Differential Requirements for Interleukin-2 Distinguish the Expression and Activity of the Cyclin-dependent Kinases CdK4 and Cdk2 in Human T Cells

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We examined the expression and activity of CdK4 and Cdk2 in resting, competent, and proliferating normal human T cells. Expression of CdK4 but not of Cdk2 was induced in competent T cells independent of an IL-2 signal. This up-regulation of CdK4 mRNA and protein was resistant to the immunosuppressant drugs cyclosporin A (CsA) and FK506. A further increase in CdK4 expression was seen upon stimulation of competent T cells by IL-2, as was de novo expression of Cdk2. Cyclin D2, a Cdk4 partner, showed similar patterns of regulation as CdK4. The increases in CdK4 and cyclin D2 expression seen in competent T cells were functionally significant since CdK4 immunoprecipitates from these cells phosphorylated recombinant RB protein in vitro. Despite the lack of an increase in the expression of Cdk2, a small pool of pre-existing Cdk2 protein detected in resting T cells could be activated upon induction of competence. These data demonstrate that 1) the signals that lead to induction of competence in T cells stimulate an IL-2-independent and CsA-resistant phase of CdK4 and cyclin D2 expression, CdK4 kinase activity, and Cdk2 kinase activity, and 2) IL-2 stimulates a second phase of CdK4 and cyclin D2 expression and de novo expression of Cdk2 in these cells. The data show that the expression and activity of these major cell cycle regulatory proteins are controlled differentially by growth factors and indicate a role for CdK4 and cyclin D2 in T-cell cycle entry and/or early G1 progression and for Cdk2 in later G1 progression and G1/S transition.

Activation of T cells by antigen or mitogens stimulates a series of nonlinear biochemical cascades that induce entry into the cell cycle. The earliest signal transduction events that follow T-cell activation have been extensively studied (1-5); however, those that regulate transit through early G1 phase and, ultimately, cell-cycle progression are less well understood. Our aim was to delineate those regulatory events which are associated with cell cycle entry and early G1 phase versus those that are necessary only for later cell cycle progression.

Two families of proteins, the cyclins and cyclin-dependent kinases (Cdk), play a major role in cell cycle progression in eukaryotic cells (6-10). The expression of some of these proteins is controlled by growth factor-derived signals in hematopoietic or fibroblastoid cell lines (11, 12). We and others have shown differential time courses of expression of Cdk and cyclin proteins in T cells during cell cycle progression (13, 14). This suggests that such proteins may respond to different environmental signals, and each may have specific regulatory functions at different stages of the mammalian cell cycle.

Despite the characterization of the kinetics of expression of the Cdkks and cyclins, little is known about the signaling mechanisms that control their expression and function. Our data show that induction of competence in normal T cells (by the transient activation of protein kinase C and increases in intracellular calcium concentrations) stimulates an IL-2-independent, cyclosporin A (CsA)-resistant phase of CdK4 and cyclin D2 expression as well as Cdk4 kinase activity. Despite the lack of an increase in Cdk2 expression, induction of competence also can activate the pre-existing Cdk2 protein. Stimulation of competent cells by IL-2 then induces a further increase in the expression of CdK4 and cyclin D2 and expression of new Cdk2 as these cells progress through G1 and into S phase.

MATERIALS AND METHODS

Cell Cultures—Peripheral blood T cells were purified from plateletpheresis residues by Ficoll-Hypaque (1.077 g/ml) density gradient centrifugation followed by depletion of adherent cells from the peripheral blood mononuclear cells and E-rosetting on neuraminidase-treated sheep erythrocytes as described (15, 16). The phenotype of the resulting T-cell population was >96% CD3+, ≤6% DR−, ≤2% CD16+, and <1% CD20− or CD14−. The IL-2-dependent Kit-225 human T-cell leukemia line was a gift from Dr. T. Uchiyama (Kyoto, Japan, Ref. 17). Cells were maintained in culture in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM l-glutamine, 2 mM sodium pyruvate, and 10 mM HEPES, in a 5% CO2 atmosphere at 37 °C. Kit-225 cell cultures were maintained in the presence of recombinant IL-2 (2 ng).

The proliferative response of T cells to mitogens was determined by the incorporation of trichloroacetic acid-precipitable [3H]thymidine into DNA, 48 h after the onset of culture, as described (18). Phorbol 12,13-dibutyrate (PDB) and ionomycin (Calbiochem) were dissolved at a concentration of 10 μM and 500 μM in dimethyl sulfoxide, respectively. Human recombinant IL-2 was obtained from Hoffman-LaRoche, Inc. through the Biologic Response Modifiers Program, Division of Cancer Treatment, NCI. Cyclin D2 (CaA, Sandoz, Basel, Switzerland) and FK506 (Fujisawa Pharmaceutical Co., Ibaraki, Japan) were prepared as 1 mg/ml stock solutions in ethanol.

Induction of Competence—Competent T cells are those that exit G0 but stop their passage through the cell cycle at a defined point before
entry into the S phase. These cells have been termed competent because the addition of a progression signal (such as IL-2) will induce the cells to progress into S phase and undergo cell division. Peripheral blood T cells were rested overnight in complete medium. These cells were rendered competent to proliferate as described (16). Briefly, following a 20-min incubation with PDB (10 nM) and ionomycin (500 nM), cells were washed three times with phosphate-buffered saline solution (pH 7.4). The competent cells were then divided into two groups. Anti-human recombinant IL-2 antibody (2 μg/ml) was added to one group, while the other group received human recombinant IL-2 (25 nM) for the duration of culture. In each experiment, an equal number of T cells as were used to render competent were allowed to remain unstimulated (received only addition of PDB and ionomycin throughout the culture period ("proliferating" T cells).

Synchronization of Kit-225 cells in early G1 was accomplished by washing the cells extensively in PBS and incubating them in complete medium deprived of IL-2 for 72-96 h as described (19). During this period, the cells were washed in PBS and citrated saline solution (pH 6.5) daily to remove any residual IL-2 and re-incubated in fresh medium. The degree of synchrony in these cells was assessed for each experiment by measuring DNA synthesis.

**Flow Cytometry—**Analyses for all the experiments were done on an Epics Profile or on a FACSort (Becton Dickinson, FL) flow cytometer. The expression of CD3 (3T-DR1, Coulter), DR (IS-3D1, Coulter), CD14 (Mo-2-FTIC, Coulter), CD16 (Leu-11b, Becton Dickinson), CD20 (B1-FTIC, Coulter), IL-2 receptor alpha chain (CD25, anti-Tac, a gift from Dr. T. Waldmann, Bethesda, MD), transferrin receptor (CD71, T9-FITC, Coulter), and p56^c{\textsuperscript{C}}yclin (Zymed, San Francisco, CA) and cellular DNA content were analyzed by single- or dual-wave flow cytometry as described (20).

**Northern Blotting—**Cytosolic RNA was isolated by the method of Wilkinson (21). Ten to twenty μg of RNA were separated electrophoretically in 1% agarose, 5% formaldehyde denaturing gels and transferred to Nytron 66 membranes (MSI, Westboro, MA) by capillary blotting. Steady state levels of mRNA expression were assessed by hybridization of human Cdk4 cDNA (PSKJ3, 22), Cdk2 cDNA (23), cyclin D2 cDNA (24), IL-2 cDNA (25), or a human IL-2 receptor cDNA (26). RNA encoding the human β2-microglobulin gene (27) was used to ensure that the amounts of mRNA present in each sample were approximately equivalent. The 32P-labeled probes were isolated by random-primer extension and hybridized to the immobilized RNA as described (28). Blots were autoradiographed on XAR-5 film (Kodak) or Reflection film (Dupont NEN), and autoradiograms were quantitated densitometrically using the ScanAnalysis software in a Macintosh IIGi computer.

**Immunoblotting—**Cells were lysed in a buffer containing 300 mM NaCl, 50 mM Tris, pH 7.6, 0.5% Triton X-100, 1 mM N-ethylmaleimide, 0.03 mM aprtinin, 6.5 μM leupeptin. Protein content in the lysates was determined using the DC Protein Assay Kit (Bio-Rad Laboratories). Ten to twenty μg of cellular protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) in 10% or 12% mini-gels and transferred to nylon-re-enforced nitrocellulose membranes (Hybond-ECL, Amersham). The blots were probed with rabbit anti-human Cdk4 antibody (directed against residues 287 to 298 of the protein, Upstate Biotechnology, Inc. (Lake Placid, NY), anti-human Cdk2 antibody (directed against residues 254 to 260 of the protein, Upstate Biotechnology, Inc.), anti-human cyclin D2 antibody (directed against residues 164 to 123 of the protein, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-human IL-2 antibody (Collaborative Biomedical, Bedford, MA), followed by secondary anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Life Sciences) or alkaline phosphatase (Promega Biotech). Detection was performed for horseradish peroxidase by the enhanced chemiluminescence (ECL, Amersham Life Sciences) method, and for alkaline phosphatase using the Protoblot AF system (Promega) according to the manufacturers' instructions. Bands of interest were quantitated by scanning autoradiograms.

**Cdk4 Kinase Assays—**Cdk4 kinase activity was determined by a modification of the method described by Matsushime et al. (29). Five to twenty million unstimulated, competent, and proliferating T cells were cultured for 12 to 36 h and lysed in a buffer containing 150 mM NaCl, 50 mM Tris, pH 7.4, 10 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40, 100 μM Na3VO4, 1 μM MgCl2, 30 μM aprotinin, 500 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride, 10 μM β-glycerophosphate, and 1 μM sodium pyrophosphate. The Cdk4-associated complexes were immunoprecipitated with rabbit anti-human Cdk4 antibody (directed against residues 254 to 260 of the protein, Upstate Biotechnology, Inc.) and blotted with rabbit anti-human Cdk4 antibody (directed against residues 287 to 298 of the protein, Zymed). The kinase activity of the immunoprecipitated complexes was assessed by transfer of phosphate from [γ-32P]ATP to truncated recombinant RB protein (p56{\textsuperscript{RB}}, QED, San Diego, CA) in a reaction buffer consisting of 50 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 2.5 mM EGTA, 10 mM β-glycerophosphate, 100 μM Na3VO4, 1 mM NaF, 20 μM ATP, 200 ng of the substrate p56{\textsuperscript{RB}} protein, and 10 μCi of [γ-32P]ATP (specific activity, 11 Ci/mmol, ICN Biochemicals). The reactions were performed for 30 min at 30 °C and stopped by the addition of SDS-sample buffer. The samples were boiled for 5 min at 65 °C, and the proteins were separated in 12% SDS-PAGE. The gels were autoradiographed on Reflection film at −70 °C.

**RESULTS**

**Progression of Competent T Cells through the Cell Cycle—**Resting peripheral blood T cells comprise a population of cells that are predominantly (96%) in the G0 phase of the cell cycle and lack significant expression of cytokine receptors (15, 16, 32-34, and data not shown). These resting T cells can be rapidly stimulated to enter the cell cycle and proliferate in a synchronous manner upon continuous stimulation with PDB and ionomycin or they can be rendered competent without proliferating by a brief stimulation with PDB and ionomycin for 20 min followed by washing (16).

The inability of the competent cells to progress through G1 phase is not due to cytotoxicity or cell death, but rather is due to their inability to sustain critical levels of lymphokine production (especially IL-2), despite the expression of cytokine receptors on the cells (15, 16, 32-34). Fig. 1A shows that resting T cells do not express IL-2 mRNA, nor did they contain any detectable IL-2 protein (Fig. 1B) as determined by Northern blotting and immunoblotting, respectively. T cells stimulated continuously by PDB and ionomycin expressed high levels of IL-2 mRNA by 2 to 4 h that were sustained over 24 h and accumulated high levels of IL-2 protein in their cytoplasm (Fig. 1, A and B). The amounts of intracellular IL-2 found in these cells have been shown to be an accurate reflection of the levels of IL-2 secreted into culture supernatants (28). In contrast to the up-regulation of receptors for IL-2 and transferrin (Ref. 16 and data not shown), neither IL-2 gene expression nor IL-2 protein accumulation was detectable in competent T cells (Fig. 1, A and B).

The human Kit-225 IL-2-dependent T cell line was used for some of the experiments detailed below. The characteristics of cell cycle progression of the human leukemia Kit-225 cell line

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stimulated by IL-2 have been described in detail previously (19). These cells arrest their growth in early G1 when they are deprived of IL-2 for 72 h and re-enter the cell cycle synchronously upon the addition of exogenous IL-2. The proliferative response of these cells was independent of changes in cytosolic Ca2+ concentrations or protein kinase C activation (19), but was inhibited by anti-IL-2 antibody.

Both IL-2-independent and IL-2-dependent Signals Regulate the Expression of Cdk4 in T Cells—The systems described above (the competence/progression system in T cells, the Kit-225 cell line, and the treatment of mitogen-stimulated T cells with the immunosuppressive drugs CsA and FK506) provided us three models to analyze IL-2-dependent versus IL-2-independent signals that may be required for the expression of cyclin-dependent kinases (Cdk) in human T cells. Of the Cdk family members studied to date, Cdk4 is among the earliest Cdk whose expression is up-regulated in mitogen-stimulated T cells.2 In the experiments described here, we first evaluated the steady state level of expression of the cdk4 gene and protein by Northern blotting and immunoblotting in resting, competent, or proliferating T cells. As shown in Fig. 2A, the Cdk4 message was undetectable in resting cells (lane 1) and was detectable in proliferating cells by 4 h (lane 6). The steady state levels of the message peaked shortly thereafter and remained at this level for at least 24 h (lane 7). Induction of competence (20-min incubation with PDB and ionomycin) increased the level of cdk4 gene expression (lane 2), but the steady state mRNA levels of Cdk4 did not increase beyond those seen at 4 to 6 h in these cells (lane 3). The addition of exogenous IL-2 to competent T cells did not affect the initial expression of Cdk4 (i.e. that seen 4 hours after stimulation; compare lane 4 versus lane 2), but the Cdk4 mRNA levels were markedly increased at 24 h (lane 5).

The treatment of mitogen-stimulated T cells with CsA (or FK506) resulted in a pattern of Cdk4 mRNA expression similar to that seen in competent T cells: Cdk4 message in these cells was only inhibited partially (Fig. 2B), further delineating the CsA-resistant or IL-2-independent component of Cdk4 mRNA expression. The addition of exogenous IL-2 restored the expression of Cdk4 to levels comparable to those seen in the absence of CsA (Fig. 2B).

We also evaluated the expression of Cdk4 in growth-arrested Kit-225 cells deprived of IL-2. Unstimulated Kit-225 cells expressed low levels of Cdk4 message (Fig. 2C), which increased markedly following stimulation with IL-2. The IL-2-stimulated increase in cdk4 gene expression was abrogated by anti-IL-2 antibody (Fig. 2C).

The pattern of Cdk4 expression in competent and proliferating T cells closely followed that of Cdk4 mRNA. As we have shown previously,2 Cdk4 protein was barely detectable or not at all in unstimulated normal T cells (Fig. 2, D and F). There was a detectable increase in the steady state levels of Cdk4 protein in proliferating T cells 6 to 8 h after stimulation with PDB and ionomycin (Fig. 2D), and Cdk4 protein continued to accumulate in these cells for at least 20 h (Fig. 2D). The 34-kDa band recognized by the anti-Cdk4 antibody in proliferating cells was effectively competed by a peptide composed of the 22 C-terminal amino acids of Cdk4, but not by a peptide composed of the 16 C-terminal amino acids of Cdk2 (Fig. 2E). Cdk4 protein also increased and accumulated in small amounts following stimulation with PDB alone or with ionomycin alone (Fig. 2F). Detectable levels of Cdk4 protein were seen in competent T cells within 8 h following stimulation (Fig. 2D), but the steady state amount of Cdk4 protein did not increase noticeably in these cells for the following 12 h (Fig. 2D). As was the case for mRNA expression, the addition of IL-2 to these cells induced a further increase in the steady state levels of Cdk4 protein (Fig. 2D). CsA and FK506 completely abrogated the accumulation of intracellular IL-2 in mitogen-stimulated T cells, but they only partially inhibited the accumulation of Cdk4 protein in these T cells (Fig. 2F).

The Expression of Cdk2 in T Cells Requires an IL-2-dependent Signal—We showed previously that Cdk2 is expressed during mid-G1 in proliferating T cells, at which time it associates with cyclin E to form a functional complex.3 We evaluated the steady state expression of the cdk2 gene and protein in unstimulated, competent, or proliferating T cells to determine whether such expression could be stimulated by signals that mediate the induction of competence. Fig. 3A shows that Cdk2 message was undetectable under any of the conditions tested, 4 h after the initial stimulatory signal in these cells (lanes 1, 2, 4, and 6). Cdk2 message was present in T cells following a 24-h stimulation by PDB and ionomycin (lane 7). Competent T cells failed to up-regulate Cdk2 mRNA even 24 h later (lane 3). In contrast, the addition of IL-2 restored the expression of Cdk2 message by competent T cells (lane 5). The restoration of Cdk2 mRNA expression and DNA synthesis correlated closely in each of the experiments. This suggested that an IL-2-mediated signal(s) were necessary for the up-regulation of Cdk2 expression (and entry into S phase), and that the process of induction of competence alone was not sufficient to stimulate expression of this gene.

Despite the absence of detectable Cdk2 mRNA, resting T cells contained low levels of Cdk2 protein2 (23) (Fig. 3B, lane 1).
The amount of Cdk2 protein increased approximately 2-fold in T cells stimulated by PDB and ionomycin for 20 h as determined by immunoblotting (lane 6). This increase in Cdk2 protein was not evident in competent T cells, and, in fact, the amount of Cdk2 protein was even decreased in some experiments (lanes 2 and 3). However, as was the case for Cdk2 message expression, the addition of exogenous IL-2 reconstituted the accumulation of Cdk2 protein by competent T cells (lane 5).

**IL-2-independent and IL-2-dependent Signals Regulate the Expression of Cdk4 in T Cells**—In parallel to the expression of Cdk4, we assessed the expression of its putative cyclin partner, cyclin D2, under the same conditions. The patterns of cyclin D2 expression were similar to those of Cdk4 itself. Aichenbaum et al. (14) previously reported that cyclin D2 mRNA was present in small amounts in unstimulated human T cells, but in our experiments cyclin D2 message was difficult to detect in these cells by Northern blotting. We did, however, find small to moderate amounts of cyclin D2 protein in unstimulated T cells (Fig. 3C). Cyclin D2 mRNA was clearly detectable in proliferating T cells 4 h after stimulation, and it peaked shortly thereafter (14 and data not shown). Increased amounts of cyclin D2 protein, especially its faster mobility form, were similarly detected in proliferating T cells 8 to 20 h after stimu-
proliferating T cells was inhibited markedly in the presence of CsA (Fig. 3D). As was the case for Cdk4, the addition of exogenous IL-2 restored the expression of cyclin D2 (Fig. 3D). These data indicate that the activation of the cells by a brief exposure to PDB and ionomycin was sufficient to induce an IL-2-independent, CsA-resistant phase of Cdk4 and cyclin D2 expression. In addition, the expression of Cdk4 and cyclin D2 increased further following an IL-2-dependent signal(s).

Induction of Competence in Normal T Cells Results in Activation of Cdk4 and Cdk2—It was important to determine whether the IL-2-independent expression of Cdk4 and cyclin D2 was functionally significant. Therefore, Cdk4 complexes were immunoprecipitated from resting, competent, or proliferating T cells 12 or 20 h following stimulation, and the activity of these complexes was examined in an in vitro assay using recombinant, truncated RB protein (p56Rb) as a substrate. The Cdk4 kinase activity present in these cells closely paralleled the expression of the Cdk4 and cyclin D2 proteins. Fig. 4 shows that Cdk4 immunoprecipitates from resting T cells contained little kinase activity (lane 1). In contrast, proliferating T cells contained detectable levels of Cdk4 kinase activity 12 h after stimulation (lane 4) that continued to increase for at least 20 h. Significant levels of Cdk4 kinase activity were detectable in competent T cells 20 h after stimulation (lane 3). The Cdk4 kinase activity in these cells after 12 h was only slightly increased over that seen in the resting cells. In other experiments, we determined that the faster mobility species of cyclin D2 was the predominant form of this protein present in association with Cdk4 in the active complexes recovered from competent or proliferating T cells, as determined by immunoblot analyses of the Cdk4 immunoprecipitates (data not shown).

As described above, even though no Cdk2 mRNA was detectable in resting T cells, these cells expressed low levels of Cdk2 protein (Fig. 3B). However, this Cdk2 protein lacked kinase activity as measured in vitro using histone H1 as a substrate (Table I). Cdk2 kinase activity increased significantly in T cells stimulated by PDB and ionomycin for 22 h (Table I). Furthermore, despite the lack of an increase in cdk2 gene expression or Cdk2 protein accumulation in competent T cells, the Cdk2 kinase activity increased on average to 53% of that seen in the proliferating cells 22 h after stimulation (Table I). Stimulation of competent T cells by IL-2 reconstituted the Cdk2 kinase activity in these cells to an average of 83% of the levels seen in proliferating cells, presumably due, at least in part, to the concurrent increase in Cdk2 protein levels (Table I).

**DISCUSSION**

We compared the requirements for cdk2 and cdk4 gene expression, protein accumulation, and activation in mitogen-activated normal human T cells. The data demonstrate the dif-
TABLE I

| Condition                        | Cdk2 kinase activity* |
|---------------------------------|-----------------------|
| Resting T cells                 | <1                    |
| Competent T cells               | 22.5                  |
| Competent T cells + IL-2        | 32.7                  |
| Proliferating T cells           | 56.6                  |

* Cdk2 kinase activity was determined in vitro in resting T cells, T cells that were rendered competent, competent T cells that were stimulated with IL-2, or T cells that were stimulated in the continuous presence of PDB and ionomycin (proliferating T cells). Cdk2 complexes were immunoprecipitated from the cells after 52 h in culture and assayed for their ability to phosphorylate histone H1 in vitro. The results shown are from one of three experiments. The percent means ± S.E. of the Cdk2 kinase activity detected in competent T cells and in competent T cells + IL-2 were 53 ± 18% and 83 ± 28% of those seen in proliferating T cells, respectively.

The expression of Cdk4 was also evaluated in the presence of CsA, a potent inhibitor of IL-2 gene transcription. This suggests that the transient activation of protein kinase C by PDB and calcium entry stimulated by ionomycin was sufficient to induce Cdk4 gene expression and protein accumulation of the Cdk4 kinase in normal human T cells. We also showed that sustained stimulation of these cells by PDB alone induced the accumulation of small amounts of Cdk4 protein. Thus, the transcription of the cdk4 gene may be regulated, at least partly, through protein kinase C-responsive transcription factors like NF-κB or AP-1 (37-40).

It is noteworthy that the Cdk4 protein induced in competent T cells could phosphorylate RB in vitro, although the appearance of Cdk4 kinase activity in competent T cells was delayed as compared to that in proliferating T cells. It is most likely that this delay could be attributable to the smaller levels of Cdk4 present in the competent T cells at the earlier time points examined (8 to 12 h); however, we cannot exclude the possibility that there also was a qualitative difference in the signaling pathways that activate this protein following stimuli that render the cells competent or induce them to undergo proliferation.

Addition of IL-2 to competent T cells induced a further increase in the steady state levels of Cdk4 mRNA and protein, approaching those levels observed in T cells cultured in the presence of PDB and ionomycin. This second increase in Cdk4 expression was clearly IL-2-dependent since it was not detected in cells continuously exposed to PDB and ionomycin and cultured in the presence of CsA. This pattern of regulation is similar to that of the α chain of the IL-2 receptor, where stimulation by antigen or mitogen induces an initial, IL-2-independent phase of IL-2 receptor gene expression that is followed by a second IL-2-dependent phase (30, 41). This IL-2-dependent stimulation of IL-2 receptor gene expression is mediated through signals associated with the activation of non-receptor tyrosine kinases, rather than through the activation of protein kinase C or calcium entry (28, 42-44). Thus, it is possible that the Cdk4 promoter may share some of the structural features characteristic of the IL-2 receptor α promoter.

The results from the analysis of cdk4 gene expression in T cells were supported by our findings with the IL-2-dependent Kit-225 cells. Kit-225 cells synchronized by IL-2 deprivation express moderate levels of IL-2 receptor mRNA and protein (19). These cells also expressed low levels of Cdk4 mRNA. Following addition of IL-2, there was a marked increase in the levels of Cdk4 that were analogous to the levels seen for the IL-2 receptor gene (19).

The regulation of expression of G1 cyclins has been analyzed previously in competent or proliferating fibroblasts stimulated by platelet-derived growth factor in the presence or absence of serum (11). Induction of competence in these cells stimulated the protein kinase C-dependent expression of cyclin D1, but a proliferative signal was required for the expression of cyclin D3. The expression of Cdk4 or Cdk2 was not evaluated in these cells. T cells do not express cyclin D1 (14); but appear to use cyclin D2 as an early G1 cyclin. In the present experiments, the expression of cyclin D2 appeared to be regulated concordantly with Cdk4. Our results show a CsA-resistant phase of cyclin D2 protein accumulation that followed T cell activation and was independent of IL-2 signaling. Furthermore, the cyclin D2 induced under these conditions associated with Cdk4 to form complexes that phosphorylated RB in vitro. As was the case for Cdk4, in addition to this initial, CsA-resistant phase of cyclin D2 induction, IL-2 stimulated a further increase in the expression of cyclin D2 mRNA and the accumulation of cyclin D2 protein. This second increase in cyclin D2 accumulation observed in T cells stimulated with PDB and ionomycin was abrogated in the presence of CsA, underscoring the role of IL-2 in triggering this phase of the response. Previous experiments also showed that CsA had no effect on cyclin D2 mRNA expression in the presence of anti-CD28 antibodies that activate a CsA-independent pathway of IL-2 production (14), and similar results were seen in mature marine IL-3-dependent hematopoietic cells that were transfected with functional, high affinity IL-2 receptor proteins and induced to proliferate in response to IL-2 (45).

In contrast to Cdk4 and cyclin D2, the expression of Cdk2 showed a dependence for IL-2-mediated signaling in the activated T cells. No aspect of the increase in Cdk2 expression observed in proliferating T cells could be detected in T cells simply rendered competent by the limited exposure to PDB and ionomycin or when CsA was included in the cultures. This suggests that the transcriptional (or post-transcriptional) regulation of Cdk2 is dependent on an IL-2-responsive element(s), or alternatively, that the expression of Cdk2 is associated with one or several progression events (through G1) and is therefore only indirectly dependent on the IL-2 signal.

Despite the lack of an increase in Cdk2 protein accumulation, induction of competence induced the activation of the pre-existing Cdk2 in normal T cells. This suggests that signals that lead to the expression and activation of Cdk4 are sufficient to activate Cdk2, and that these two events may be causally related. These data also are consistent with the findings of Ewen et al. (46) that showed transforming growth factor β1 inhibited the expression of Cdk4 and the activation of Cdk2 in mink lung epithelial cells, and that overexpression of Cdk4 restored the activation of Cdk2 in these cells. Similar to the effects seen in the capacity of competent cells to proliferate in response to

* J. F. Modiano, J. J. Lucas, and E. W. Gelfand, unpublished data.
IL-2, this progression signal increased the Cdk2 kinase activity in competent T cells to levels comparable to those seen during late G1 phase (20 to 24 h after stimulation) in the proliferating T cells.

Our results thus distinguish early cell cycle regulatory events that follow T cell activation from those that control later cell cycle progression. A model based on these data would predict that protein kinase C-dependent pathways, at least in part, initiate IL-2-independent increases in Cdk4 and cyclin D2 gene expression, and for Cdk4 activation that in turn may lead to an initial phase of Cdk2 activation. The interaction of IL-2 with its receptor initiates a second wave of biochemical events that activate increased expression of Cdk4 and cyclin D2, as well as de novo expression of Cdk2 with a concomitant increase in the activity of both proteins. Whether or not the initial IL-2-independent increases in Cdk4 kinase activity are prerequisites for the subsequent IL-2-dependent increases in activity of this enzyme and of Cdk2-cyclin complexes remains to be determined.

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