or forskolin. In contrast, stimulation of PKA did result in an increase in p27kip1 protein levels. Induction of p27kip1 in response to activation of PKA was visible after 2 h of stimulation with forskolin, indicating that this is a rather rapid response (Fig. 4E). We next checked expression of p21cip1 but could not detect p21cip1 in control or forskolin-stimulated Jurkat cells (data not shown), nor in cells treated with anti-CD3 mAbs (19). Thus, it seems unlikely that reduced expression of p21cip1 causes the observed inhibition of cyclin/Cdk complexes in response to TCR or PKA activation, but it appears that PKA-mediated growth inhibition is due to combined regulation of both cyclin D3 and p27kip1 expression.

**Ectopic Expression of Cyclin D3 Partially Overrides Growth Suppression by cAMP**—We have previously shown that the TCR-induced growth arrest in Jurkat cells is critically dependent on down-regulation of cyclin D3. In order to investigate if the PKA-induced growth suppression shows a similar depend-
ence, we studied PKA-mediated growth inhibition in Jurkat-derived cell lines stably overexpressing cyclin D3. To this end, we transfected a plasmid encoding human cyclin D3 into Jurkat cells and isolated single cell colonies by limiting dilution. Of several lines isolated, two lines were selected for further studies, JD3.I and JD3.II, which express moderate levels of exogenous cyclin D3 (13). To confirm that cyclin D3 was indeed constitutively expressed in these cell lines, also in the presence of forskolin, we analyzed cyclin D3 expression by Western blot analysis. Whereas cyclin D3 is efficiently down-regulated in the parental Jurkat cell line, no effect on cyclin D3 expression by forskolin or anti-CD3 mAbs is seen in JD3.I or JD3.II (Fig. 5A). As can be seen in Fig. 5C, the growth inhibitory response to anti-CD3 mAbs was almost completely abolished when we measured [3H]thymidine incorporation in the JD3.I and JD3.II clones, consistent with our previous report (13). In contrast, although a significant reduction in the forskolin-induced inhibition of thymidine incorporation was observed in JD3.I and JD3.II, ectopic expression of cyclin D3 was unable to fully revert growth inhibition by forskolin. These data show that down-regulation of cyclin D3 plays an important role in both TCR- and PKA-mediated growth suppression, but also indicate that PKA must affect other cell cycle regulatory proteins besides cyclin D3. Our data suggest that this other pathway involves regulation of p27kip1 (see Fig. 4D). p27kip1 binds to multiple G1 cyclin/Cdk complexes and is a potent Cdk inhibitor for complexes containing Cdk2, although it does not seem to inhibit cyclin D/Cdk complexes (19). As such, p27kip1 can inhibit cell cycle progression in G1, by inhibition of cyclin/Cdk complexes other than cyclin D-Cdk4 or Cdk6, such as cyclin E-Cdk2. Thus, regulation of p27kip1 expression, independent from cyclin D, could very well constitute a separate pathway of growth inhibition in these PKA-stimulated T cell lines. Indeed, when analyzing p27kip1 expression, we found that the ability of forskolin to induce p27kip1 expression was retained in the JD3.I and JD3.II clones (Fig. 5B), demonstrating that regulation of p27kip1 by PKA occurs independently of its effects on cyclin D3 expression.

**DISCUSSION**

Agents that activate the PKA signaling cascade act *in vivo* to down-regulate the immune response in order to prevent excessive reactivity. In this report we have addressed the mechanism of suppression of T cell proliferation in response to activation of PKA. To this end, we have used leukemic T cell lines that are no longer dependent on activating stimuli for their proliferation. Rather, these T cell lines resemble activated mature T cells, in that apoptosis can be induced upon activation of the TCR complex, and their proliferation is dramatically inhibited by agents that cause a rise in the intracellular cAMP levels. These properties allowed us to use these cell lines to study PKA-mediated immunosuppression in more detail and to compare this to immunosuppression by stimulation of the TCR complex on the same T cell lines. We could show that the PKA-mediated block in cell proliferation was due to an arrest of cell cycle progression at some point in the G1 phase, but no dramatic reduction in cell viability was observed. The G1 arrest induced by forskolin 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. 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 late in G1 as a function of cell cycle progression. No reduction in the expression level of cyclin E

**FIG. 4.** PKA activation results in the induction of p27kip1 and a reduction in cyclin D3 expression. Total lysates prepared from Jurkat or H9 cells were separated on SDS-PAGE gels for the analysis of cyclin D3 expression (A, B), pSer780 phosphorylation of pRb (C), or p27kip1 expression levels (D, E) on Western blots. Jurkat (A – E) or H9 cells (A, D) were left untreated or stimulated with forskolin, α-CD3 for 24 h (A, D) or a time course of induction (B, C, E) with forskolin or a-CD3 was performed as indicated. Equal amounts of protein were loaded in each lane. Kinetics of the loss of Ser780 phosphorylation of pRb was analyzed in Jurkat cells after stimulation with forskolin or anti-CD3 mAbs is seen in JD3.I or JD3.II (Fig. 5A). As can be seen in Fig. 5C, the growth inhibitory response to anti-CD3 mAbs was almost completely abolished when we measured [3H]thymidine incorporation in the JD3.I and JD3.II clones, consistent with our previous report (13). In contrast, although a significant reduction in the forskolin-induced inhibition of thymidine incorporation was observed in JD3.I and JD3.II, ectopic expression of cyclin D3 was unable to fully revert growth inhibition by forskolin. These data show that down-regulation of cyclin D3 plays an important role in both TCR- and PKA-mediated growth suppression, but also indicate that PKA must affect other cell cycle regulatory proteins besides cyclin D3. Our data suggest that this other pathway involves regulation of p27kip1 (see Fig. 4D). p27kip1 binds to multiple G1 cyclin/Cdk complexes and is a potent Cdk inhibitor for complexes containing Cdk2, although it does not seem to inhibit cyclin D/Cdk complexes (19). As such, p27kip1 can inhibit cell cycle progression in G1, by inhibition of cyclin/Cdk complexes other than cyclin D-Cdk4 or Cdk6, such as cyclin E-Cdk2. Thus, regulation of p27kip1 expression, independent from cyclin D, could very well constitute a separate pathway of growth inhibition in these PKA-stimulated T cell lines. Indeed, when analyzing p27kip1 expression, we found that the ability of forskolin to induce p27kip1 expression was retained in the JD3.I and JD3.II clones (Fig. 5B), demonstrating that regulation of p27kip1 by PKA occurs independently of its effects on cyclin D3 expression.
These data indicate that the arrest in G1 is caused by the reduction in expression by cyclin D3, which will result in inhibition of proliferation because a failure to down-regulate cyclin D3 does not result in a complete loss of responsiveness to forskolin. Thus, regulation of p27kip1 expression appears to distinguish the PKA-mediated growth suppression from TCR-mediated inhibition of leukemic T cell growth. Taken together, our data indicate that PKA activation in leukemic T cell lines affects multiple pathways to elicit an efficient inhibition of proliferation. Interference with one of the PKA targets, namely cyclin D3, is not sufficient to fully override growth suppression, indicating that other targets play an equally important role in the PKA response.

It was recently reported that PKA activation can also affect cyclin D3 expression in primary T lymphocytes (21). However, p27kip1 levels were not rapidly induced after PKA activation in primary T lymphocytes, although we find induction of p27kip1 expression as early as 2 h after stimulation with forskolin in leukemic T cells. This discrepancy could be due to a fundamental difference in PKA-mediated growth suppression in primary lymphocytes versus the leukemic T cell lines studied here. Unfortunately, the importance of down-regulation of cyclin D3 to PKA-mediated growth suppression of primary T lymphocytes has not been addressed, making it impossible to draw conclusions as to the existence of other potential PKA targets in primary T cells. Nevertheless, our experiments have clearly shown that down-regulation of cyclin D3 is not solely responsible for PKA-mediated growth suppression of leukemic T cells, because a failure to down-regulate cyclin D3 does not result in a complete loss of responsiveness to forskolin. The data presented here suggest that one other crucial target may be p27kip1, which is induced upon activation of PKA in the leukemic cell lines studied here.

Regulation of p27kip1 expression in a fashion independent from cyclin D expression, as seen here for PKA activation in Jurkat T cells, truly constitutes a separate pathway of growth suppression. Although p27kip1 binds with high affinity to cyclin D/Cdk complexes, it is clear that these complexes remain catalytically active, even in the presence of high levels of p27kip1 (22, 23). In contrast, cyclin E-Cdk2 complexes are very efficiently inhibited by p27kip1 (19). Based on these observations, it has been proposed that cyclin D/Cdk complexes function to sequester p27kip1 away from cyclin E-Cdk2 complexes (19). Therefore, whereas down-regulation of cyclin D3 expression by PKA will inhibit G1 progression at the level of cyclin D activity, up-regulation of p27kip1 will exert its inhibition at the level of cyclin E--associated kinase activity. This way, activation of PKA can lead to a more tight suppression of T cell proliferation, responsible for the efficient immunosuppressive activities of agents known to activate PKA in T lymphocytes. It will be interesting to determine at which point after PKA activation this bifurcation toward cyclin D3 and p27kip1 occurs, and future experiments will be directed toward a better understanding of the mechanism by which PKA can exert its effects on p27kip1 expression.

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Analysis of the expression of other cell cycle regulatory proteins that play a role in the progression from G1 to S phase showed that expression of the Cdk inhibitor p27kip1 is induced after stimulation with forskolin. This rise in p27kip1 occurs independently of the reduction of cyclin D3, since it is not seen in response to TCR activation and, more importantly, is not affected in the JD3.I and JD3.II cell lines that fail to down-regulate cyclin D3 in response to forskolin. At this point, we cannot completely rule out the possibility that the effects of PKA on p27kip1 are an indirect consequence of cell cycle arrest. However, expression of p27kip1 is not induced by TCR activation, although this leads to a similar block in proliferation. Also, the rapid kinetics of p27kip1 induction would argue against this possibility. Thus, regulation of p27kip1 expression appears to distinguish the PKA-mediated growth suppression from TCR-mediated inhibition of leukemic T cell growth. Taken together, our data indicate that PKA activation in leukemic T cell lines affects multiple pathways to elicit an efficient inhibition of proliferation. Interference with one of the PKA targets, namely cyclin D3, is not sufficient to fully override growth suppression, indicating that other targets play an equally important role in the PKA response.

FIG. 5. Ectopic expression of cyclin D3 is unable to fully override growth inhibition in response to PKA activation. A and B, effect of PKA activation on cyclin D3 expression (A) and p27kip1 expression (B) was compared in parental Jurkat cells versus two cell lines stably overexpressing cyclin D3, JD3.I and JD3.II. Cells were stimulated with forskolin or α-CD3 mAb for 24 h, and total lysates were prepared to determine cyclin D3 (A) and p27kip1 (B) protein levels by Western blotting. C, rescue from PKA- or TCR-mediated growth suppression by ectopic expression of cyclin D3 was determined by analysis of [3H]thymidine incorporation in parental Jurkat versus the JD3.I and JD3.II cell lines. Cells were stimulated for 24 h with forskolin or α-CD3 before the addition of [3H]thymidine. Bars indicate the percentage of inhibition of thymidine incorporation relative to the untreated control. Error bars represent the S.E. determined from three independent experiments.
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Brigit A. van Oirschot, Marie Stahl, Susanne M. A. Lens and René H. Medema

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