Independent Regulation of Human D-type Cyclin Gene Expression during G1 Phase in Primary Human T Lymphocytes*

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Cyclins and cyclin-dependent kinases are critically involved in controlling cell cycle progression in virtually all cells. The recent identification of candidate G1 cyclins in mammalian cells has been a major advance in this field, but the exact functions of these cyclins are unknown. The expression of three D-type cyclins (D1, D2, and D3) was investigated in primary human T lymphocytes as these cells were induced to leave G0, traverse G1, and enter S phase by T cell-specific mitogens. Go phase T cells expressed low levels of cyclin D2, but not cyclin D3. Treatment of these cells with phytotoxaphagglutinin and 12-O-tetradecanoylphorbol-13-acetate in the presence of fetal calf serum resulted in rapid induction of cyclin D2 RNA in early G1 and slower induction of cyclin D3 in late G1. Cyclin D1 was not detected in T cells under any condition tested. Treatment of T cells with hydroxyurea to arrest cells at G1/S did not block induction of either D2 or D3. However, arrest of cells in mid G1 with deferoxamine blocked D3 expression without affecting D2. Cyclosporin A blocked the induction of both cyclin D2 and D3. Polyclonal antisera were prepared in rabbits against both cyclin D2 and cyclin D3 glutathione S-transferase fusion proteins and used to examine cyclin D2 and D3 proteins in [35S]methionine-labeled T cells. Protein levels were found to correlate closely with RNA levels for both cyclins. No detectable histone H1 kinase activity could be precipitated with either cyclin. However, several cellular proteins were observed to coprecipitate with the cyclins, including several proteins that were observed to associate only with D3. These results indicate that striking differences exist in the induction and regulation of two candidate G1 cyclins in human T cells and suggest that these cyclins could participate in multiple cell cycle checkpoints during Go, G1, or S phase.

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**Cyclosporin A (Sandimmune®) was generously provided by Sandoz, Inc.
phages, the CYL proteins were found to associate with a CDC2-related protein, although the identity and functions of any associated kinases have not been determined (Matsushima et al., 1991). Human cyclin E was shown to interact with human P42 and cdk2 to perform Start in yeast (Koff et al., 1991) and to form a complex with cdk2 during the G1 phase of the human cell cycle (Koff et al., 1992). Taken together, these data indicate that cyclins C, D, and E are each candidate G1 cyclins for human cells and could regulate one or more stages of cell cycle progression.

In addition to the immortal or transformed cell lines used to study the mammalian cell cycle, primary T lymphocytes have proven to be a useful model of normal cells. During the process of differentiation, T cells spontaneously arrest in G0 and may remain quiescent for long periods of time until exposed to specific antigen or mitogens. Previous studies have indicated that the transitions from G0 to G1 and from G1 to S are accompanied by an orderly sequence of interdependent events (Crabtree, 1989). For example, transition from G0 to G1 is marked by transcriptional activation of the IL-2 receptor and IL-2 genes (Crabtree, 1989). Subsequent G1 events and initiation of DNA synthesis are dependent on induction of IL-2 receptor and on a supply of IL-2 from autocrine or external supply (Kumagai et al., 1988). T cells have a prolonged G1 phase compared to immortal or neoplastic cells (typically 24–30 h for T cells).

In this study, we have examined the expression and regulation of the human D cyclin genes in T cells and examined the role of IL-2 in their regulation. The results indicate an unexpected degree of diversity, suggesting that the functions of the D cyclins may be distinct and non-redundant in these cells.

**MATERIALS AND METHODS**

**Cell Preparation and Culture**—Leukocytes were obtained from normal healthy volunteers by leukopheresis. All tissue samples were obtained after informed consent of donors and under Institutional Review Board-approved protocols. Mononuclear cells were isolated by Ficoll/Hypaque gradient centrifugation (Pharmacia LKB Biotechnology Inc.). After depletion of adherent cells on plastic dishes, T cells were obtained after informed consent of donors and under Institutional Review Board-approved protocols. Mononuclear cells were isolated by Ficoll/Hypaque gradient centrifugation (Pharmacia LKB Biotechnology Inc.). After depletion of adherent cells on plastic dishes, T lymphocytes were isolated by erythrocyte rosetting. The erythrocyte rosetting substrates were washed 5 times in the same buffer, suspended in sample buffer and protein A-Sepharose beads (Sigma) for 2 h at 4°C. The beads were washed 5 times in the same buffer, suspended in sample buffer as described (DeCaprio et al., 1992), heated at 95°C for 5 min, and the eluted proteins applied to SDS-polyacrylamide gels and detected by autoradiography. In some experiments, the immune complexes were collected on protein A beads, washed 5 times with lysis buffer, suspended in kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol, 10 mM ATP), and incubated with γ-[32P]ATP (24 nmoI/µl, 7000 Ci/mmol, Du Pont-New England Nuclear) for 20 min at 30°C, in the presence or absence of histone H1 (1 µg) as an exogenous substrate.

**RESULTS**

The Expression of D Cyclins Is Cell-cycle-dependent in T Cells—Resting T lymphocytes were isolated from the peripheral blood of normal donors, and induced to enter the cell cycle by exposure to a combination of phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate (PMA), and ionomycin, at 1 µg/ml, 0.3 ng/ml, and 0.12 µg/ml, respectively. Under these conditions, T lymphocytes enter G0 phase in 2–4 h, enter S phase after approximately 18–24 h, and reach G1/M by 36–48 h (Furukawa et al., 1990). After stimulation, aliquots of cells were harvested at various time points, analyzed for DNA content by flow cytometry, and for cyclin mRNA expression by Northern blot analysis (Fig. 1). Of the six cyclins studied, only cyclin C and cyclin D2 mRNA were detected in resting (G0) cells, although in both cases the level of RNA was low. Cyclin D1 was not detected at any time of the cell cycle in T lymphocytes. Cyclin A, cyclin C, cyclin D2, cyclin D3, and cyclin E were each inducible by >10-fold following treatment with mitogens, but the kinetics of induction varied markedly. In resting T cells, cyclin D2 mRNA was present in a very low amount, whereas cyclin D3 mRNA was undetectable. Cyclin D2 expression increased >5-fold within 2 h after stimulation, reached a plateau after 24 h, and did not change thereafter. Cyclin D3 could first be detected typically at 12–16 h after stimulation, reached maximal levels after approximately 30 h, and remained stable thereafter.

**RNA extraction and northern blotting**—Total cellular RNA was extracted from fractions (2 x 10^6 cells) using the guanidium thiocyanate method. Samples (10 µg) were subjected to 1% MOPS/formaldehyde-agarose gel electrophoresis and blotted onto nitrocellulose membranes. The blots were hybridized with 32P-labeled cDNA probes. Post-transfection, D2 and D3 murine cDNAs containing plasmids have been described (Matsushima et al., 1991) and were gifts from Drs. A. Koff and J. M. Roberts, Fred Hutchinson Cancer Research Center, Seattle, WA. A cyclin C cDNA (Lew et al., 1991) was a gift from Dr. Steve Reed, Scripps Research Institute, LaJolla, CA. Inserts were labeled with 32P[αCTP] by the hexanucleotide primer technique.
Cyclin D Expression in T Cells

Hydroxyurea—In order to assess more precisely the kinetics of induction in relation to the onset of S phase, cell cycle progression of T cells was blocked at G1/S by hydroxyurea. Hydroxyurea was added at the same time as PHA, PMA, and ionomycin, and cells were cultured for 0–48 h. Propidium iodide staining of an aliquot of the samples confirmed that the cells were blocked at the G1/S transition (<5% S phase cells, data not shown). Hydroxyurea addition had no effect on the appearance or maintenance of cyclin D2, cyclin D3, cyclin C, or cyclin E mRNA expression, but partially inhibited accumulation of cyclin A mRNA (Fig. 1). This result suggests that the inducible accumulation of cyclins is not dependent on the initiation or completion of DNA synthesis, with the possible exception of cyclin A. Expression of cyclin mRNAs after release from hydroxyurea block was also investigated (Fig. 1). Expression of cyclin D2, cyclin D3, and cyclin C changed very little at 8 or 30 h after release, while cyclin E decreased rapidly and cyclin A increased slightly.

Induction of D Cyclin RNA in T Cells Is Differentially Affected by Deferoxamine—Deferoxamine is an iron chelator that interferes with cell metabolism and blocks lymphocyte proliferation in late G1, possibly through inhibition of ribonucleotide reductase (Lederman et al., 1984). Cells were cultured with PHA, PMA, and ionomycin in the presence or absence of deferoxamine (100 μM) for 48 h, then released from deferoxamine blockade by extensive washing. DNA content analysis and measurement of [3H]thymidine incorporation indicated that the cells were >95% arrested in G1 by deferoxamine. The presence of deferoxamine had no effect on the expression of cyclin D2 and cyclin C mRNA expression, whereas it completely inhibited the appearance of cyclin D3 and cyclin E mRNA (Fig. 2). Following release by washing, cyclin D3 and cyclin E mRNAs were first seen between 10 and 24 h, coincident with the onset of S phase measured by DNA histogram analysis (Fig. 2 and data not shown). The long delay between entry into S phase and release from deferoxamine is consistent with previous studies (Terada et al., 1991). Taken together with the absence of inhibition by hydroxyurea, this experiment suggests that the onset of expression of cyclin D3 and cyclin E genes takes place in late G1, but before the G1/S transition, while the further induction of cyclin D2 and cyclin C occurs at an early time in G1, well before G1/S. Cyclin A was not studied in these experiments. As a control, cdk2 was found to be induced at 24 h of culture.

Cyclin A and cyclin E were first detected at 18–24 h, at approximately G1/S. DNA histogram analysis showed that virtually all the cells were still in G1 at the time of cyclin D3 and cyclin E induction (data not shown).

Induction of D Cyclin RNA in T Cells Is Not Blocked by

![Fig. 1. Expression of cyclin D RNA in primary human T lymphocytes.](image-url)

![Fig. 2. Differential effects of deferoxamine on cyclin D gene expression in T lymphocytes.](image-url)
and its expression was partially blocked by deferoxamine.

**Effects of Cyclosporin A on Cyclin Gene Expression**—Cyclosporin A (CsA) blocks T cells early in G1, at least in part by inhibiting the transcriptional activation of IL-2 (June et al., 1989). Addition of IL-2 overcomes CsA inhibition (June et al., 1987). T cells were cultured with PHA, PMA, and ionomycin in the presence or absence of CsA for 48 h. Cyclin D3 and cyclin E mRNAs were blocked by the presence of CsA. CsA also blocked the induction of cyclin D2 and cyclin C mRNA, which is usually observed between 2 and 24 h (Fig. 3). This experiment suggests that although cyclin D2 and cyclin C are detectable in resting, G0 T cells, further induction and expression of these cyclins in late G1 or S phase is at least partly IL-2-dependent.

In an effort to better define the complex blocking effects of CsA on cyclin D2 expression, we examined the effects of adding exogenous IL-2, and also triggered T cells to produce IL-2 through a CsA-independent pathway (cross-linking of the cell surface antigen CD28) (June et al., 1987, 1990). Expression of cyclin D2 mRNA was inhibited by CsA in both PHA- and PMA/ionomycin-stimulated T cells (data not shown). Addition of IL-2 at time zero of the culture restored expression of cyclin D2 mRNA. However, in the presence of anti-CD28 and PMA, CsA had no effect on cyclin D2 expression.

**Cell Cycle-dependent Expression of D Cyclin Genes in Rapidly Proliferating Cultures of T Cells**—The studies above examined cyclin gene expression during the initial entry of resting cells into the cell cycle. In order to better compare our results on resting T cells with those of immortal cell lines, T cells were stimulated for 72 h and separated by centrifugal elutriation into fractions enriched for G1 cells (fractions 1–3), S phase (fractions 5 and 6), and G2/M phase (fractions 8–10).

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1 The abbreviations used are: CsA, cyclosporin A; IL, interleukin; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13-acetate; MOPS, 4-morpholinoethanesulfonic acid.
found to precipitate with several high molecular mass proteins (140-240 kDa; (Fig. 5B).

**Immune Complex Kinase Assays**—In order to determine if either cyclin D2 and D3 coprecipitated with a kinase capable of phosphorylating histone H1, an immune complex kinase assay was performed with the anti-D2 and D3 antisera described above. Neither cyclin D2 or D3 were found to coprecipitate with any histone H1 kinase activity (Fig. 6). However, a polyclonal antibody against cdk2 precipitated an histone H1 kinase that was inducible in T cells (Fig. 6).

**DISCUSSION**

In human and other higher eukaryotic cells, identification of the genes that control passage through G1 is of particular interest because virtually all external events that regulate proliferation act primarily or exclusively during G1. In hematopoietic cells, for example, growth factors such as interleukin-2 (IL-2) or colony stimulating factor 1 are required to induce commitment of responsive cells to enter S phase, but are not again necessary until the next G1 phase, a situation analogous to Start in yeast. Similarly, growth inhibitory cytokines such as transforming growth factor β or interferons cause cells to arrest in G1 (Barnard et al., 1990). The molecular mechanisms involved in regulating passage through G0 and G1 remain largely unknown, and are likely to be complex. Data from many lower organisms indicate that cyclins are likely to play a regulatory role in all phases of the cell cycle. Although cell cycle dependent expression of cyclin A and B has been well documented in mammalian cells (Pines and Hunter, 1991), the kinetics of expression and regulation of other cyclin genes has not been extensively investigated.

In this study, we have examined expression of various candidate D cyclins in primary human T lymphocytes as they were induced to traverse G0. It has been demonstrated in many previous studies with T cells that a series of genes believed to be involved in growth control, such as IL-2, IL-2 receptor, transferrin receptor, c-myc, and c-myc, are induced in a carefully controlled order as the cells pass through G0, G1, and S phase (Shipp and Reinherz, 1987; Crabtree, 1989). It is likely that the order of induction of such genes is determined, at least in part, by when a particular gene product is first needed within the cycle.

The data presented here indicate that the kinetics of expression of two candidate G1 cyclin genes, cyclin D2 and D3, are strikingly different during G1 phase in human T cells. Freshly isolated, normal T lymphocytes did not express detectable levels of RNA for cyclin D3, but did express low levels of cyclin D2 and cyclin C. Activation of resting T cells was associated with rapid induction (2 h) of cyclin D2 and delayed induction (18-24 h) of cyclin D3, cyclin C, cyclin E, and cyclin A expression. Cyclin D2 was induced in less than 2 h, at a time approximating the G0 to G1 transition, while cyclin D3 and cyclin E RNAs were not detected until late G1, about 18-24 h after stimulation, and cyclin C at 24-36 h. Cyclin C was present at low level at time zero, but was also induced >10-fold between 8-24 h of stimulation. It is possible that the low level expression of cyclin D2 and C in freshly isolated T cells is due to the presence of a small population of T cells that were activated in vivo. However, reprobing the Northern blots indicated that there was no detectable expression of IL-2 in the populations of resting T cells studied here. Likewise, expression of the β5 IL-2 receptor could not be detected by flow cytometry. The considerable differences in the kinetics of induction of these cyclins suggest that the events which regulate expression of each gene are distinct and non-overlap-
ping. These results also suggest that the functions of each cyclin in Go, G1, and S phase may be at least partially distinct. For example, it is unlikely that cyclin D3, cyclin A, and cyclin E play an important role in G0 or early G1, since their earliest detectable expression is in late G1 or S. The product of the retinoblastoma susceptibility gene, pRB, is an attractive candidate as a substrate for a G1 cyclin-associated kinase (DeCaprio et al., 1989; Lyn et al., 1991). We have previously shown that pRB is phosphorylated in at least three stages in primary T cells, with the first phosphorylation occurring in mid G1 (about 12 h) (DeCaprio et al., 1992). This phosphorylation occurs before detectable expression of cyclin D3, E, A, and B, and is not blocked by deferoxamine treatment, which blocks expression of cyclin D3, E, A, and B. However, definitive studies on where in the cell cycle these cyclins are important will require identification of their functions.

In order to better define the stage of the cell cycle during which each cyclin first appeared, T cells were blocked at different stages by various agents, allowing one to temporally order the induction of cyclin gene expression with more precision. Hydroxyurea, which arrests cells at G1/S by reversibly inhibiting DNA synthesis (Adams and Lindsey, 1967), did not block expression of any of the cyclins studied here, including the "last" cyclin to appear, cyclin A. These results are interpreted to mean that these cyclins are induced in S phase or earlier and that completion of DNA synthesis is not required. Since hydroxyurea did not help distinguish among the cyclins, two other agents that arrest T cells earlier in the cell cycle were examined. Deferoxamine, which blocks cells in mid-G0 phase (Lederman et al., 1984; Terada et al., 1991), was found to have markedly differential effects, blocking induction of cyclin D3, cyclin A, and cyclin E, but not cyclin D2 or cyclin C. These results were largely consistent with the kinetic analysis of these cyclins, which suggested that cyclin D2 and cyclin C were induced much earlier than the other cyclins.

The results with hydroxyurea and deferoxamine were extended by studies with cyclosporin A, which induces arrest early in G1, by inhibiting IL-2 transcription (Emmel et al., 1989; June et al., 1989). The induction of each of cyclins was blocked by CsA. However, since cyclin C and D2 are apparently expressed in resting T cells (prior to IL-2 gene expression) there may be both IL-2-dependent and independent regulation of these two genes. Overall, the order of cyclin gene induction in primary T cells is cyclin D2, cyclin C, cyclin D3, cyclin E, cyclin A, and cyclin B. Our kinetic data would suggest that cyclin D3 and cyclin E could play a role at the mammalian equivalent of Start in yeast, or during S phase. In contrast, cyclin D2 or cyclin C could play roles at either the Go/G1 or G0/G1 transition or early in G1.

Surprisingly, when we separated unsynchronized, rapidly proliferating cultures of primary T cells by centrifugal elutriation, none of the cyclins studied showed any dramatic level of cycling at the RNA level. In multiple experiments, cyclin D3, cyclin C, and cyclin E RNA expression tended to be highest in S/G2/M phases, however, expression was readily detected during all phases of the cell cycle. Although cyclin A and B show considerable variation through the cell cycle at the level of RNA, protein, or both, at least one other cyclin, CLN 3, does not cycle in yeast (Wittenberg et al., 1990). Cyclin E RNA was the most cyclical in our experiments, consistent with previous observations (Koff et al., 1991). The expression of cyclin D2 and D3 proteins were also studied in T cells. Cyclin D2 appeared to cycle, peaking at approximately G1/S, at least through the first cell cycle after stimulation. Elutriated samples were not studied, however, and whether or not cycling is maintained is not known.

Although not studied in detail here, preliminary studies of the induction of cdc2-related kinases in T cells suggest that there are some parallels with induction of the cyclin genes. p34\(^{src}\) mRNA was not detectable in resting T cells and was not induced until 24 h after stimulation. We and others have previously shown that p34\(^{src}\) mRNA first appears in T cells at about 24 h and is not blocked by hydroxyurea (Furukawa et al., 1991; Terada et al., 1991). Deferoxamine blocked both cdk2 and cdc2 mRNA (Fig. 2 and data not shown). If cyclin D2 and cyclin C interact with a cdc2-related kinase in early G1, our results suggest that p34\(^{src}\) or cdk2 are unlikely candidates. Cyclin A can associate with and activate p34\(^{src}\) in many species, including clam (Rosenthal et al., 1980), Drosophila (Lehner and O’Farell, 1989), Xenopus (Draetta et al., 1989), and humans (Draetta and Beach, 1988; Pines and Hunter, 1989, 1990a). Cyclin A and cyclin E can also associate with p34\(^{src}\), a p34\(^{src}\)-related kinase (Koff et al., 1991; Tsai et al., 1991). The kinetics of induction of cyclin A, cyclin E, cdk2, and cdk2 are similar, again consistent with the notion that the kinetics of induction may reflect similarities in gene regulation.

Cyclin D2 and cyclin C mRNA are present 0–2 h after stimulation, considerably earlier than either the other cyclins or the p34\(^{src}\)-related kinase, p33\(^{src}\). Although cyclin C mRNA is present at or before that of cyclin D2, the further induction of RNA induced by mitogenic stimulus occurs later than cyclin D2 induction, at about 8–12 h. Thus, cyclin D2 is the first of these cyclin genes to be induced in T cells, several hours before the other cyclin genes respond to mitogens. The induction of cyclin D2 is blocked by cycloheximide treatment (data not shown), suggesting that cyclin D2 is not a classical "early response gene." The timing of induction of cyclin D2, and its partial IL-2-independence suggests a potential role for this cyclin at the G0/G1 transition, a point in the cell cycle not previously associated with control by cyclins or cdc2-like kinases. Although cyclin D2 was expressed in the absence of IL-2 expression (in the presence of a cyclosporin A block), its further induction and expression in late G1 appeared to be dependent on a supply of IL-2. In studies not shown, we observed that activation of T cells by PMA alone or by ionomycin alone were not sufficient to promote IL-2 secretion and did not lead to cyclin D2 expression. Simultaneous exposure to both PMA and ionomycin bypasses the requirement for T cell receptor activation, triggers IL-2 expression, and induces cyclin D2 RNA.

Expression of a third D-type cyclin gene, cyclin D1, was not detected in T cells, consistent with the previous studies of Matsushime et al. (1991), who showed that cyclin D1 was inducible in murine macrophages by colony stimulating factor-1 but was not present in murine T cells. This heterogeneity of CYL gene expression is unexpected from previous studies with other cyclins or from studies with cell cycle control genes in general, where conservation of function among different lineages is typical (Nurse, 1990). The reasons for the differential usage of D cyclins in T cells and macrophages are unknown. It is not due to differences between species since the human equivalent of cyclin D1, PRAD1, has been shown to be expressed in several human cell types (Matsushime et al., 1991; Withers et al., 1991), and in unpublished studies with normal murine T cells, we again observed induction of cyclin D2 and cyclin D3, but not cyclin D1. It is possible that this differential usage reflects the diversity of positive and negative growth signals in various cell lineages that are operative in controlling cell cycle progression during G1. However, it will be necessary to identify specific functions of each
D cyclin before the real significance of this observation can be evaluated. In preliminary studies with 24 different transformed human hematopoietic cell lines, cyclin D3 was found to be widely expressed in both myeloid and lymphoid cell lines, whereas cyclin D2 was expressed only in a small fraction, without regard to whether the line was myeloid, T cell or B cell in origin. Since all of the lines were factor-independent, it is possible that the "phenotype" of the D cyclins expressed may be a reflection of the generally unknown mutations in signaling or regulatory pathways that have led to factor independence.

Using polyclonal antisera raised against cyclin D2 and D3, we could not detect histone H1 kinase activity in multiple experiments at any time point between 0 and 48 h after stimulation, while control experiments demonstrated that anti-cdk2 precipitated an inducible histone H1 kinase in T cells (Fig. 6). This is consistent with older studies, which demonstrated that H1 kinase activity is minimal in G0 and G1 but is induced rapidly at G2/M coincident with the activation of p34^Cdc2. If a kinase coprecipitates with any of the D cyclins, other substrates such as pRb would be potentially useful for immune complex assays. In fact, Matsushima et al. (1992) have recently identified a novel kinase, cdk4, which can form complexes with D cyclins. These complexes lack histone H1 kinase activity but can phosphorylate pRb in vitro.

We did, however, detect specific coprecipitation of several potentially interesting cellular proteins with both of the cyclins (Fig. 5). It is noteworthy that the proteins which coprecipitate with cyclin D3 differ, in part, from the proteins which coprecipitate with cyclin D2, suggesting that the functions of these two cyclins in G0 may be distinct. The identification of these coprecipitating proteins may be useful in elucidating the functions of the D cyclins in T cells.

Taken together, our results indicate that striking differences exist in the induction and regulation of two candidate G1 cyclins in human T cells and suggest that these cyclins could participate in multiple cell cycle checkpoints during G0, S phase, and even potentially in G1. Detailed studies of gene regulation, identification of associated kinases, and identification of any other cellular proteins which interact with these cyclins, such as Rb or p107, are likely to be informative as to the exact role each of these cyclins play in regulating the growth of human T cells.