Cyclin T1 Expression Is Regulated by Multiple Signaling Pathways and Mechanisms during Activation of Human Peripheral Blood Lymphocytes

Renée M. Marshall, Dominic Salerno, Judit Garriga and Xavier Graña

*J Immunol* 2005; 175:6402-6411; doi: 10.4049/jimmunol.175.10.6402
http://www.jimmunol.org/content/175/10/6402

---

**References**  This article cites 61 articles, 38 of which you can access for free at: http://www.jimmunol.org/content/175/10/6402.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Cyclin T1 Expression Is Regulated by Multiple Signaling Pathways and Mechanisms during Activation of Human Peripheral Blood Lymphocytes

Renée M. Marshall, Dominic Salerno, Judit Garriga, and Xavier Graña

Stimulation of primary human T lymphocytes results in up-regulation of cyclin T1 expression, which correlates with phosphorylation of the C-terminal domain of RNA polymerase II (RNAP II). Up-regulation of cyclin T1 and concomitant stabilization of cyclin-dependent kinase 9 (CDK9) may facilitate productive replication of HIV in activated T cells. We report that treatment of PBLs with two mitogens, PHA and PMA, results in accumulation of cyclin T1 via distinct mechanisms. PHA induces accumulation of cyclin T1 mRNA and protein, which results from cyclin T1 mRNA stabilization, without significant change in cyclin T1 promoter activity. Cyclin T1 mRNA stabilization requires the activation of both calcineurin and JNK because inhibition of either precludes cyclin T1 accumulation. In contrast, PMA induces cyclin T1 protein up-regulation by stabilizing cyclin T1 protein, apparently independently of the proteasome and without accumulation of cyclin T1 mRNA. This process is dependent on Ca\(^{2+}\)-independent protein kinase C activity but does not require ERK1/2 activation. We also found that PHA and anti-CD3 Abs induce the expression of both the cyclin/CDK complexes involved in RNAP II C-terminal domain phosphorylation and the G\(_r\)-S cyclins controlling cell cycle progression. In contrast, PMA alone is a poor inducer of the expression of G\(_r\)-S cyclins but often as potent as PHA in inducing RNAP II cyclin/CDK complexes. These findings suggest coordination in the expression and activation of RNAP II kinases by pathways that independently stimulate gene expression but are insufficient to induce S phase entry in primary T cells. The Journal of Immunology, 2005, 175: 6402–6411.

Cyclin T1 is one of four regulatory cyclins (cyclin T1, T2a, T2b, or K) that bind to and activate cyclin-dependent kinase 9 (CDK9) (1–4). Each T-type cyclin/CDK9 complex constitutes a distinct positive transcription elongation factor b (P-TEFb) (1, 3). These complexes have been implicated in stimulating elongation upon initiation, of otherwise paused transcripts, by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (RNAP II) and negative transcription elongation factors (5–12). It has also been proposed recently that the Saccharomyces cerevisiae and Drosophila melanogaster CDK9 orthologs recruit polyadenylation factors linking transcription with RNA processing (13, 14). Surprisingly, in these studies CDK9/Cdk1 was not required for transcriptional elongation (13, 14).

The cyclin T1/CDK9 complex is also known as Tat-associated kinase (15, 16). Tat is a HIV protein essential for viral replication that binds a RNA structure in the nascent HIV transcript called trans-activation response and recruits the cyclin T1/CDK9 complex but not other T-type cyclin/CDK9 complexes (17, 18). Tat-mediated recruitment of the cyclin T1/CDK9 complex stimulates processive transcription by RNAP II by phosphorylating its CTD and negative transcription elongation factors (reviewed in Ref. 19). CD4-positive T lymphocytes are one of the primary targets for HIV and are fundamental to viral pathogenesis. Interestingly, HIV replicates more efficiently in activated, as opposed to resting, T cells, as the viral genome integrates poorly in quiescent T cells. Once integrated, replication depends on viral and host cellular factors, which may become limiting, as these cells become resting memory cells (20). GST-Tat pull-down experiments suggested that during activation of PBLs, TAK activity was up-regulated (21). Subsequently, we and others (22–24) showed that cyclin T1 expression was up-regulated following stimulation of PBLs by mitogens, such as PHA and PHA, as well as by costimulation with anti-CD3/anti-CD28 Abs or cytokines. Cyclin T1 protein up-regulation correlated with hyperphosphorylation of RNAP II and HIV replication in PMA/PHA-treated PBLs (22). Studies using flavopiridol, a potent inhibitor of CDK9 activity, have suggested that P-TEFb activity is required for transcription of several genes (25). HIV replication is also very sensitive to flavopiridol and a dominant-negative form of CDK9 (25–27). Thus, up-regulation of cyclin T1 expression during T cell activation may serve to keep pace with the increase in transcription and/or increased recruitment of the cyclin T1/CDK9 complex by inducible transcription factors to specific promoters. The increase in cyclin T1 expression may also facilitate HIV replication during reactivation of T cells harboring integrated latent HIV genomes.

Primary T cells are fully activated by engagement of the TCR and CD28 costimulation (reviewed in Ref. 28). This process is mimicked by costimulation with mitogens, PHA and PMA, which raise intracellular Ca\(^{2+}\) levels and activate protein kinase C (PKC),
respectively (28). Given the potential importance of cyclin T1 up-regulation in the expression of cellular genes and HIV replication during T cell activation/reactivation, we have dissected the signaling pathways required and the mechanisms responsible for up-regulation of cyclin T1 via two distinct T cell mitogens, which also stimulate HIV replication (22). We demonstrate that PHA and PMA independently up-regulate cyclin T1 protein levels through different mechanisms. We have also identified key signaling steps required for cyclin T1 up-regulation by each mitogen. In this regard, PHA-mediated up-regulation of cyclin T1 mRNA and protein requires the activation of both calcineurin and JNK, as inhibition of either event prevents cyclin T1 accumulation. In contrast, PMA-mediated stabilization of cyclin T1 protein requires calcium-independent PKCs but does not require ERK activity. Our results also show coordination of the expression of NAP-2 cyclin/CDKs with signals that induce gene expression.

Materials and Methods

Cell culture

PBLs were isolated as previously described with some modifications (22). Briefly, 500 ml of whole blood was collected from donors and layered onto Ficoll-Paque Plus (Amersham Biosciences) and centrifuged at 650 × g for 45 min. The mononuclear layer was isolated, transferred to a fresh tube, and washed twice with room temperature PBS and resuspended in RPMI 1640 supplemented with 10% heat-inactivated FBS at a concentration of 1.5 × 10^6 cells/ml. PBLs were obtained after monocyte depletion using plastic adherence and adjusted to a concentration of 1.0 × 10^6 cells/ml, plated in 10-cm plates, and incubated overnight at 37°C and 5% CO2. The next day PBLs were activated with PHA (Sigma-Aldrich) at a final concentration of 1 μg/ml and/or PMA (Sigma-Aldrich) at a final concentration of 1 ng/ml, unless otherwise indicated. To activate PBLs via CD3/CD28 costimulation, 1 ml of PBS containing 10 μg/ml human anti-CD3 (217669; EMD Biosciences) was incubated in 6-cm plates at 37°C for 30 min to allow the Ab to attach to the plastic. PBS was removed and cells plated at 1.0 × 10^6 cells/ml, and 10 ng/ml anti-CD28 (D23648; EMD Biosciences) was immediately added to the appropriate plates. Cells were collected 48 h later.

Pharmacological inhibitors

The following pharmacological inhibitors were used to inhibit particular signaling pathways: SP600125 (10 μM), a JNK inhibitor (BIOMOL) (29); FKS06 (20 μM), a calciuminhibitor (Calbiochem) (30); PD98059 (30 μM), a MEK inhibitor (Calbiochem) (30); and rottlerin (5 μM), a Ca2+-independent PKC inhibitor (Calbiochem) (31). In addition, cycloheximide (CHX) (50 μg/ml) (Sigma-Aldrich) was used to inhibit protein translation (32), actinomycin D (1 μg/ml) was used to inhibit cellular transcription for the β-actin stability assays (33) and lactacystin (10 μM) (Sigma-Aldrich) to inhibit proteasome activity (34).

Protein assays

Whole cell lysates were prepared under non-denaturing and denaturing conditions. Cells were lysed in non-denaturing lysis buffer (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM sodium vanadate, 1 mM PMSF, 10 μg/ml leupeptin, 4 μg/ml aprotinin, and 40 μg/ml pepstatin) as reported previously (22). Protein concentration was determined by the method of Bradford in the cell lysates. Standards were normalized for SDS concentration. For Western blot analysis, 20 μg of protein was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane in 10 mM CAPS (pH 11.0) containing 10% methanol. The following Abs were used: anti-cyclin T1 (SC-10750), CDK9 (SC-494), cyclin H (SC-609), cyclin D2 (SC-593), cyclin D3 (SC-182), cyclin E (SC-247), cyclin A (SC-596), p53 (SC-6243), and total RB SC-50 were purchased from Santa Cruz Biotechnology. Total ERK1/2 (9102), phospho-ERK1/2 (9101), and Rb-780 (9307) Abs were obtained from Cell Signaling Technology. Rb-826 (PC644-50UG) was purchased from Oncogene.

Northern blot analysis

Total RNA was isolated using the QiAshredder and RNasey Mini kit from Qiagen. Prime RNase inhibitor (Eppendorf) was added at a final concentration of 1.0 U/μl. Five micrograms of total RNA was resolved by formaldehyde-denaturing 1% agarose electrophoresis and transferred overnight to nitrocellulose (Hybond-N; Amersham Biosciences) by capillarity in 10× SSC as described previously (36). Membranes were incubated overnight with random-primer labeled probes at 42°C in hybridization solution (50% formamide, 5× SSC, 1× Denhardt, 100 μg/ml SDS, 20 mM NaPO4, and 10 μg/ml denatured herring sperm DNA). Membranes were washed at 42°C twice with 2× SSC/0.1% SDS and then with 0.1 SSC/0.1% SDS until the wash was free of radioactivity. Bands were visualized by autoradiography.

JNK assay

Cells were lysed in JNK lysis buffer (25 mM HEPES (pH 7.6), 0.1% Triton X-100, 300 mM NaCl, 1.5 mM MgCl2, and 0.2 mM EDTA with 20 mM β-glycerophosphate, 2.0 μM DTT, 0.2 mM sodium vanadate, 2 μg/ml leupeptin, and 4 μg/ml aprotinin freshly added). Two hundred micrograms of lysate was incubated with ~5 μg of GST-c-Jun (1-79)-bound-Sepharose beads (see below) rocking for 2 h at 4°C. c-Jun-bound complexes were pelleted and washed five times with cold PBS and then incubated in 40 μl of JNK reaction buffer (20 mM HEPES (pH 7.6), 10 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM sodium vanadate, and 20 μM [γ-32P]-ATP (5000 cpm/μmol) (PerkinElmer)) for 20 min at 30°C. The reaction was stopped by addition of 2× Laemmli’s sample buffer. Samples were resolved by 12% SDS-PAGE, and gels were stained, dried, and visualized by autoradiography. GST-c-Jun (1-79) was expressed and purified as described previously (37).

Transduction of PBLs with luciferase reporter viral vectors

PCEIII-CMV-LacZ vector was derived from pHR-CMV-LacZ (38) as described previously (39). PCEIII.cyclinT1.Luc was generated by removing the cyclin T1 promoter-luciferase cassette from PGL3.cyclinT1.Luc by digesting with XbaI/KpnI. The cyclin T1 promoter spans 2220 bp upstream of the ATG initiation codon and was fully sequenced (40). PCEIII-CMV-LacZ was digested ClaI/XbaI to remove the CMV-LZ cassette. Both digests were blunt ended and ligated. 293T cells were cotransfected with PCEIII.cyclinT1.Luc (transfer), gag/pol (packaging), and vesicular stomatitis virus-G (VSV-G) (envelope) constructs to generate cyclin T1-luciferase lentiviruses or NL-A-Luc.R.E- (41, 42) and VSV-G envelope constructs to generate long terminal repeat (LTR)-luciferase viruses by calcium-phosphate precipitation. Medium was changed 16–20 h following transfections, and medium containing viruses was collected at 24 and 48 h later. The virus containing medium was centrifuged at 652 × g for 5 min and filter sterilized using a 0.22-μm filter to remove cellular debris. The viral medium was ultracentrifuged at 25,000 rpm for 90 min at 4°C. Pellets were resuspended in 1 ml of PBS/0.1% BSA and then subjected to a second round of ultracentrifugation to further concentrate the sample. Pellets were then resuspended in 200 μl of PBS/0.1% BSA. A total of 2.0 × 10^6 PBLs was resuspended in 500 μl of RPMI 1640 and 10% heat-inactivated FBS in 24-well plates, Sequabrene (Sigma-Aldrich) was added to a final concentration of 8 μg/ml, virus was added, cells were centrifuged at 244 × g for 90 min, and then incubated at 37°C. After 16 h of infection, 1.5 ml of medium was added to bring the concentration to 1.0 × 10^6 cells/ml, cells were activated with PHA (1 μg/ml) or vehicle and collected 48 h later. Cells were lysed under non-denaturing conditions and luciferase activity measured using the Promega luciferase assay kit as per manufacturer directions.

Results

Cyclin T1 protein expression is independently up-regulated in PBLs by signals that activate T cells

It has been previously shown that cyclin T1 protein expression is regulated in PBLs stimulated with PMA and/or PHA as well as via costimulation of CD4 positive cells with anti-CD3/anti-CD28 Abs or cytokines (22–24). However, these findings were later challenged by Martin-Serrano et al. (35), who suggested that the apparent increase in cyclin T1 protein levels was likely due to proteolytic degradation during the preparation of the cell lysates. We have carefully re-examined this issue and found that cyclin T1 is
clearly up-regulated during T cell activation. Fig. 1A shows up-regulation of cyclin T1 following stimulation of quiescent PBLs with PHA and PMA in whole protein lysates prepared under denaturing conditions immediately after cell harvesting. The samples were immediately boiled after resuspending cells in denaturing lysis buffer containing 2% SDS and a mixture of protease inhibitors (see Materials and Methods). Parallel examination of cyclin T1 levels in cell lysates obtained under nondenaturing conditions showed that the levels of cyclin T1 were relatively reduced in unstimulated PBLs, suggesting the possibility that a protease could be degrading cyclin T1 during cell lysis. To examine this possibility, we mixed 293 cells, which contain stable cyclin T1, with unstimulated quiescent PBLs and immediately collected them. Whole cell lysates were prepared under nondenaturing conditions. Fig. 1B shows that significantly less cyclin T1 was detected in the lysates of mixed cells than in lysates of 293 cells (cell mixtures were calculated such that approximately half the amount of protein came from each cell type), indicating that a protease is present in unstimulated PBLs, which degrades a portion of cyclin T1 during cell lysis. This protease is inactivated soon after lysis as no further degradation occurs in cell lysates incubated at 37°C (data not shown). Similar results were obtained by mixing nonstimulated (unstable cyclin T1) and stimulated (stable cyclin T1) PBLs (data not shown). Thus, while it is clear that cyclin T1 is up-regulated several fold during T cell activation, previous studies slightly overestimated the magnitude of cyclin T1 up-regulation (see quantitation; Fig. 1A).

Having established that stimulation of PBLs with both PHA and PMA leads to increased cyclin T1 expression, we re-examined the extent to which each signal induces cyclin T1 protein expression. Cells were stimulated with PMA (1 or 25 ng/ml), PHA (1 μg/ml), or both signals for 24 and 48 h and then lysed under denaturing conditions. Two different PMA concentrations were used to ensure that the discrepancies between previous studies were not due to differences in the potency of the stimulatory signal (22, 23, 35). Stimulation of PBLs led to up-regulation of cyclin T1 steady-state protein levels by 48 h in all the above conditions (Fig. 1C). CDK9 levels mirrored the increase in cyclin T1 expression, which is consistent with CDK9 stabilization dependence on binding to cyclin T1 (43–45). As a control, we measured cyclin A expression, which is only up-regulated by PHA, as well as ERK 1/2 phosphorylation, which is only induced by PMA. To demonstrate that cyclin T1 expression is similarly up-regulated independently of donor variability, we simultaneously analyzed expression in five different donors (Fig. 1D).

Cyclin T1 mRNA is induced in PBLs stimulated with PHA but not PMA

Previous studies have suggested that cyclin T1 mRNA levels are modestly induced by stimulation of PBLs with PMA, which correlates with cyclin T1 protein up-regulation (23). However, it is not known whether PHA-mediated up-regulation of cyclin T1 is mediated by similar mechanisms. Thus, we measured the effects of PMA and/or PHA on cyclin T1 mRNA levels. Northern blot analysis was performed on total RNA isolated from PBLs stimulated for 24 and 48 h, as indicated in Fig. 2A, using a specific cyclin T1 probe as well as a 7SK small nuclear RNA (snRNA) probe as a loading control and visualized by autoradiography. Interestingly,
were preincubated with CHX (50 ng/ml) or vehicle for 1 h and then collected or activated with PHA or vector and collected at the indicated time points. Five micrograms of total RNA was analyzed by Northern blot using the indicated probes and visualized by autoradiography. rRNA bands showing equal loading were visualized by ethidium bromide staining.

FIGURE 2. Cyclin T1 mRNA is up-regulated in response to PHA, but not PMA, stimulation of human PBLs. A, PBLs were activated with PMA (1 ng/ml), PHA (1 μg/ml), and PMA/PHA and collected at the indicated time points. Northern blot analysis was performed using the indicated probes, and bands were visualized by autoradiography. B and C, PBLs were preincubated with CHX (50 μg/ml) or vehicle for 1 h and then collected or activated with PHA or vector and collected at the indicated time points. Five micrograms of total RNA was analyzed by Northern blot using the indicated probes and visualized by autoradiography. rRNA bands showing equal loading were visualized by ethidium bromide staining.

Cyclin T1 mRNA accumulation is due to cyclin T1 mRNA stabilization rather than cyclin T1 promoter activation

PHA-mediated up-regulation of cyclin T1 mRNA could be a result of increased RNA transcription, RNA stability, or both. To determine whether PHA stimulation positively regulates the activity of the cyclin T1 promoter, we infected PBLs with a lentivirus expressing a luciferase reporter gene under the control of the cyclin T1 promoter (~2.2 kb upstream of the ATG initiation codon). This promoter region has been reported previously to direct potent reporter activity in a variety of transfected cells (40). As a positive control for regulation of promoter activity, we infected PBLs with a VSV-G-pseudotyped, replication-defective HIV virus expressing a luciferase reporter gene inserted in the Nef gene (41, 42). Thus, luciferase expression is under the control of the HIV LTR. To generate VSV-G-pseudotyped cyclin T1-luciferase lentiviruses, 293T cells were cotransfected with PCEIII.cyclinT1.Luc, Gag-pol, and VSV-G envelope constructs by calcium-phosphate precipitation (Fig. 3A). Viruses were then isolated and concentrated as described in Materials and Methods. HIV-luciferase control VSV-G-pseudotyped viruses were similarly generated by cotransfecting the NL4-3.Luc.R-E- and VSV-G envelope constructs into 293T cells. As seen in Fig. 3B, cyclin T1 promoter activity was not stimulated by PHA. In contrast, and as expected, when PBLs infected with control LTR-luciferase viruses were stimulated with PHA, a 3-fold increase in luciferase activity was detected. These data show that cyclin T1 promoter activity is not regulated by PHA stimulation in primary lymphocytes and suggest that the mechanisms leading to cyclin T1 mRNA accumulation are posttranscriptional.

To determine whether PHA stimulation of PBLs results in an increase in cyclin T1 mRNA stability, we either activated PBLs with PHA (1 μg/ml) or vehicle for 48 h and applied actinomycin D for the last 30 or 90 min before collection. Addition of actinomycin D (1 μg/ml) blocks cellular transcription, allowing determination of mRNA stability via Northern blot analysis (33). RNA was isolated, Northern blot analysis was performed, and bands were visualized by autoradiography. Fig. 3C shows that PHA activation of PBLs results in an increase in cyclin T1 mRNA half-life. These data, along with data from Figs. 2A and 3B, demonstrate that PHA activation of PBLs results in increased cyclin T1 mRNA stability, which leads to steady accumulation of cyclin T1 mRNA by 48 h.
JNKs and calcineurin are required for PHA-mediated up-regulation of cyclin T1 in PBLs

PHA is a T cell mitogenic plant-lectin, which functions, at least in part, by binding component chains of the TCR (48). PHA stimulation of PBLs induces passage through the G0-G1 transition, but entry and progression through S phase requires IL-2 or PMA stimulation (22, 49). Both calcineurin and JNK are activated by engagement of the TCR, resulting in transduction of signals that mediate changes in gene expression (reviewed in Ref. 28). As such, we proceeded to dissect the components of this pathway that are required for cyclin T1 protein and mRNA up-regulation following PHA-mediated stimulation of PBLs. We started by performing JNK assays to determine whether these kinases are activated in response to PHA and/or PMA stimulation in PBLs under our experimental conditions. PBLs were activated with PMA, PHA, or both and collected at the indicated time points (Fig. 4A). Cells were lysed under non-denaturing conditions, and luciferase activity was measured. C, PBLs were stimulated with vehicle or PHA for 48 h. Actinomycin D (1 μg/ml) was added for the last 30 or 90 min of activation. RNA was isolated, and 15 μg of total RNA was analyzed by Northern blot using a cyclin T1 probe. Bands were visualized by autoradiography and quantified using MAC BAS. Lower panel, The average of two independent experiments.

FIGURE 4. PHA-mediated up-regulation of cyclin T1 requires JNK activation. PBLs were incubated with vehicle or SP600125 (10 μM), a specific JNK inhibitor, for 30 min and then collected or activated with PMA, PHA, or PMA/PHA for the indicated times. A, Cells were lysed under non-denaturing kinase assay buffer, JNK assays were performed as in A. B, Total RNA was isolated, and Northern blots were performed. Cyclin T1 mRNA levels were quantified using MAC BAS (lower panel). C, Cells were lysed under denaturing lysis conditions, and Western blots (WB) were performed as in Fig. 1 with the indicated Abs. D, Cells were lysed under non-denaturing kinase assay buffer, and JNK assays were performed as in A.
the ERKs, as has been suggested previously (50). To determine whether JNK activation is important for PHA-mediated up-regulation of cyclin T1, unstimulated PBLs were pretreated for 30 min with vehicle or SP600125 (10 μM) and then stimulated with PHA. As seen in Fig. 4, B and C, up-regulation of cyclin T1 mRNA and protein is prevented in the presence of SP600125, which effectively inhibited JNK activity (Fig. 4D), suggesting that JNK activation controls cyclin T1 expression by stabilizing cyclin T1 mRNA. CDK9 protein up-regulation mirrored that of cyclin T1 (Fig. 4C), which is consistent with stabilization of CDK9 by the increased availability of the newly synthesized, limiting, cyclin T1 subunit (43, 44). Up-regulation of cyclin H, the regulatory subunit of CDK7, which phosphorylates the CTD of RNAP II during transcriptional initiation, was also prevented. Additionally, the expression and phosphorylation status of proteins involved in cell cycle entry was also determined as a control. Fig. 4C shows that PHA also stimulated accumulation of cyclin D2, cyclin D3, and p107, as well as hyperphosphorylation of both p107 and pRB, all of which was prevented by SP600125 treatment, clearly showing that critical events associated with progression through the G1 phase of the cell cycle depend on JNK activity. However, in contrast to cyclin T1, the expression of p107 and cyclins D2 and D3 is regulated at the transcriptional level during cell cycle entry.

Next, we examined the dependence of cyclin T1 mRNA and protein up-regulation on the activation of calcineurin. As calcineurin is believed to act upstream of JNK, we anticipated that inhibition of calcineurin using the chemical inhibitor FK506 would block cyclin T1 up-regulation (reviewed in Ref. 28). As anticipated, FK506 blocked cyclin T1 mRNA and protein up-regulation (Fig. 5, A and B). FK506 also prevented accumulation of CDK9, the CTD kinase subunits cyclin H and CDK7, as well as the up-regulation of cell cycle regulators. Consequently, pRB phosphorylation was inhibited. Surprisingly, JNK activity was minimally affected by inhibition of calcineurin to levels that were sufficient to block the expression of cyclin T1, CDK9, cyclin H, CDK7, and all examined cell cycle regulatory proteins (Fig. 5C). Altogether, these data show that there are two parallel pathways, one JNK dependent and the other calcineurin dependent, that are both required for cyclin T1 mRNA stabilization following PHA stimulation of PBLs. These two pathways appear to independently control the expression of RNAP II CTD kinases and numerous cell cycle control proteins as well.

**Calcium-independent PKCs, but not ERKs, are required for PMA-mediated up-regulation of cyclin T1**

It is known that PMA induces downstream events by activating PKCs (51, 52). The most abundant PKC in T cells is PKC-θ, a calcium-independent PKC, whose activity is up-regulated 30-fold during T cell activation. The activity of PKC-δ, a calcium-dependent PKC, is up-regulated 4-fold, and all other known PKCs, calcium dependent and independent, have either negligible or no increase in activity (53). Rottlerin is a calcium-independent PKC inhibitor (54). As expected, inhibition of PKC-θ using rottlerin blocked cyclin T1 up-regulation (Fig. 6A). In contrast, inhibition of calcium-dependent PKC using PD98059 stimulated cyclin T1, pERK1/2 (Fig. 6B). These results demonstrate that calcium-independent PKCs, but not ERKs, are required for PMA-mediated up-regulation of cyclin T1.

**FIGURE 5.** PHA-mediated up-regulation of cyclin T1 requires calcineurin activation. PBLs were incubated with vehicle or FK506 (20 nM), a calcineurin inhibitor, for 30 min and then collected or activated with PHA for the indicated times. Cell lysates were analyzed by Northern blot (A) and Western blot (B). A, Total RNA was isolated, and Northern blots were performed. rRNA is used as loading control. Cyclin T1 mRNA levels were quantified using MAC BAS. B, Cells were lysed under denaturing lysis conditions, and 20 μg of protein lysate was analyzed by Western blot using the indicated Abs. C, Cells were lysed in non-denaturing kinase assay buffer, and JNK assays were performed as in Fig. 4.

**FIGURE 6.** PMA-mediated up-regulation of cyclin T1 protein levels requires Ca2+-independent PKC activation but not ERK1/2 activation. PBLs were incubated with rottlerin (5 μM), a Ca2+-independent PKC inhibitor (A), or PD98059 (30 μM), a MEK1 inhibitor (B), for 30 min and then collected or activated with PMA (1 ng/ml) for the indicated times. Cells were lysed under denaturing lysis conditions, and cyclin T1, phospho-ERK1/2, and total ERK1/2 protein levels were analyzed by Western blot (WB).
PMA-mediated up-regulation of cyclin T1 is a result of increased protein stability

As demonstrated in Fig. 2A, PMA-mediated up-regulation of the cyclin T1 protein is not due to an increase in cyclin T1 mRNA levels. Therefore, one possibility is that the increase in cyclin T1 steady-state levels is due to protein stabilization. To test this possibility, we determined whether stimulation of PBLs with PMA results in an increase in cyclin T1 protein half-life. PBLs were stimulated with PMA or vehicle for 48 h, treated with CHX (50 µg/ml), and collected 12 and 24 h later. Whole cell lysates obtained under denaturing conditions were analyzed by Western blot analysis. A representative experiment is shown in Fig. 7A, and the steady-state levels of cyclin T1 were quantified following densitometry analysis from three separate experiments using three different donors. Expression values were normalized using total ERK1/2 as a loading control. Cyclin T1 protein half-life was increased by 12 h in PBLs stimulated with PMA vs control unstimulated PBLs (Fig. 7, A and B). Because the kinetics of cyclin T1 up-regulation is slow, this increase in stability is likely sufficient to explain cyclin T1 accumulation over time in the absence of cyclin T1 mRNA up-regulation. Although our data show that PHA induces cyclin T1 mRNA stabilization, it is conceivable that PHA could concomitantly stabilize cyclin T1 protein. Thus, we determined the half-life of cyclin T1 in cells treated with PHA or PMA and in control PBLs. In contrast to PMA, our results clearly show that PHA does not result in stabilization of cyclin T1 protein (Fig. 7C).

We next examined whether the increase in cyclin T1 protein half-life was due to decreased degradation of cyclin T1 by the proteasome in PMA-stimulated cells, as it has been shown previously that the CDK9/cyclin T1 complex can be found in complexes with the SCF<sub>skp2</sub> ubiquitin ligase (44, 54). Our results show no significant change in cyclin T1 protein levels in either unstimulated or PMA-stimulated PBLs treated with proteasome inhibitors but dramatic stabilization of a known proteasomal substrate, p53 (Fig. 7D). These results indicate that cyclin T1 protein stabilization following PMA stimulation is independent of proteasomal degradation.

Engagement of the TCR via anti-CD3 stimulation is sufficient to induce cyclin T1 expression

The signaling pathways and the mechanisms involved in up-regulation of cyclin T1 expression following stimulation of PBLs with...
the expression of cyclins and CDKs involved in stimulation of correlation in the expression and phosphorylation of RNAP II with nated following stimulation with PMA and/or PHA. A striking whether the expression of cyclins and CDKs involved in the con-

we performed an experiment with multiple donors to determine cyclin T1 and other proteins upon stimulation of PBLs with PHA, Finally, as we have seen a correlation between up-regulation of

Expression of RNAP II kinases is coordinated following stimulation with T cell mitogens

Finally, as we have seen a correlation between up-regulation of cyclin T1 and other proteins upon stimulation of PBLs with PHA, we performed an experiment with multiple donors to determine whether the expression of cyclins and CDKs involved in the control of RNAP II phosphorylation and cell cycle entry was coordinated following stimulation with PMA and/or PHA. A striking correlation in the expression and phosphorylation of RNAP II with the expression of the cyclins and CDKs involved in stimulation of

Discussion

We and others (22, 23) have previously reported that cyclin T1 expression is up-regulated following stimulation of human PBLs with PMA and PHA. However, the mechanisms leading to up-regulation of cyclin T1 by independent mitogens have not been studied in detail. In the present study, we report that cyclin T1 expression is regulated independently by two mitogenic pathways via distinct mechanisms. Although PHA induces accumulation of both cyclin T1 mRNA and protein, PMA induces cyclin T1 protein up-regulation via stabilization without modulating cyclin T1 mRNA levels. We have also identified the mechanisms and components in each mitogenic signaling pathway that are required for up-regulation of cyclin T1 (Fig. 8).

Cyclin T1 expression is up-regulated during T cell activation

We and others (22, 23) have shown that cyclin T1 expression is up-regulated following mitogenic stimulation of human PBLs and that this correlates with phosphorylation of RNAP II in vivo and HIV replication (22). However, these studies were later challenged by a report suggesting that the apparent increase in cyclin T1 expression was due to degradation of cyclin T1 in cell lysates of unstimulated PBLs (35). We have carefully re-examined this issue and found that cyclin T1 protein is clearly up-regulated during costimulation of quiescent PBLs with both PMA and PHA as well as by each mitogen singly. Using stringent denaturing conditions, we typically find that cyclin T1 expression is up-regulated several fold, with minor variation among donors (Fig. 1). We suggest that the modest increases detected in cyclin T1 protein expression in the Martin-Serrano study (35) may be due to the use of PBMCs, which contain a very significant percentage of monocyte/macrophages, as well as stimulation with a single mitogen, which leads to lower induction of cyclin T1 expression than when both mitogens are applied together. In any case, it is clear that lysates should be prepared under denaturing conditions, as there is a protease in quiescent PBLs that apparently targets cyclin T1 for degradation during cell lysis under non-denaturing conditions, even in the presence of protease inhibitors. Unfortunately, this prevents an accurate measurement of cyclin T1-associated kinase activity, which can only be measured in non-denaturing lysates. Thus, we conclude that, while previous studies may have slightly overestimated the fold increase in cyclin T1 expression and associated activity, the notion that cyclin T1 expression is up-regulated during T cell activation and its likely relevance in this process remains valid.

PHA-mediated regulation of cyclin T1 expression

Analysis of cyclin T1 mRNA expression following stimulation with PHA/PMA singly, or in combination, showed that PHA, but not PMA, induces cyclin T1 mRNA. However, costimulation with PHA and PMA accelerated up-regulation of cyclin T1 mRNA significantly but not cyclin A mRNA expression, suggesting that downstream effectors of PMA synergize with PHA signaling in inducing cyclin T1 mRNA. It is unlikely that the increased expression of cyclin T1 simply reflects acceleration of steps leading
to T cell activation because this is not observed for cyclin A mRNA. We also report that the accumulation of cyclin T1 mRNA is due to message stabilization and not increased transcription. Interestingly, our data also shows that CHX itself induces cyclin T1 mRNA accumulation, suggesting that cyclin T1 mRNA stabilization involves depletion of a short-lived protein that stimulates cyclin T1 mRNA degradation. However, given the effects of CHX in the absence of mitogens, we cannot conclude that cyclin T1 mRNA stabilization does not require de novo protein synthesis in PHA-stimulated PBLs. Using PBMCs, as opposed to PBLs, others have reported that cyclin T1 mRNA levels are up-regulated by PMA (23, 35). Given that PMA does not induce cyclin T1 mRNA accumulation in PBLs, the effects observed in PBMCs by others are likely indirect, possibly due to signaling induced by the monocye/macrophage fraction present in PBMCs. In agreement with this proposal, stimulation with PMA is sufficient to induce T cell activation in PBMCs (35), but PBLs require costimulation with a second mitogen (22).

The downstream signaling steps that mediate T cell activation in response to engagement of the TCR have been studied in some detail (reviewed in Ref. 28). Previous reports have indicated that calcineurin is upstream of JNK, whose activation leads to AP-1-dependent stimulation of gene expression (55, 56). Using PBLs, we found that PHA stimulation was sufficient in inducing JNK activation and as potent as costimulation with PHA and PMA (Fig. 4). Our experiments demonstrate that the activities of both calcineurin and JNK are required for PHA-induced up-regulation of cyclin T1 mRNA. Interestingly, we found that an inhibitor of calcineurin prevents cyclin T1 mRNA up-regulation without inhibiting JNK activation (Fig. 5). This suggests two possibilities: either JNK is activated by a mechanism independent of calcineurin and is part of an independent pathway or JNK is upstream of calcineurin. As calcineurin has been shown to be directly activated by intracellular Ca²⁺ (reviewed in Ref. 57), we favor the first possibility (Fig. 9). This study has also revealed that several markers of cell cycle entry and progression through mid-G1 are induced by PHA. This includes up-regulation of a number of G1-S cyclins and phosphorylation of pRb and p107, which mark passage through the restriction point. Interestingly, inhibition of either JNK or calcineurin activities prevents up-regulation of cell cycle markers and pocket protein phosphorylation. Up-regulation of RNAP II CTD kinases (cyclin T1/CDK9 and cyclin H/CDK7) and G1-S cell cycle control proteins also occurs via stimulation with anti-CD3 Abs. Altogether, this indicates that increased cyclin T1 and CDK9 expression are coordinated with the expression of cell cycle regulators following TCR engagement of quiescent T cells. Of note, cell cycle entry in other quiescent cell types, such as primary fibroblasts or T98G cells, does not involve up-regulation of CDK9 and cyclin T1 (22, 44).

**PMA-mediated regulation of cyclin T1 expression**

Our results show that stimulation of PBLs with PMA does not result in up-regulation of cyclin T1 mRNA, but cyclin T1 protein levels are up-regulated. These effects are mediated by a Ca²⁺-independent PKC, likely PKC-θ, independently of ERK activation. In most donors, PMA induces cyclin T1 protein up-regulation to levels slightly lower than or comparable to those induced by PHA (Fig. 1, C and D). Our results show that PMA results in a reproducible increase in the half-life of the cyclin T1 protein (from 12 to 24 h). The cyclin T1/CDK9 complex associates with the SCF⁻skp² E3 ubiquitin ligase, which is involved in targeting multiple cell cycle regulatory proteins for proteosomal degradation, including p27 (58, 59), p130 (34, 60), and myc (61, 62). Although we have shown that SKP2 does not regulate CDK9 expression during the cell cycle (44), the possibility that this ubiquitin ligase regulates cyclin T1 expression in certain cell types has not been ruled out. Thus, we tested the possibility that the proteasome could be involved in the rapid degradation of cyclin T1 in unstimulated PBLs. However, a number of experiments using proteasome inhibitors (lactacystin and MG132) failed to show any significant stabilization of the cyclin T1 protein in quiescent or PMA stimulated PBLs (Fig. 7 and data not shown). Incubations with proteasome inhibitors were performed for short periods of time (up to 5 h) to minimize the possibility of secondary effects caused by stabilization of a protein(s) involved in regulating cyclin T1 stability in unstimulated PBLs. Thus, it appears that the low levels of cyclin T1 in unstimulated PBLs are not the result of rapid proteosomal-dependent degradation.

Finally, it is worth noting that, following stimulation of primary T cells with PHA or anti-CD3 Abs, there is a coordinated increase in the expression of both cell cycle control proteins and RNAP II kinases, coinciding with cell cycle entry into the G1 phase. In contrast, in PMA-stimulated PBLs, RNAP II CTD kinases are up-regulated, but events associated with entry into the G1 phase of the cell cycle, such as increased expression of G1-S cyclins (Fig. 10) and pockets protein phosphorylation (data not shown), are poorly induced. Thus, in PMA-stimulated cells, the up-regulation of RNAP II CTD kinases is likely in place to keep up with a gene expression program independent of cell proliferation. It is also important to note that the increases in cyclin T1 and CDK9 expression are exclusively associated with stimulation of quiescent T cells, as transformed T cells proliferate in the absence of PHA/PHA mitogens if provided with serum and natively express high levels of cyclin T1. Primary T cells are quiescent and respond to TCR/CD28 stimulation by entering the cell cycle and expressing molecules important for T cell function. The coordinated increase in the expression of cyclin T1 and transcriptional control proteins by both mitogens is likely in place to keep pace with the increased program of gene expression typical of rapidly proliferating and fully functional activated T cells.

**Acknowledgments**

We thank Qiang Zhou, Scott Shore, Danny Dhanasekaran, Alex Tsygankov, Muneer Hasham, and Clement Lee for cDNA constructs. PMLA-3.Luc.8.5E-plasmid was obtained through the National Institutes of Health AIDS Research Reference Reagent Program (National Institute of Allergy and Infectious Diseases) from Dr. Nathaniel Landau. We thank Dr. Gunther Boden, May Truongcao, and the Temple University General Clinical Research Center staff for assisting in the blood collections. We also thank Arun Jayaraman, Danny Dhanasekaran, and Alex Tsygankov for reading the manuscript and comments.

**Disclosures**

The authors have no financial conflict of interest.

**References**


28. Isakov, N., and A. Altman. 2002. Protein kinase C


8. Isakov, N., and A. Altman. 2002. Protein kinase C


5. Isakov, N., and A. Altman. 2002. Protein kinase C


2. Isakov, N., and A. Altman. 2002. Protein kinase C