

# CDK4 Expression and Activity Are Required for Cytokine Responsiveness in T Cells<sup>1</sup>

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Stimulation of lymphocytes through the Ag receptor can lead to cytokine responsiveness or unresponsiveness. We examined the importance of cyclin-dependent kinase (CDK)4 to establish and maintain IL-2 responsiveness in human T cells. Our results show that a herbimycin A- and staurosporine-sensitive phase of CDK4 expression and activity preceded the acquisition of IL-2-responsiveness in mitogen-stimulated peripheral blood T cells. Intriguingly, CDK4 expression and activity were demonstrable in purified unstimulated peripheral blood T cells from ~30% (5/16) of healthy individuals examined for this study. These T cells proliferated in response to IL-2 without additional mitogens, and both the expression and activity of CDK4 and the ability to respond to cytokines were resistant to herbimycin A and staurosporine. The pattern of CDK4 expression and response to IL-2 in this subset of individuals resembled that seen in the human IL-2-dependent Kit-225 T cell line. However, in contrast to normal T cells, Kit-225 cells were rendered unresponsive to IL-2 by stimulation through the Ag receptor. In these cells, PHA, anti-CD3, or PMA induced marked reductions of CDK4 expression and activity that paralleled IL-2 unresponsiveness, and these effects were not reversible by IL-2. Furthermore, IL-2-dependent proliferation could be similarly inhibited in Kit-225 cells by overexpression of the CDK inhibitors p16/Ink4-a or p21/Waf-1a or by overexpression of a kinase-inactive CDK4 mutant. The data indicate that CDK4 expression and activity are necessary to induce and maintain cytokine responsiveness in T cells, suggesting that CDK4 is important to link T cell signaling pathways to the machinery that controls cell cycle progression. *The Journal of Immunology*, 2000, 165: 6693–6702.

Stimulation of lymphocytes through the Ag receptor can lead to cytokine responsiveness or unresponsiveness (1). The mechanisms that mediate these diametrically opposite responses are incompletely understood. Stimuli that promote cytokine responsiveness are known to activate the cell cycle machinery, including cyclin-dependent kinase (CDK)<sup>7</sup>4, CDK6, and CDK2, along with their respective cyclin partners (2, 3). Alternatively, Ag receptor-mediated stimuli that block T cell proliferation induce sustained activation of mitogen-activated protein kinase

(MAPK) pathways as well as robust expression of the CDK inhibitor (CDKI) p21/Waf-1 with the consequent inhibition of CDK2 (4).

A central role for CDK4 in the acquisition of lymphocyte cytokine responsiveness seems likely. CDK4 may be the principal Rb kinase in mammalian cells. Rb, the retinoblastoma susceptibility protein, is the 110-kDa product of the prototypical tumor suppressor gene *RB-1*, a member of a family of nuclear pocket proteins that corral transcription factors linked to the G<sub>1</sub>/S transition (5, 6). Active, hypophosphorylated Rb sequesters these transcription factors and acts as a cell cycle brake (7). Phosphorylation of Rb leads to the functional collapse of the “pocket”, releasing the latent transcription factors and allowing their activation. CDK4 phosphorylates at least two distinct residues of Rb (S795 and T826) that are required for Rb inactivation (8, 9), and this CDK4-mediated Rb phosphorylation is required to render Rb sensitive to CDK2-mediated phosphorylation (10). Furthermore, CDK4 may serve as a sink for CDKIs, allowing for CDK2 activation and the resultant events that lead to S phase entry (11).

For the studies described in this report, we evaluated the importance of CDK4 expression and activity in T cells during the acquisition of a cytokine-responsive state and in the induction of cytokine unresponsiveness. We observed a direct correlation between CDK4 expression and activity, and the capacity of normal human T cells to respond to IL-2. Moreover, stimuli that led to cytokine unresponsiveness in T cell lines caused down-regulation of CDK4 expression and CDK4 activity, and overexpression of CDK4 antagonists impaired the ability of T cells to proliferate in response to IL-2. These results indicate that CDK4 expression and activity are necessary to establish IL-2 responsiveness in T lymphocytes, and they suggest this protein may play an important role in proliferative disorders of the immune system.

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<sup>7</sup> Abbreviations used in this paper: CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PTK, protein tyrosine kinase

## Materials and Methods

### Chemicals and reagents

Tissue culture materials were obtained from Nalge Nunc (Naperville, IL); chemicals were obtained from Sigma (St. Louis, MO) unless otherwise specified. PHA (PHA-P, Sigma) was dissolved in sterile water; PMA (Sigma), herbimycin A (LC Laboratories, Woburn, MA), and staurosporine (Calbiochem, La Jolla, CA) were dissolved in DMSO and diluted at least 1000-fold in the cell cultures. The addition of 0.1% sterile water or DMSO to T cell cultures as controls did not affect any of the parameters measured. Human rIL-2 was obtained from Hoffman-La Roche (Nutley, NJ) through the Biologic Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute (Frederick, MD).

### Cell cultures

Peripheral blood T cells were purified from apheresis residues by Ficoll-Hypaque (1.077 g/ml) density gradient centrifugation followed by depletion of adherent cells from the PBMC and E-rosetting on neuraminidase-treated sheep erythrocytes as described previously (3). The human IL-2-dependent Kit-225 T cell line, and the murine IL-2 dependent CTLL-2 cell line were maintained in tissue culture (3, 12). Proliferative responses of T cells or Kit-225 cells to mitogens were determined by the incorporation of [<sup>3</sup>H]thymidine (NEN Life Sciences, Boston, MA) into DNA 48 or 24 h after the onset of culture, respectively.

### Induction of competence

Human peripheral blood T cells were rendered competent to proliferate (cytokine-responsive) by stimulation with a submitogenic concentration of PHA (0.5 μg/ml) for 1 h followed by extensive washing (13). For each submitogenic stimulation experiment, an equal number of T cells was allowed to remain unstimulated, or was stimulated to proliferate by a mitogenic concentration of 10 μg/ml PHA. To confirm the acquisition of the cytokine-responsive state, proliferation was examined in cultures with or without 25 nM IL-2. Staurosporine and herbimycin A were added to the T cell cultures 30 min before stimulation. The inhibitors were washed along with the mitogen at the end of the competence induction period, and were not replenished for the duration of culture.

### Immunoblotting

Immunoblotting was performed as described previously (12). Briefly, cultured T cells were lysed in a buffer containing 300 mM sodium chloride, 50 mM Tris (pH 7.6), 0.5% Triton X-100, 1 mM *N*-ethylmaleimide, 30 mM aprotinin, and 500 nM leupeptin. Insoluble material and nuclei were removed by centrifugation, and protein concentrations of the cell lysates were determined using the bicinchoninic acid method (Bio-Rad, Hercules, CA). Cellular proteins (3 μg/lane) were separated by SDS-PAGE in 7–12% mini-gels, transferred to nitrocellulose membranes (Hybond; Amersham, Arlington Heights, IL), and probed using Abs against CDK4, cyclin D2, p27/Kip-1, p16/Ink4-a, p21/Waf-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Rb (PharMingen, San Diego, CA), or β-actin (Sigma). Digital images of immunoblots were used for quantification with NIH Image software version 1.6.2.

### Gene expression

**Northern blotting.** Cytosolic RNA was isolated from T cells as previously described (12). RNA (10 μg) was separated electrophoretically in 1% agarose and 5% formaldehyde denaturing gels and transferred to nylon 66 membranes (Gene Screen Plus; DuPont-NEN, Boston, MA) by capillary blotting. Steady-state levels of mRNA expression were assessed using a human CDK4 cDNA or a human *c-myc* cDNA. cDNA encoding the human β<sub>2</sub>-microglobulin gene was used as a loading control. Autoradiography was performed at –80°C using a single amplifying screen and reflection film (DuPont-NEN). Quantification of relative changes in mRNA expression was performed on digital images using NIH Image and normalized to the levels of β<sub>2</sub>-microglobulin.

**RT-PCR.** Messenger RNA levels also were analyzed by reverse transcription and amplification of cDNA by PCR (14). Briefly, 20 ng of total RNA were incubated with CDK4 or β-actin-specific primers in 50 μl of a reaction buffer containing 10 mM Tris (pH 8.3), 50 mM potassium chloride, 2.5 mM magnesium chloride, 0.001% (w/v) gelatin, 200 μM of each deoxynucleotide and 50 pM of oligonucleotide primer. cDNAs were generated using 25 U of murine leukemia virus reverse transcriptase (Roche Molecular Systems, Branchburg, NJ) for 15 min at 48°C followed by heating to 99°C for 5 min. PCR amplification of the resultant cDNA was conducted with 10 U *Taq* polymerase (Promega, Madison, WI) per reaction. Thirty cycles of amplification in a thermocycler (Lab-Line Instru-

ments, Melrose Park, IL) were used; the annealing temperatures for CDK4 and β-actin were 58°C and 60°C for 1 min, respectively. A melting temperature of 95°C for 1 min and an extension temperature of 72°C for 2 min were used for all reactions. PCR products (15 μl) were separated in 8% polyacrylamide gels and visualized by staining with ethidium bromide. The oligonucleotide primers sets used to amplify CDK4 and β-actin, respectively, were 5'-CTGAGAATGGCTACCTCTCGATATG-3' (forward) and 5'-AGAGTGTAAACAACACCGGGTGTAAAG-3' (reverse), 5'-ATGTTT GAGACCTTCAACACCCC-3' (forward) and 5'-GCCATCTCTTGCTC GAAGTCCAG-3' (reverse). The resultant PCR products were 541 bp (CDK4) and 317 bp (β-actin).

### Cyclin-dependent kinase assays

The kinase activity of CDK4 was determined as previously described (3). Briefly, cyclin-dependent kinase complexes were immunoprecipitated from cultured T cells and immune complex kinase assays were performed using [<sup>γ</sup>-<sup>32</sup>P]ATP and p56/Rb (a truncated rRb protein) as a substrate. The reactions were terminated by addition of SDS sample buffer and separated by SDS-PAGE. Autoradiography was performed at room temperature for 1–2 h. Quantification was performed by densitometric analysis of band densities, and for some experiments, the levels of CDK4 kinase activity were confirmed by excision of bands from the gels followed by liquid scintillation counting. In the latter cases, 1 U/min of CDK4 kinase activity was defined as the incorporation of 1 fmol of phosphate/min into the substrate.

### Transfections

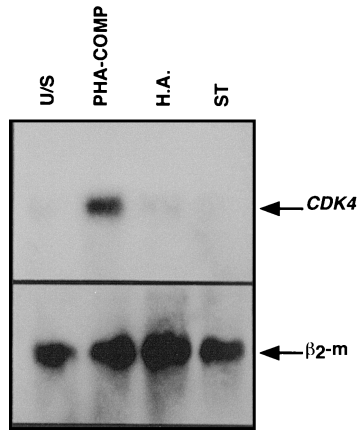
Transient transfections were performed using cationic liposomes (Dosper; Boehringer Mannheim, Indianapolis, IN; Ref. 14). Genes encoding wild-type CDK4 and kinase-inactive CDK4 (15), p16/Ink-4a (16), or p21/Waf-1 (17) were subcloned into the multiple cloning site of the pBK-RSV eukaryotic expression vector (Stratagene, La Jolla, CA). The pGL3 expression vector (Promega) encoding the firefly luciferase gene under the control of a constitutive promoter was used to monitor transfection efficiencies. Twenty micrograms of each experimental expression vector and 0.5 μg of pGL3 were mixed with 50 μg of the cationic liposome reagent in 100 μl of HEPES-buffered saline and incubated for 15 min at 25°C. The empty pBK-RSV vector was used as a negative control. These mixtures were added in 1 ml OptiMEM serum-free medium (Life Technologies) to 1 × 10<sup>7</sup> IL-2-deprived Kit-225 cells. The cell-DNA-liposome mixtures were incubated for 6 h at 37°C, followed by the addition of 3 ml of supplemented media, and incubation overnight at 37°C. Luciferase activity was examined using a commercial assay kit (Promega) as described (14).

## Results

### CDK4 expression and activity in competent human T cells

We showed previously that induction of T cell competence stimulated expression of CDK4 mRNA that was independent of cytokine signals and resistant to cyclosporin A (3, 18). The increased CDK4 gene expression was abrogated by cycloheximide, suggesting it required new protein synthesis (19). To analyze the activation signals responsible for the cytokine-independent phase of CDK4 expression, we evaluated the effect of signaling inhibitors that are known to impair protein kinase activities on CDK4 gene expression in competent primary human T cells. There was no detectable CDK4 mRNA in unstimulated, cytokine-unresponsive (resting) T cells (Fig. 1). To ensure that the inability to detect CDK4 message in these cells was not due to technical limitations of the Northern blot analysis, the experiments were repeated using qualitative RT-PCR analysis and the same results were obtained (Fig. 2A). As shown previously (3, 19), small amounts (ranging from 0 to 30% of the levels seen in competent T cells) of pre-existing CDK4 protein were detectable in resting unstimulated T cells (Figs. 2B and 3A), and immunoprecipitated CDK4 complexes from these cells revealed marginal kinase activity (ranging from 7 to 20% of the levels seen in competent T cells) *in vitro* (Fig. 2C). Unstimulated, resting T cells did not proliferate spontaneously or in response to exogenous IL-2 (Figs. 2D and 3A).

CDK4 mRNA was detectable within 3–6 h in T cells that were rendered cytokine responsive (competent to proliferate) by stimulation with submitogenic concentrations of PHA (Figs. 1 and 2A).



**FIGURE 1.** Ag receptor signaling pathways required for CDK4 gene expression in competent T cells. RNA was isolated from resting peripheral blood T cells (U/S), T cells rendered competent by stimulation with a submitogenic concentration of PHA (0.5  $\mu\text{g}/\text{ml}$  for 1 h, PHA-COMP), or T cells stimulated with submitogenic PHA in the presence of 3  $\mu\text{M}$  herbimycin A (H.A.) or 10 nM staurosporine (ST). CDK4 gene expression was assessed by Northern blotting 5 h after stimulation. Messenger RNA levels of  $\beta_2$ -microglobulin were measured as loading controls.

Induction of T cell competence increased the levels of CDK4 protein in whole cell lysates (Figs. 2B and 3A). For purposes of quantification, the levels of CDK4 expression in competent T cells were considered to be equal to 1.0, because CDK4 protein was undetectable in resting cells from some individuals. The increase in CDK4 was variable, but it appeared to correlate with the capacity of cells to proliferate in response to exogenous IL-2. These conditions similarly increased CDK4 kinase activity as determined by an *in vitro* immune complex assay (Fig. 2C). Furthermore, this stimulation enabled these cells to proliferate in response to IL-2 (Fig. 2D).

CDK4 message was readily detectable by RT-PCR in T cells stimulated by a mitogenic concentration of PHA (Fig. 2A), although this assay is not quantitative. Nevertheless, mitogenic stimulation resulted in higher levels of CDK4 protein accumulation (1.25- to 4.2-fold greater than those seen in competent T cells; Figs. 2B and 3A) and CDK4 activity (1.25- to 5.4-fold greater than those seen in competent T cells; Fig. 2C). Under these conditions, T cells produce saturating levels of IL-2 (3, 13), which leads to proliferation without the need for exogenous IL-2 (Fig. 2D).

The steady-state levels of CDK4 mRNA in T cells provided with the competence-inducing stimulus were significantly reduced or abrogated by herbimycin A, a protein tyrosine kinase (PTK) inhibitor and by staurosporine, a protein kinase C (PKC) inhibitor (Figs. 1 and 2A). In parallel with these effects, CDK4 protein and CDK4 activity in T cells that received the competence-inducing stimulus in the presence of herbimycin A and staurosporine were comparable to those seen in resting T cells (Figs. 2, B–C, and 3A). In cells treated with herbimycin A and staurosporine, respectively, the levels of CDK4 protein ranged from 4 to 50%, and from 19 to 54%, and the levels of CDK4 kinase activity ranged from 14 to 44%, and from 21 to 52% of those seen in competent T cells. The reduced CDK4 activity in T cells stimulated in the presence of herbimycin A or staurosporine was not due to direct inhibition of CDK4 by these compounds (data not shown). Moreover, both of these compounds prevented T cells from acquiring a cytokine-responsive state (Fig. 2D).

The functional significance of these findings was illustrated by 5 healthy individuals (of 16 examined) who had a detectable proportion of peripheral blood T cells that responded to IL-2 without

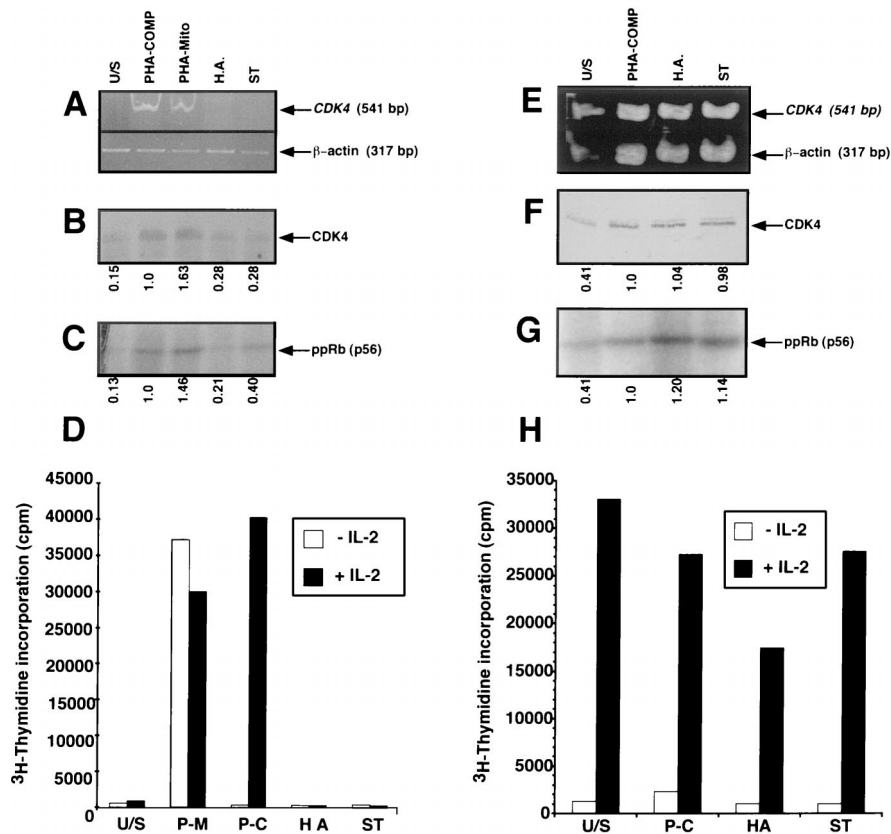
exogenous stimuli (Figs. 2H and 3B). Pre-existing CDK4 mRNA (Fig. 2E), protein (Figs. 2F and 3B), and activity (Fig. 2G) were detectable in unstimulated cells from these individuals. The levels of CDK4 protein and activity in unstimulated T cells ranged from 41 to 80% of those seen in cells from these individuals following competence-inducing stimuli (Figs. 2, F–G, and 3B), but the responses to IL-2 were not enhanced significantly beyond those seen in the unstimulated T cells (Figs. 2H and 3B). CDK4 expression and activity in these cells were refractory to the effects of herbimycin A and staurosporine (Figs. 2, E–G, and 3B). These observed levels of CDK4 expression and activity in this case were not significantly different; cells treated with herbimycin A and staurosporine, respectively, had levels of CDK4 protein ranging from 60 to 126%, and from 90 to 98%, and levels of CDK4 kinase activity ranging from 88 to 120%, and from 90 to 114% of those seen in competent T cells. This suggests that the expression and activity of CDK4 were not limiting in these cases (see also Fig. 3B); however, the observation that herbimycin A frequently inhibited IL-2-dependent proliferation of these cells by 20–50% indicates that this compound may affect additional events associated with IL-2 signaling.

Assembly of an active CDK4 holoenzyme complex requires association with a D-type cyclin and dissociation from CDKI (20). We were able to evaluate expression of cyclin D2 (the initial partner for CDK4), p27/Kip-1, p21/Waf-1, and p16/Ink4-a in T cells from individuals representative of the resting phenotype ( $n = 2$ ) and the preactivated phenotype ( $n = 1$ ). Consistent with previous reports (21, 22), cyclin D2 was absent and p27/Kip-1 was abundant in unstimulated T cells from individuals with the resting phenotype. Induction of competence led to elevated levels of cyclin D2 (considered as 1.0 for purposes of quantification) within 6 h of stimulation, as well as disappearance of p27/Kip-1 from the cells (Fig. 3A). Predictably, even higher levels of cyclin D2 (~6-fold greater than those seen in the competent T cells) were seen in cells that received mitogenic stimuli that promoted IL-2 production and proliferation (Fig. 3A). Similar to the block of CDK4 expression, herbimycin A and staurosporine largely prevented accumulation of cyclin D2 in T cells that received competence-inducing stimuli (to levels that were less than 10% of those seen in the competent T cells), although treatment with these kinase inhibitors did not prevent disappearance of p27/Kip-1. Expression of p16/Ink4-a and p21/Waf-1 proteins was detectable at low levels and restricted, respectively, to unstimulated T cells and to T cells that received a mitogenic stimulus.

Along with elevated levels of pre-existing CDK4 protein, unstimulated cells with the preactivated phenotype also had detectable levels of cyclin D2 with remarkably reduced p27/Kip-1 (Fig. 3B). Intriguingly, the levels of CDK4 and cyclin D2 in cells from these individuals decreased following stimulation with mitogenic levels of PHA, with no appreciable effect on proliferation, supporting the notion that CDK4 activity was no longer limiting for proliferation of these cells (Fig. 3B). Cyclin D2 expression also persisted longer (>20 h), particularly in the stimulated cells.

#### *Reduction of CDK4 expression and activity lead to IL-2 unresponsiveness*

We used IL-2-dependent T cell lines to evaluate the significance of the correlation between CDK4 expression and activity and cytokine responsiveness. Human Kit-225 cells have wild-type *RB-1* ( $Rb^{+/+}$ ) and express surface CD3, CD4, CD28, and CD25. These cells undergo growth arrest under conditions of IL-2-deprivation and proliferate in response to IL-2 stimulation, much as normal competent T cells do (Refs. 3 and 23; Fig. 4A). The proliferative response of Kit-225 cells to IL-2 was specific, as it could be



**FIGURE 2.** Direct correlation between CDK4 expression and activity and cytokine responsiveness in primary T cells. CDK4 gene expression (A and E), CDK4 protein accumulation (B and F), CDK4 kinase activity (C and G), and competence to proliferate in response to IL-2 stimulation (D and H) were measured in resting peripheral blood T cells (U/S), T cells stimulated with a mitogenic concentration of PHA (10  $\mu\text{g}/\text{ml}$ , PHA-Mito), T cells stimulated with submitogenic PHA (0.5  $\mu\text{g}/\text{ml}$  for 1 h, PHA-COMP), or T cells stimulated with submitogenic PHA in the presence of 3  $\mu\text{M}$  herbimycin A (H.A.) or 10 nM staurosporine (ST) as indicated. Expression of the CDK4 and  $\beta$ -actin genes was assessed by RT-PCR 5 h after stimulation; cell lysates were prepared 15 h after stimulation for immunoblotting and for immunoprecipitation of CDK4 complexes for *in vitro* kinase activity assays by phosphorylation of a truncated rRb protein (p56/Rb); and IL-2 responsiveness was determined by [ $^3\text{H}$ ]thymidine incorporation 48 h after stimulation. Twelve donors showed the resting phenotype shown in A–D. The donor shown is representative of data from 3 (A and C), 9 (B), and 11 (D) individuals, respectively. Five donors showed the preactivated phenotype shown in E–H. The donor shown is representative of data from two (E and G), three (F), and five (H) individuals, respectively. Densitometric data provided under the immunoblots and kinase autoradiographs are normalized to a level of 1.0 present in competent T cells. Equivalent loading was confirmed by staining gels after transfer and membranes after immunoblotting with Coomassie blue.

blocked by anti-IL-2 Abs, but not by nonimmune rabbit IgG (Fig. 4A). Growth-arrested Kit-225 cells contained detectable levels of CDK4 message (Fig. 4B), CDK4 protein (Fig. 4C), and CDK4 activity (Fig. 4D) that were comparable to those seen in normal competent T cells, although most of the Rb in these cells accumulated in a hypophosphorylated state (Fig. 4E). The expression of CDK4 mRNA in growth-arrested Kit-225 cells did not result from a generalized state of activation, as expression of *c-myc* was undetectable in these cells, and was up-regulated by IL-2 stimulation (Fig. 4B).

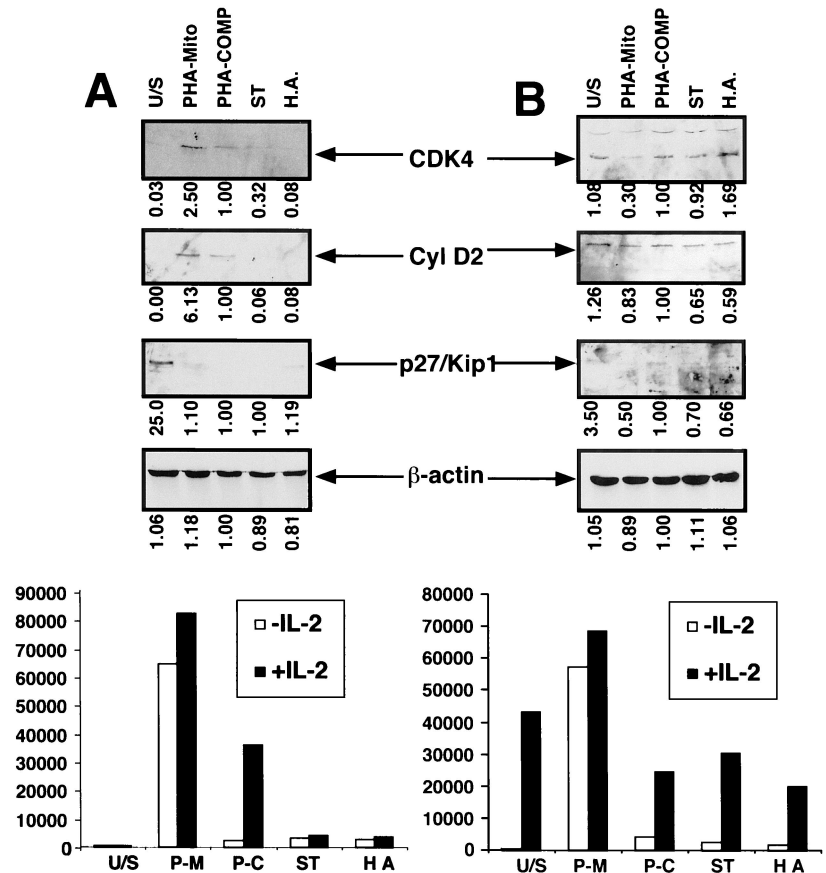
IL-2 stimulation led to an  $\sim 4$ -fold increase in the steady-state levels of CDK4 mRNA in Kit-225 cells (Fig. 4B), along with a modest (10–24%) increase in CDK4 protein (Fig. 4C). IL-2 stimulation similarly led to increased CDK4 activity in Kit-225 cells by 2- to 8-fold (Fig. 4D), and hyperphosphorylation of Rb within 1 h of stimulation (Fig. 4E). Hyperphosphorylated Rb was the predominant form of the protein in both Kit-225 cells progressing through the  $G_1$  phase 16 h after stimulation and Kit-225 cells cultured under conditions of asynchronous growth (Fig. 4E).

Ligation of the Ag receptor/CD3 complex in Kit-225 cells (using PHA or anti-CD3), with or without concomitant ligation of CD28, impaired their capacity to proliferate in response to IL-2 (Fig. 5A). Similar results were obtained when the murine IL-2-

dependent CTLL2 cell line was stimulated by anti-CD3. These stimuli did not lead to a detectable increase in expression of Fas or a detectable decrease in the expression of IL-2 receptors. Cytokine unresponsiveness was neither due to increased cell death, nor to polarization of the cells to a Th2 phenotype, as the cells did not proliferate in response to IL-4 (data not shown). In Kit-225 cells, IL-2 unresponsiveness could also be induced by PMA (Fig. 5A), but not by the PKC inhibitors staurosporine or H7, by PTK inhibitors genistein or tyrphostin 25, or by EDTA.

Steady-state levels of CDK4 mRNA were reduced by 60–95% in Kit-225 cells that were rendered cytokine unresponsive by stimulation with either PHA or PMA (Fig. 5B). Similarly, stimulation with anti-CD3 led to a 10–20% decrease in the levels of CDK4 protein (Fig. 5C); and PHA, anti-CD3, and PMA significantly reduced or abrogated the activity of CDK4 in these cells (Fig. 5D). This observed reduction in CDK4 activity could only be partially reversed by the addition of exogenous IL-2 (Fig. 5E). It is noteworthy that stimulation of Kit-225 cells by PMA, PHA, or anti-CD3 (in the presence or absence of anti-CD28) increased MAPK activity, indicating that these stimuli were able to deliver positive activation signals to Kit-225 cells. This was confirmed by the observed elevation of cyclin D2 protein levels in Kit-225 cells treated with anti-CD3 (Fig. 5C). The magnitude by which treatment with

**FIGURE 3.** Direct correlation between expression of CDK4-associated proteins and cytokine responsiveness in primary T cells. CDK4, cyclin D2 (Cyl D2), and p27/Kip-1 protein accumulation, along with competence to proliferate in response to IL-2 stimulation were measured in resting peripheral blood T cells (U/S), T cells stimulated with a mitogenic concentration of PHA (10  $\mu$ g/ml, PHA-Mito), T cells stimulated with submitogenic PHA (0.5  $\mu$ g/ml for 1 h, PHA-COMP), or T cells stimulated with submitogenic PHA in the presence of 10 nM staurosporine (ST) or 3  $\mu$ M herbimycin A (H.A.) as indicated. A donor with the resting phenotype is shown in *A*. A donor with the preactivated phenotype is shown in *B*. Densitometric data provided under the immunoblots are normalized to a level of 1.0 present in competent T cells in relation to the expression of  $\beta$ -actin in each sample.



PHA, anti-CD3, or PMA reduced CDK4 activity could not be completely explained by changes in the levels of CDK4 protein. Messenger RNA for p21/Waf-1 and p16/Ink4-a were present in unstimulated Kit-225 cells, although the proteins were undetectable by immunoblotting. In preliminary experiments, the levels of both of these CDKI, as well as p27/Kip-1, appeared to increase upon treatment of Kit-225 cells with PMA, PHA, or anti-CD3 (data not shown).

To confirm the importance of CDK4 activity in T cell cytokine responsiveness, we evaluated the effects of ectopic overexpression of CDK4 antagonists (a catalytically inactive CDK4 mutant with a disrupted ATP binding site, or wild-type genes encoding the CDKIs p16/Ink-4a or p21/Waf-1) on IL-2-dependent Kit-225 cell proliferation. Because selection of cells expressing molecules that suppress growth can be difficult, these experiments were done using transient transfection conditions. The transfection efficiency for each condition in these experiments was determined by the luciferase activity achieved from a cotransfected luciferase expression vector (pGL3) also under the control of the RSV promoter. IL-2-dependent proliferation of Kit-225 cells was not affected by transfection with a control vector (RSV neo) or wild type CDK4 (Fig. 6A). Conversely, each of the CDK4 antagonists decreased IL-2-stimulated proliferation of Kit-225 cells by 60–80% (Fig. 6A).

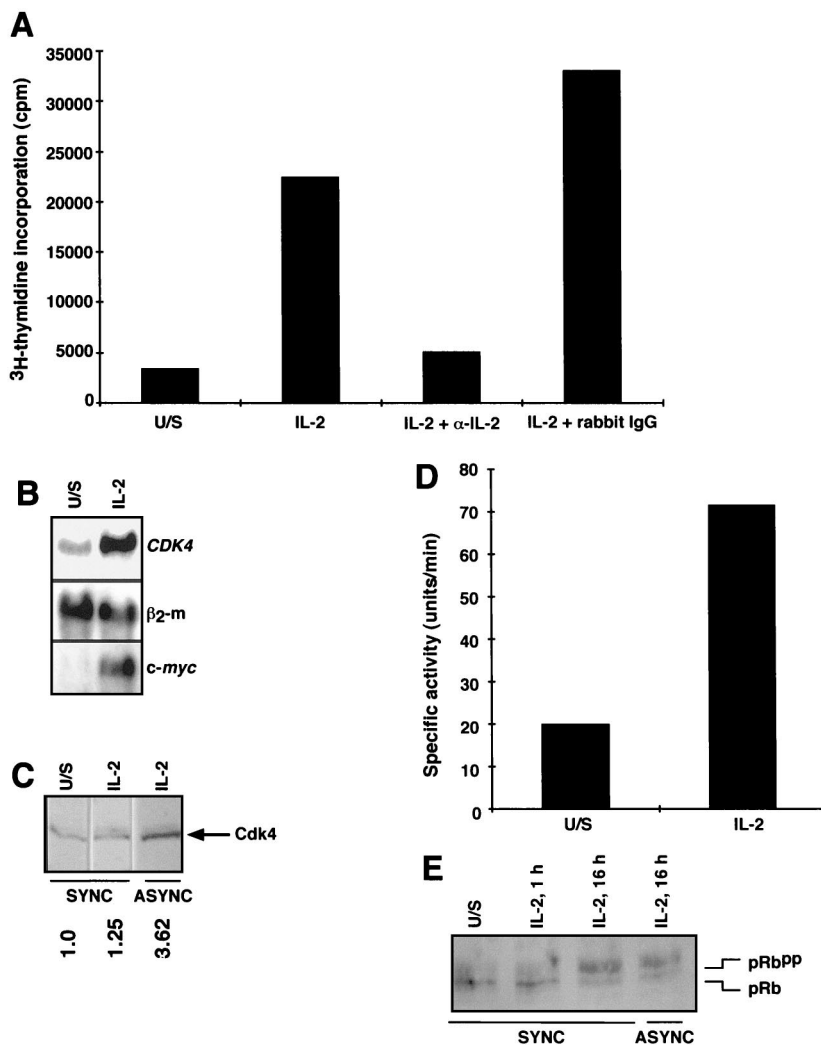
The levels of immunoreactive CDK4 were higher (by ~1.2- to 2-fold) in the cells transfected with the CDK4 constructs (Fig. 6B). In contrast, CDK4 appeared to be reduced in Kit-225 cells transfected with either p16/Ink-4a or p21/Waf-1 (Fig. 6B, lanes 3 and 4); and both basal and IL-2-stimulated CDK4 activity were reduced by >50% in Kit-225 cells transfected with any one of the three CDK4 antagonists (Fig. 6C).

## Discussion

A finding common to growth-arrested, growth factor-responsive T cells (2, 3), B cells (24, 25), fibroblasts (26), and other cell types (27, 28) is the expression of the cyclin-dependent kinases CDK4 and/or CDK6 and of the D-type cyclins, their preferred partners during the early G<sub>1</sub> phase. Conversely, decreased expression and activity of CDK4 have been correlated with cell cycle exit and terminal differentiation in hematopoietic cells (29, 30). Previous work from our group showed that the initial induction of CDK4 (and cyclin D2) expression and activity during the acquisition of the competent state in T cells was independent of cytokine-mediated (progression) signals (3, 18). The studies described in this report were undertaken to examine the role of CDK4 in the establishment of a cytokine-responsive state in T cells.

The experiments described in this report show that there is a direct correlation between CDK4 expression and activity and T cell cytokine responsiveness. A critical role for CDK4 in cell cycle entry and the early stages of progression through the G<sub>1</sub> phase have been demonstrated in various cultured cell lines (31, 32). However, it is generally believed that a growth factor signal is required to promote assembly and activation of active CDK4 complexes (33–36). Resting primary T cells that are unable to respond to cytokines appear to have little or no CDK4 mRNA, and the presence of small amounts of pre-existing CDK4 protein in these cells appears to be inconsequential as its potential for activation is repressed by the presence of CDKI such as p27/Kip-1 and p16/Ink-4a and by the absence of D-type cyclins (2, 3, 37). Our results show that activation of PTK and PKC were required to promote expression of CDK4 and cyclin D2, as well as the cytokine-responsive state in mitogen-stimulated T cells. These data are in

**FIGURE 4.** Kit-225 cells resemble competent T cells and can be rendered unresponsive to cytokines by down-regulating CDK4 expression and activity. **A**, Kit-225 cells were deprived of IL-2 for 72 h, and incubated in the presence or absence of IL-2, anti-IL-2 Ab, or control rabbit IgG as indicated. Proliferation was evaluated by [ $^3$ H]thymidine incorporation. **B**, Expression of CDK4 and *c-myc* was evaluated by Northern blotting in growth-arrested Kit-225 cells (U/S), or Kit-225 cells stimulated for 3 h with IL-2. The expression of  $\beta_2$ -microglobulin was used as a loading control. **C**, CDK4 protein accumulation was evaluated in growth-arrested Kit-225 cells before (U/S) or 16 h after IL-2-stimulation (SYNC), as well as in Kit-225 cells growing asynchronously in the presence of IL-2 (ASYNC). **D**, The kinase activity of Cdk4 was assessed in immunoprecipitates from growth-arrested (U/S) Kit-225 cells or Kit-225 cells stimulated by IL-2 16 h after the onset of culture using a truncated form of the rRb protein (p56/Rb) as a substrate. One unit/minute of specific activity was defined as the incorporation of 1 fmol of phosphate per min into the substrate. **E**, Rb phosphorylation was evaluated in growth-arrested Kit-225 cells before (U/S) or after 1 or 16 h of IL-2-stimulation that elicits synchronous cell cycle entry (SYNC), and in asynchronously growing Kit-225 cells (ASYNC). Hypophosphorylated Rb exhibits a faster electrophoretic mobility ( $M_r \sim 110$  kDa); Rb that is hyperphosphorylated migrates slower ( $M_r \sim 115$ – $120$  kDa). Densitometric data provided under the immunoblot are normalized to a level of 1.0 present in growth-arrested, unstimulated Kit-225 cells.



agreement with a previous report showing that both calcium-dependent signals and PKC-dependent signals are required for CDK4 expression in human T cells (38).

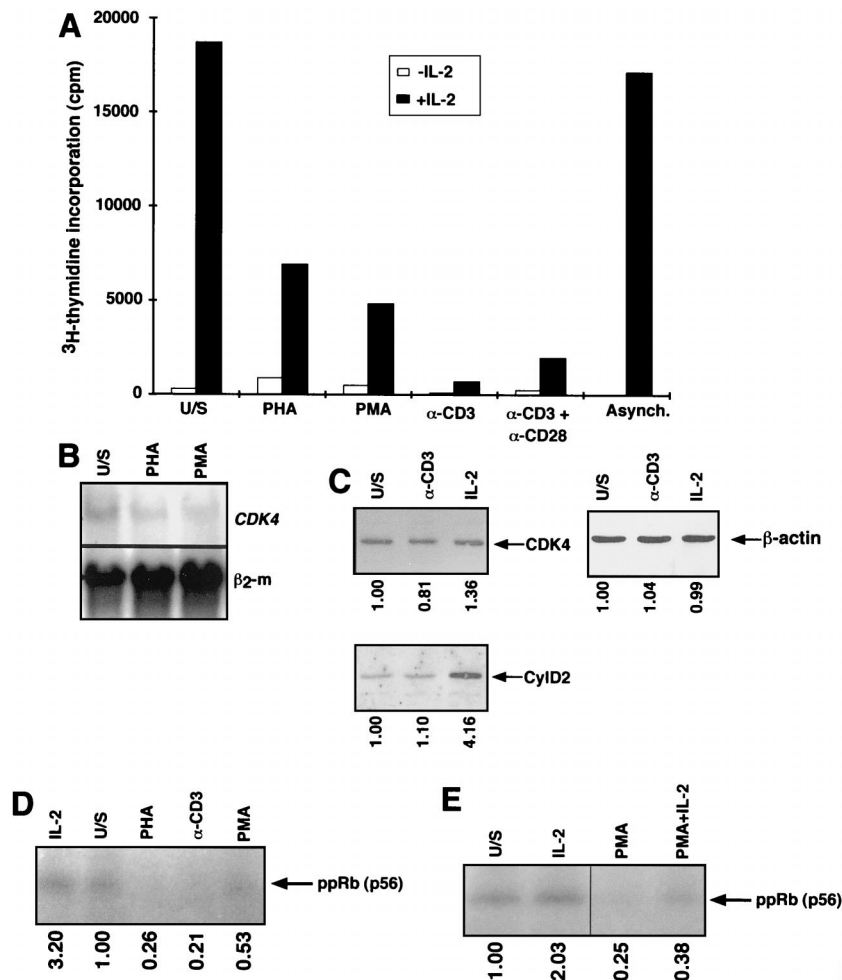
In this study, we also identified 5 individuals (of 16 examined) whose T cells could respond to IL-2 *in vitro* in the absence of prior stimulation. T cells from these individuals were characterized by detectable levels of CDK4 message, pre-existing cyclin D2, reduced p27/Kip-1, and higher constitutive CDK4 activity than that seen in cytokine unresponsive resting T cells. In addition, the expression and activity of CDK4 in these cells, and their capacity to respond to IL-2 without additional mitogenic stimuli, were refractory to PTK or PKC inhibitors. CDK4 activity and IL-2-dependent proliferation of Kit-225 cells were similarly unaffected by inhibitors of PTK or PKC. Underlying differences that could account for the discrepancy in CDK4 expression and activity, and cytokine responsiveness between these two groups of individuals include atopic disease, allergies, or other minor immune disturbances. The individuals for this study were recruited from pools of healthy apheresis donors in the states of Colorado and Texas; however, donors' identification remained confidential and clinical histories were not reviewed. Nevertheless, the data suggest that CDK4 activity may be a useful marker for the T cell-competent state.

Stimulation through the Ag receptor can lead to cytokine unresponsiveness in T cell lines and B cell lines that require cytokines for growth (1, 39, 40). We took advantage of this to examine how CDK4 was affected in cells that lost their ability to respond to IL-2.

The importance of CDK4 to establish cytokine responsiveness was reiterated by the observation that stimuli that reduced CDK4 expression and activity also impaired proliferation of the IL-2-dependent T cell lines Kit-225 and CTLL-2. CDK4 expression and activity were detectable in these IL-2-dependent cell lines under conditions of asynchronous growth. Under conditions of IL-2 withdrawal, CTLL-2 cells lost CDK4 activity within 3–6 h and underwent apoptosis after 6–24 h. CDK4 activity in CTLL2 cells was rapidly restored by IL-2 stimulation. In contrast, IL-2-deprived Kit-225 cells were more resistant to cytokine withdrawal. Upon removal from IL-2-containing media, Kit-225 cells underwent growth arrest near the  $G_0/G_1$  boundary with accumulation of the hypophosphorylated form(s) of Rb, but they retained CDK4 expression and activity at levels similar to those seen in competent peripheral blood T cells. These cells remained viable for up to 96 h, suggesting that CDK4 expression and activity may increase the resistance of lymphoid cells to apoptosis.

The presence of CDK4 message in Kit-225 cells was unlikely to be due to residual stimulation or a reservoir of proliferating cells, as *c-myc* expression was absent in these cells. Stimulation by anti-CD3 inhibited the capacity of both CTLL-2 cells and Kit-225 cells to respond to IL-2. PHA and PMA reduced CDK4 gene expression in Kit-225 cells by at least 65%, and in most cases by >80%. This is in contrast to stimulation of Kit-225 cells by IL-2, which increased the steady-state levels of CDK4 mRNA by ~4-fold. Small

**FIGURE 5.** Kit-225 cells can be rendered unresponsive to cytokines by down-regulating CDK4 expression and activity. *A*, IL-2-deprived Kit-225 cells were stimulated by PHA, anti-CD3 (OKT3), anti-CD28 (Ab 9.3), and PMA with or without IL-2 as indicated. The proliferation of asynchronously growing Kit-225 cells in the presence of IL-2 is shown for comparison. Proliferation was evaluated by [ $^3$ H]thymidine incorporation. *B*, Expression of CDK4 was evaluated by Northern blotting in growth-arrested Kit-225 cells (U/S), or Kit-225 cells stimulated for 3 h with PHA or PMA. The expression of  $\beta_2$ -microglobulin was used as a loading control. *C*, Accumulation of CDK4 and cyclin D2 proteins was evaluated by immunoblotting in growth-arrested Kit-225 cells before (U/S) or 5 h after anti-CD3 or IL-2-stimulation. The expression of  $\beta$ -actin was used as a loading control. *D*, Kit-225 cells were synchronized by IL-2 deprivation and lysates were prepared from unstimulated cells (U/S), cells stimulated by IL-2, PHA, anti-CD3, or PMA for 4 h. CDK4 complexes were immunoprecipitated and kinase activity was determined in vitro by phosphorylation of a truncated rRb protein (p56/Rb). *E*, Kit-225 cells were synchronized by IL-2 deprivation and lysates were prepared from unstimulated cells (U/S), cells stimulated by IL-2, PMA-treated cells, and PMA-treated cells stimulated by IL-2 after 4 h in culture. CDK4 activity was determined as in Fig. 3*D*. Densitometric data provided under the immunoblots are normalized to a level of 1.0 present in growth-arrested, unstimulated Kit-225 cells in relation to the expression of  $\beta$ -actin in each sample; data under the kinase autoradiographs are normalized to a level of 1.0 present in growth-arrested, unstimulated Kit-225 cells.



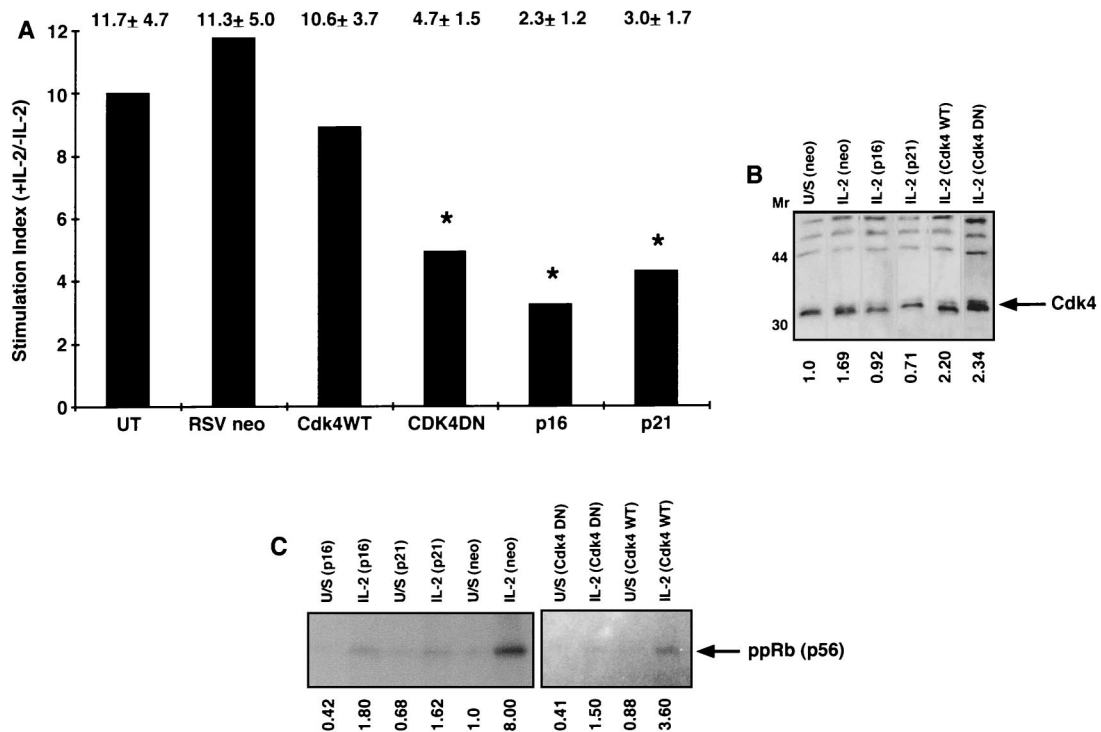
increases in CDK4 protein were observed in Kit-225 cells stimulated by IL-2 for 1–3 h, but CDK4 activity increased by ~2- to 8-fold, suggesting IL-2 mediates additional events required for assembly and activation of active holoenzyme complexes (31, 33). CDK4 activity was essentially abrogated in Kit-225 cells treated with anti-CD3, PHA, or PMA, and it could only be partially rescued by IL-2. The reduction in CDK4 activity could be due in part to the observed attenuation in CDK4 message, leading to decreased CDK4 protein synthesis and increased turnover. However, we observed only a small reduction in the levels of CDK4 protein under these conditions. Thus, the inhibitory effects are likely to have also been mediated by inactivation of cofactors required for assembly and activation of CDK4/cyclin D holoenzyme complexes. A similar phenomenon was reported in MEL erythroleukemia cells induced to terminal differentiation by treatment with hexamethylene bisacetamide. In these cells, hexamethylene bisacetamide treatment increased expression of D-type cyclins, reduced the levels of CDK4 and diminished Rb phosphorylation, and these effects could be reversed by ectopic overexpression of CDK4, but not CDK2. However, the reduction in CDK4 was mediated by decreased stability of the protein apparently without affecting protein synthesis (30).

To confirm the importance of CDK4 in establishment of the cytokine-responsive state in T cells, we ectopically expressed either of two CDKIs in Kit-225 cells before stimulation by IL-2. Both p16/Ink4-a and p21/Waf-1 decreased the levels of CDK4 protein, CDK4 activity and IL-2-dependent proliferation in Kit-225 cells. The decreased proliferation observed under these con-

ditions also could have been due to inhibition of CDK6 or CDK2. However, the reduction of CDK4 activity and IL-2-dependent proliferation by overexpression of a catalytically inactive CDK4 mutant supports the notion that CDK4 is critical for cell proliferation in response to IL-2. We obtained similar results in preliminary experiments where we used antisense oligonucleotides to suppress synthesis of this protein in Kit-225 cells.

Precisely how CDK4 participates in the response to growth factors remains incompletely understood. CDK4 is an important Rb kinase during G<sub>1</sub> phase progression (9). There also is evidence to suggest that CDK4 may serve to remove CDKIs from CDK2 complexes in response to IL-2 (11, 41) or serum (35). Given the dominant-negative effect of inactive CDK4, and the fact that IL-2 responsiveness was not enhanced in cells expressing approximately twice the normal levels of wild-type CDK4, it is likely that the role of this enzyme during proliferative responses to growth factors must be more substantial than to serve to remove CDKIs from other CDKs.

Other mechanisms may contribute to cytokine unresponsiveness in T cell and B cell lines stimulated through the Ag receptor. One mechanism postulated to account for this anergic state in anti-CD3-stimulated T cells is the induction of calcium mobilization in the absence of costimulation by CD28 (42–44). However, anti-CD3, PHA, or PMA did not induce calcium mobilization in Kit-225 cells, and costimulation by anti-CD28 was unable to prevent or reverse the cytokine-unresponsive state in these cells. This suggests that costimulation may be more important in the activation of



**FIGURE 6.** CDK4-associated kinase activity is necessary for IL-2-dependent Kit-225 cell proliferation. **A**, Kit-225 cells ( $1 \times 10^7$ /condition) were deprived of IL-2 for 72 h and transfected with expression vectors (20  $\mu\text{g}$ /condition) encoding wild-type CDK4 (Cdk4WT), kinase-inactive CDK4 (Cdk4DN), or the CDKIs p16/Ink-4a or p21/Waf-1. The same vector encoding the neomycin resistance gene (RSV neo) was used for control for the effects of transfection. Untransfected (UT) or transfected Kit-225 cells were then cultured with or without IL-2 for 24 h. The stimulation index was calculated as the ratio of [ $^3\text{H}$ ]thymidine incorporation (proliferation) in IL-2-stimulated cells over that in unstimulated cells and in the case of transfected cells, normalized to the measurable luciferase activity. The data show one representative experiment from a set of three. The mean  $\pm$  SEM of the three experiments is shown parenthetically above each bar. \*, The results are significantly different ( $p < 0.05$ ) from the untransfected controls. **B**, The accumulation of CDK4 was evaluated in cells transfected as described for **A**. **C**, CDK4 kinase activity was evaluated in cells transfected with the control vector or those transfected with the control vector were comparable to those seen in untransfected cells (see Fig. 3). Similar results were seen in three experiments. Densitometric data provided under the immunoblot are normalized to a level of 1.0 present in growth-arrested, unstimulated Kit-225 cells; data under the kinase autoradiographs are normalized to a level of 1.0 present in growth-arrested, unstimulated Kit-225 cells.

pathways leading to cytokine production than in events that promote cytokine responsiveness. More recently, it was shown that an inhibitor of MEK could reverse anti-CD3-induced unresponsiveness, and that sustained activation of MAPK pathways by overexpression of active Raf-1 was sufficient to induce cytokine unresponsiveness in T cells (4). The observation that anti-CD3, PHA, or PMA, which rendered Kit-225 cells unresponsive to IL-2, increased MAPK activity, supports these findings. However, we did not determine how long MAPK was active in these cells, or whether inhibition of MAPK was sufficient to reverse the cytokine-unresponsive state.

It is possible that the reduction in CDK4 expression and activity could be mediated by altered MAPK activation. We have recently cloned the genomic CDK4 5'-flanking sequence and identified potential transcriptional control elements within the first 900 bp upstream from the start site. E2F-like (ctTTTCGCtCc) and AP-1-like (cattTGtGTCA) binding sites are present at positions -477 to -487 and -509 to -519, respectively. The underlined segments represent consensus binding sequences. Preliminary experiments suggest that higher order complexes containing AP-1 and E2F bind to the CDK4 promoter and may be important in the transcriptional control of this gene (C. Su and J. F. Modiano, unpublished observations). Because distinct patterns of MAPK activation may induce formation of qualitatively different forms of AP-1 (45), it is possible that sustained activation of MAPK upon stimulation of IL-2-dependent T cells by anti-CD3 results in the assembly of AP-1

factors that repress CDK4 expression. MAPK-dependent pathways are also known to increase expression of D-type cyclins and lead to CDK4 activation (46–49). We observed an increase in the levels of cyclin D2 in Kit-225 cells stimulated by anti-CD3, albeit smaller than that reported with anti-CD3-induced, or Raf-1-induced cytokine unresponsiveness in D10 T cells (4). CDK4 expression was not evaluated in the D10 cells, but there was increased association of CDK2 with cyclin D2, suggesting the levels of CDK4 may have been reduced. It is possible that conditions that promote formation of CDK2 complexes with D-type cyclins also contribute to cell cycle arrest in anti-CD3-stimulated cytokine-dependent T cell lines, as CDK2 is not phosphorylated by CDK-activating kinase when bound to cyclin D (50). Finally, it is also possible that the temporally inappropriate stimulation of the T cell Ag receptor (that is, in T cells that have achieved the capacity to respond to cytokine growth factors) may lead to increased expression of CDKI that, in turn, may reverse the cytokine responsiveness and promote anergy.

In summary, we have shown that CDK4 expression and activity are necessary for IL-2-mediated T cell proliferation. Dysregulation of this pathway may be important in proliferative disorders of lymphocytes by eliminating important constraints of cell cycle progression and apoptosis, thereby allowing promiscuous proliferation in response to cytokines. This state may contribute to lymphoproliferative disorders seen upon loss of CDKI such as

p16/Ink4-a and p27/Kip-1 (51–58), promotion of autoimmune disease (59), and impaired lymphocyte responses in the aged, where there may be increased accumulation of CDKs (60).

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