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CDK4 Expression and Activity Are Required for Cytokine Responsiveness in T Cells

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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.

Materials and Methods

Tissue culture materials were obtained from Naige 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), herbycin 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 IL-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 [3H]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 suboptimal concentration of PHA (0.5 μg/ml) for 1 h followed by extensive washing (13). For each suboptimal 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 mM IL-2. Staurosporine and herbycin 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, 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/Kip1, p16/Ink-4a (16), or p21/Waf-1 (17) (Promega, Madison, WI). Digital images of immunoblotts 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-NEON, 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 β2-microglobulin gene was used as a loading control. Autoradiography was performed at ~80°C using a single amplifying screen and reflection film (DuPont-NEON). Quantification of relative changes in mRNA expression was performed on digital images using NIH Image and normalized to the levels of β2-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 95°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 Instruments, 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’-AGAGTGTAAC AACCGGTTGTAATG-3’ (reverse), 5’-ATGGTTGAGAGCTTCAACACC-3’ (forward) and 5’-GCCATCTTGGCTGAAGTCCAG-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 [γ-32P]ATP and p56/Rb (a truncated Rb protein) as a substrate. The reactions were terminated by addition of SDS sample buffer and separation 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 pBKB-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 lipidosome reagent in 100 μl HEPESBuffered saline and incubated for 15 min at 25°C. The empty pBKB-RSV vector was used as a negative control. These mixtures were added in 1 ml OptiMEM serum-free medium (Life Technologies) to 1 × 10^5 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 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 subliminogenic concentrations of PHA (Figs. 1 and 2A).
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 herbinycin 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 herbinycin A and staurosporine were comparable to those seen in resting T cells (Figs. 2B–C, and 3A). In cells treated with herbinycin 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 herbinycin 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 herbinycin A and staurosporine (Figs. 2, F–G, and 3B). These observed levels of CDK4 expression and activity in this case were not significantly different; cells treated with herbinycin 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 herbinycin 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 δ-type cyclin and dissociation from CDKI (20).

We were able to evaluate expression of cyclin D2 (the initial part of the preactivated 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, herbinycin 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 markedly 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-1) 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
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 ~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 G1 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 PHA, 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
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 CDKIs, 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 G1 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 G1 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 [3H]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 β2-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 Rb 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 (Mr ~110 kDa); Rb that is hyperphosphorylated migrates slower (Mr ~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.

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 G0/G1 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 [3H]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 β2-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 β-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, 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).

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 β2-microglobulin was used as a loading control. From these experiments where we used antisense oligonucleotides to suppress kinase during G1 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 at least 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
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 (cTTCGC(Ac)) 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 δ-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 δ-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-dependent Kit-225 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...
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