Effects of Iron-Depletion on Cell Cycle Progression in Normal Human T Lymphocytes: Selective Inhibition of the Appearance of the Cyclin A-Associated Component of the p33cdk2 Kinase

By Joseph J. Lucas, Agota Szepesi, Joanne Domenico, Kozo Takase, Attila Tordai, Naohiro Terada, and Erwin W. Gelfand

Iron removal by the chelating-agent deferoxamine (DFO) arrests cell cycle progression of activated human T cells in late G1 phase, before the G1/S border. The effects of the drug on molecules that regulate progression through the cell cycle were defined. DFO (10 μmol/L) inhibited induction of transcription of the cdc2 gene, but had no effect on accumulation of cdk2, cdk4, or interleukin (IL)-2 transcripts. No detectable p34cdc2 protein accumulated, but synthesis of the p33cdc2 protein was begun. It accumulated to normal levels during the first 20 to 30 hours of incubation in the presence of DFO. Furthermore, p33cdc2 was activated as an H1 histone kinase. As active p33cdc2 primarily represents complexes of the p33 protein with cyclin E or cyclin A, the effects of DFO on these cyclins were examined. Although the induction of synthesis and early accumulation of cyclin E and cyclin E-associated kinase activity appeared normal, the appearance of cyclin A and cyclin E-associated kinase activity were inhibited by DFO. However, the production of cyclin A mRNA appeared to be normal in the presence of DFO. A major effect of DFO in blocking cell cycle progression may be mediated through inhibition of the appearance of cyclin A protein and, therefore, a major component of p33cdc2 activity. The results also indicate that the p33cdc2/cyclin E activity produced in the presence of DFO was not sufficient for completion of the G1 phase of the cell cycle.

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depletion. The first component, due to a complex of p33Cdk2 and cyclin E, appears in nearly normal amounts and with normal kinetics. However, the complete inhibition of cyclin A synthesis, combined with a block to the further S-phase-associated accumulation of the p33Cdk2 and cyclin E proteins, prevents normal induction of the second wave of p33Cdk2 activity.

The system of DFO-arrested T cells thus permits examination of the differential roles and effects of the cyclin E- and cyclin A-associated p33Cdk2 kinases. Furthermore, as overproduction and/or activation of cyclins and cyclin-dependent kinases has been increasingly observed in certain tumors and tumor-derived cell lines, the possibility of using iron-chelating agents to target cells with such aberrations is raised.

MATERIALS AND METHODS

Cell preparation and culture. Peripheral blood T cells isolated from healthy volunteers were used in all experiments. Mononuclear cell suspensions were prepared by Ficoll-Hypaque gradient centrifugation, and T cells (E") were obtained by E-rosette enrichment. This population contained greater than 95% T cells as determined by immunofluorescence with anti-CD3 antibody. Cells were cultured in RPMI medium containing 10% (vol/vol) fetal calf serum and 2 mmol/L glutamine. When using normal lymphocytes, experiments are routinely repeated at least three times using cells from different donors to establish the generality and reproducibility of the findings.

Representative results are presented in the figures. T cells were activated by addition of phorbol dibutyrate (PDB; 10 nmol/L) and ionomycin (0.5 mmol/L). PDB, obtained from Sigma (St Louis, MO), was dissolved in dimethyl sulfoxide. Ionomycin, obtained from Calbiochem (San Diego, CA) was prepared as a 5 mmol/L stock solution in dimethyl sulfoxide. DFO was obtained from Ciba-Geigy (Mississauga, Canada) and prepared as a stock solution in distilled water at a concentration of 200 mmol/L. Metabolic labeling of cellular proteins was performed by preincubating cells in methionine-free medium containing dialyzed fetal calf serum for 30 minutes, adding 150 μCi/mL of [35S]-methionine (specific activity, 1,200 Ci/mmol; Dupont NEN, Wilmington, DE), and incubating for the times indicated.

Simultaneous flow cytometric determination of DNA and p34CdK2 content. The method described by Takase et al was used. After a 60-minute fixation with 70% ethanol at 4°C, cells (about 10^6) were pelleted and washed. After a 5-minute incubation in 250 μL of phosphate-buffered saline (PBS) containing 0.5% Tween 20, 0.5% bovine serum albumin, and 1.5% human γ-globulin (Sigma) at room temperature, 2 μg of mouse monoclonal antibody p34CdK2 antibody (Zymed, San Francisco, CA) was added, and the mixture was incubated at 4°C for 120 minutes.

Immunoblot analysis. For immunoblot analysis, cells were washed with PBS and then lysed at 4°C in a buffer containing 25 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 50 μg/mL aprotinin, 50 μg/mL leupeptin, and 100 mmol/L sodium orthovanadate. After removal of cellular debris by centrifugation, lysates were prepared for electrophoresis, and polyclonal antibodies were used.

Immunoprecipitation of metabolically ([35S]-methionine) labeled proteins. After labeling of cells, as described above, they were collected by centrifugation and washed twice with cold PBS. Cells were disrupted by a 15-minute incubation in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris pH 7.4, 0.1% Triton X-100, and 0.5% sodium deoxycholate) containing 1 mmol/L PMSF, 10 μg/mL leupeptin, 20 μg/mL aprotinin, 6.1 mmol/L sodium orthovanadate, and 50 mmol/L sodium fluoride. Cells were centrifuged, and supernatants were incubated with Protein G coupled to Sepharose beads (Zymed) for 30 minutes at 4°C. After removal of the Protein G-beads, supernatants were incubated with the appropriate primary antibody, as follows: for detection of p34CdK2, a mouse monoclonal antibody directed against a peptide comprising the C-terminal 15 amino acids of the protein (Zymed, San Francisco, CA); for p33Cdk2, a rabbit polyclonal antibody directed against a peptide comprising the C-terminal 12 amino acids of the protein (UBI, Lake Placid, NY); for cyclin A, a rabbit polyclonal antibody directed against a peptide comprising a C-terminal portion of the protein (residues 359-432; UBI); for cyclin E, a mouse monoclonal antibody (HE 12) prepared using recombinant cyclin E protein (PharMingen, San Diego, CA); and for p110α, a mouse monoclonal antibody (1 F8) prepared using recombinant Rb protein (Santa Cruz Biotech, Santa Cruz, CA). Proteins were detected by an enhanced chemiluminescence (ECL) method using reagents and protocols provided by Amersham International (Arlington Heights, IL). The secondary antibodies used were either a sheep antirabbit or donkey antirabbit antibody, linked to horseradish peroxidase.

Northern blot analysis. Total cellular RNA was extracted from 2 × 10^7 cells in 300 mmol/L sodium acetate (pH 5.5), 10 mmol/L EDTA (pH 8.0), 2% sodium dodecyl sulfate (SDS), 1 mg/mL heparin sodium (Grade I; obtained from Sigma), which was added as an RNase inhibitor. Five microgram amounts of RNA were electrophoresed in a 1% formaldehyde-agarose gel, blotted onto a nylon membrane (MSI, Westboro, MA), and hybridized with multiple primed-radioiodinated probes, as described previously. Equal loading of gel lanes was confirmed by monitoring 28S and 18S ribosomal RNAs after ethidium bromide-staining of the gels. The probes used here were the excised inserts from plasmids (pSE999 and pSE1000) containing full-length cDNA copies of the human p34CdK2 and p33Cdk2 mRNAs, as described by Elledge and Spottswood. For detection of cdk4 transcripts, the excised insert from a full-length human PSK-J/Cdk4 cDNA probe, provided by Dr S.K. Hanks (Duke University, Durham, NC), was used. Interleukin (IL)-2 mRNA was detected as described previously. For detection of cyclin A and E transcripts, excised inserts from plasmids containing the human cyclin A and cyclin E sequences, which were provided by Drs Tony Hunter (Salk Institute, San Diego, CA) and Steven Reed (Scripps Research Institute, LaJolla, CA), respectively, were used.

Immunoprecipitation analysis. For immunoprecipitation analysis, cells were washed with PBS and then lysed at 4°C in a buffer containing 25 mmol/L Tris-HCl pH 7.4, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 50 μg/mL aprotinin, 50 μg/mL leupeptin, and 100 mmol/L sodium orthovanadate. After removal of cellular debris by centrifugation, lysates were prepared for electrophoresis, and polyclonal antibodies were used.

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antigen. Immunocomplexes were recovered by binding to protein G-beads. Complexes were washed five times with RIPA buffer before being suspended in electrophoresis sample buffer. Samples were heated for 5 minutes at 90°C and beads were pelleted by centrifugation and discarded. The denatured proteins were resolved by SDS-PAGE and visualized by fluorography.

Immunoprecipitation of active cyclin-dependent kinase/cyclin complexes. For isolation of active complexes for in vitro kinase assays, the method of Howe et al was used, with some modifications. Cells (2 x 10⁶ to 5 x 10⁶) were washed with PBS and then lysed in a buffer containing 50 mmol/L Tris-HCl pH 7.4, 5 mmol/L EDTA, 250 mmol/L NaCl, 1 mmol/L PMSF, 50 μg/mL aprotinin, 50 μmol/L leupeptin, 100 μmol/L sodium orthovanadate, and 0.1% Nonidet-P40 (NP-40 lysis buffer). Complexes were precipitated with the same antibodies used for immunoblotting, except in the case of cyclin E, for which the mouse monoclonal antibody HE 67 (Pharmino) was used. Optimal amounts of primary antibodies were used, as determined by prior immunoprecipitation experiments using radiolabeled (35S-methionine) protein extracts. Complexes were recovered by precipitation with protein G chemically bound to Sepharose beads (Zymed).

Enzymatic assay for cdk activity. The complexes, prepared by immunoprecipitation, were washed first with the NP-40 lysis buffer and then with a solution containing 50 mmol/L Tris-HCl pH 7.4, 10 mmol/L magnesium chloride, and 1 mmol/L dithiothreitol. They were incubated in a kinase reaction buffer (25 μL per sample) containing 50 mmol/L Tris-HCl pH 7.4, 10 mmol/L magnesium chloride, 1 mmol/L dithiothreitol, 80 μmol/L adenosine triphosphate (ATP), 10 μg of histone H1 (Boehringer Mannheim, Indianapolis, IN), and 40 μCi of [γ-32P]-ATP at 30°C for 10 minutes. All reactants were in excess, and the reactions were stopped by addition of an SDS-containing electrophoresis sample buffer while in a linear range of reaction. After heating at 90°C for 5 minutes and then cooling, the protein G-immunobeads were pelleted by centrifugation, and proteins in the reaction mixtures were resolved by SDS-PAGE. The histone H1 band was visualized by a brief staining of the gels with Coomassie Blue (R-250) in fixative (10% acetic acid/40% methanol/50% water). The gels were then dried, and the labeled histone bands were detected by autoradiography. The Coomassie-stained histone bands were excised from the dried gels and quantitated by scintillation counting. Unless noted in the text, values were corrected for nonspecific activity, which was determined from duplicate samples in which the primary antibody step was omitted. As comparisons are made between levels of activities obtained using complexes immunoprecipitated using different antibodies (eg, cyclin A- vs cyclin E-associated activities), data are expressed as the percentage of maximal activity obtained with a particular antibody. Because many factors associated with the reactions, for example, the differential effects of the antibodies on reactions performed by particular cdk/cyclin complexes, are unknown, direct comparisons of the absolute values (counts per minute incorporated into the H1 histone substrate) are not made.

RESULTS

Effects of DFO on cell cycle progression in normal human T lymphocytes. As shown previously, dual flow cytometric analysis of DNA and RNA contents have permitted a detailed delineation of the kinetics of cell cycle progression in normal human T lymphocytes. In brief, it was shown that T cells activated with the potent combination of PDB and ionomycin (PDBI) rapidly enter the G1 phase of the cell cycle, as indicated by increasing content of RNA. S-phase entry, indicated by increased DNA content, begins at about 30 hours after activation. By performing flow cytometric analysis of cells that have been activated with PDBI and then accumulated about the G2/M border with demecolcine, it has been estimated that 80% to 90% of activated T cells enter and progress through the cell cycle (Takase et al, unpublished results, April 1994). DFO has been shown to arrest T-cell proliferation before S-phase entry. Determinations of RNA content indicated that this point occurred in mid- to late G1 phase, as cells possessed an overall RNA content significantly lower than T cells poised to enter S phase.

T-cell progression and the effects of DFO on the cell cycle were further delineated using a new method for the simultaneous determination of DNA and p34cdc2 cellular contents. As shown in Fig 1, resting T cells represent a homogeneous population of cells containing a G1 content (2N) of DNA and low p34cdc2 content. As shown previously, resting T cells isolated from peripheral blood, in fact, contain no p34cdc2, as measured by sensitive immunoblotting or immunoprecipitation methods, and so the levels of fluorescence indicated in Fig 1A permit definition of background levels for the technique. As shown in Fig 1B, increased DNA and p34cdc2 contents are clearly detectable at 30 hours and are dramatically enhanced by 40 hours (Fig 1D). Little change is seen in either DNA or p34cdc2 content in DFO-treated cells (Fig 1C and E), in agreement with biochemical analysis presented elsewhere and below. Because p34cdc2 content does not return to the low levels characteristic of T cells in their first G1 phase, cells that have progressed through the cell cycle and re-entered G1 phase appear, as in Fig 1D, as cells with a G1 (2N) content of DNA but high p34cdc2 content. Therefore, these results provide confirmation that a large portion of PDBI-activated T cells successfully and synchronously progress through the cell cycle during the first 40 hours after activation and that DFO effectively blocks cells before both p34cdc2 accumulation and DNA replication.

Effects of DFO on expression of T-cell cycle regulatory genes. In previous reports, we showed that DFO inhibited cell cycle progression at a point in late G1 phase, before the G1/S-phase transition, which was earlier than and distinguishable from the arrest point induced by either aphidicolin or hydroxyurea. T lymphocytes arrested by DFO contained no detectable p34cdc2 protein, a protein whose synthesis begins just before and independently of S-phase entry. In the first cell cycle of activated human T lymphocytes, the cdk2 and cdc2 genes are first expressed beginning at about 8 and 24 hours after activation, respectively. As shown in Fig 2, accumulation of transcripts of these two genes shows a differential sensitivity to the chelating agent. The steady-state levels of cdk2 mRNAs accumulated are the same in the presence or absence of the drug, even at a concentration of 100 μmol/L. Accumulation of cdc2 mRNA, on the other hand, is almost completely suppressed by DFO, even at the low dose of 10 μmol/L. As shown in Fig 3, expression of the cdk4 and IL-2 genes, which normally is first detected within 3 to 4 hours after activation, was also not affected by DFO.

Effects of DFO on accumulation of cell cycle regulatory proteins. Stimulation of T cells with PDBI induces a large portion of the cells to synchronously enter the cell
Fig 1. Simultaneous flow cytometric detection of DNA and p34cdc2 content in human T lymphocytes activated in the presence or absence of DFO. Normal human T lymphocytes were activated with PDB/1 (P/I) in the absence (A, B, and D) or presence (C and E) of DFO and harvested at 0 (A), 30 (B and C), and 40 (D and E) hours after treatment. Cells were fixed and stained for flow cytometric analysis as described in Materials and Methods. In these two-dimensional cytograms, cellular p34cdc2 content (horizontal axis) is presented on a logarithmic scale, whereas DNA content (vertical axis) is on a linear scale.

cycle, with DNA synthesis first beginning at about 30 hours after cell activation is begun. The appearance of four cell cycle regulatory proteins (p34cdc2, cyclin A, p33cdc2, and cyclin E) and the effects of DFO on their accumulation are shown in the immunoblotting results presented in Fig 4. The p34cdc2 protein first appears in activated T cells at 30 to 40 hours after activation, almost concomitant with but independent of the onset of DNA replication. Its appearance is completely inhibited by 10 μmol/L DFO, as late as 40 hours after stimulation. The normal pattern of accumulation of cyclin A, which forms complexes with both the p34cdc2 and p33cdc2 proteins, is similar to that of the p34cdc2 protein. Its appearance is also completely prevented by DFO. The p33cdc2 and cyclin E proteins comprise a pair of proteins whose patterns of synthesis also appear concordant. Both are present in resting T cells at very low levels. The basal level of p33cdc2 appears to be inactive as a functional kinase. These proteins begin to increase in amount in early to mid-G1 phase (at 10 to 15 hours after stimulation), about the time at which enzymatically active p33cdc2 kinase is first detected. As shown in Fig 4, increased amounts of the two proteins appear in the presence of DFO. However, only levels of the proteins characteristic of those seen at about 30 hours after normal activation (ie, in the absence of DFO) are achieved in the presence of the drug. Thus, although induction of synthesis of the proteins occurs and appears to proceed normally for the first 30 hours after activation, continued production of the proteins, characteristic of S-phase progression, is partially inhibited. It is
also demonstrated in Fig 4 that normal levels of the p33<sup>cdk2</sup> and cyclin E proteins accumulate in T cells that are activated in the presence of DFO and an excess of iron.

Also, it was determined that phosphorylation of the p110<sup>Rb</sup> protein (shown by the appearance of the slower mobility forms of the protein) was initiated in the presence of DFO (Fig 4). However, levels of phosphorylation of p110<sup>Rb</sup> achieved in the presence of DFO were characteristic of those normally seen after about 30 hours after activation; attainment of the normal pattern of hyperphosphorylation, characteristic of S-phase entry and progression, was partially suppressed. Also shown in Fig 4, addition of excess iron to the cultures almost completely restored the normal pattern of accumulation and phosphorylation of p110<sup>Rb</sup>, indicating that the effects of DFO were, indeed, due to chelation of the metal.

**Effects of DFO on metabolism of the p33<sup>cdk2</sup> protein.** The results described above indicate that the induction of cdk2 gene expression and p33<sup>cdk2</sup> protein synthesis occurred normally in the presence of DFO and that the point of arrest in late G1 phase, before the first appearance of the p34<sup>cdk2</sup> and cyclin A proteins, which normally begin just before the G1/S-phase transition. The exact role(s) of the p33<sup>cdk2</sup> kinase in cell cycle progression is still unclear. It has been proposed that the p33<sup>cdk2</sup> kinase, which has been found to be associated with both cyclin A and cyclin E, may regulate passage through the so-called "restriction point," entry into S phase, and/or progression through S phase. A possible role for p33<sup>cdk2</sup> at the G2/M phase transition has also been discussed. As DFO arrested cell cycle progression in late G1 phase, apparently without suppressing the initial production of the p33<sup>cdk2</sup> protein, the effects of the drug on this protein were examined in more detail.

First, as shown in Fig 5A, the levels of p33<sup>cdk2</sup> protein accumulated in the presence or absence of DFO were compared. In this experiment, the p33<sup>cdk2</sup> protein was prepared by immunoprecipitation. After resolution of proteins by electrophoresis, the protein was detected by immunoblotting. As shown by comparison of lanes 1 and 2 in Fig 5A, the amount of p33<sup>cdk2</sup> present at 30 hours after activation (characteristic of late G1 phase) were very similar in cells incubated in the absence (lane 1) or presence (lane 2) of DFO. However, continued accumulation was suppressed, as shown by comparison of lanes 3 and 4. By 40 hours after activation, the amount of p33<sup>cdk2</sup> present in DFO-treated cells (lane 4) was less than that detected in untreated cells (lane 3). As shown in lane 5, the inhibitory effect of DFO on accumulation of the p33<sup>cdk2</sup> protein by 40 hours was effectively overcome by the addition of excess iron.

As noted above, levels of cdk2 mRNA appeared to be normal in cells activated in the presence of DFO, even as late as 48 hours of incubation. To determine whether the decreased accumulation of the protein was due to decreased synthesis or enhanced degradation of the protein, the follow-
CELL CYCLE ARREST BY IRON DEPLETION

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Fig 4. Effect of DFO on accumulation of the p34cdc2, cyclin A, p33cdk2, cyclin E, and p110Rb proteins in activated human T cells. T cells were stimulated with PDB/I and incubated in the absence (lanes 1 through 4) or presence (lanes 5 through 8) of 10 μmol/L DFO. Samples were harvested at initiation of the experiment (0 hours, lane 1) and at 20 (lanes 2 and 5), 30 (lanes 3 and 6), and 40 (lanes 4 and 7) hours of incubation. Samples were also prepared at 40 hours from a culture activated with PDB/I in the presence of both DFO and an excess of iron, provided by 500 μmol/L ferric citrate (lane 8). Samples were analyzed by immunoblotting for the p34cdc2, cyclin A, p33cdk2, cyclin E, and p110Rb proteins. Immunoreactive protein bands of appropriate size, as compared with marker proteins of known molecular weight included in the same gels, are indicated. Protein extracts prepared from equal numbers of cells were loaded in the lanes.

The absence or presence of DFO. A portion of each culture was harvested at 45 hours. The remaining cells were washed free of the radioisotope, and portions were incubated for an additional 2 or 4 hours in normal growth medium. The p33cdc2 protein was isolated from the six samples and visualized by autoradiography after electrophoresis. As shown in Fig 5C, the amount of label incorporated during the labeling period was, as expected, somewhat reduced in the presence of DFO (lane 4) as compared with the control cells activated in the absence of the drug (lane 1). However, the kinetics of decay appeared to be essentially the same in the presence or absence of DFO. A slight loss of radioactivity associated with p33cdc2 was detected by 2 hours (lanes 2 and 5) after the labeling period and became more pronounced by 4 hours (lanes 3 and 6). It appeared that the protein was a relatively long-lived one in the absence or presence of DFO. No rapid or dramatic degradation of p33cdc2 occurred in the presence of the drug, supporting the notion that the increased synthesis normally occurring as cells entered and progressed through S phase was, indeed, curtailed.

The effects of DFO on H1 histone kinase activities in T cells. The levels of H1 histone kinase activity due to p34cdc2 and p33cdk2 after T-cell activation in the presence or absence of DFO were compared. In the experiment shown in Fig 6, kinase activities associated with specific immunoprecipitates of the two kinases were measured at 55 hours after stimulation. At this late time point, normal levels of activity due to both kinases were very high. As expected considering its inhibitory effect on the appearance of the protein, DFO almost completely prevented the appearance of p34cdc2 kinase activity. Activity due to p33cdk2 was also substantially reduced, to about 8% of its control level, as determined using the extract prepared from cells activated in the absence of the drug. However, that this represents a substantial level of activity above background control levels or the low residual level of p34cdc2 activity is apparent from a comparison of the two autoradiograms presented as insets in Fig 6.

As DFO appeared to inhibit the late accumulation of p33cdk2, but not its initial synthesis, p33cdk2 activity was next measured at earlier times after activation in the presence or absence of the drug. In these experiments, active cyclin/p33cdk2 complexes were isolated by immunoprecipitation from cells activated in the presence or absence of DFO. Histone H1 kinase activities were determined using the in vitro kinase reaction method described above. The normal kinetics of activation of p33cdk2 after T-cell stimulation occurs in two distinguishable phases (Fig 7, solid bars). Activity is first detectable at about 15 to 20 hours after stimulation. It increases slowly for up to 30 hours, until the G1/S-phase transition, reaching about 10% of subsequent maximal levels. After entry into S phase, the second phase of activation, marked by a rapid 10-fold increase in activity, begins. In the presence of DFO (Fig 7, open bars), the initial phase of activation of the kinase was observed; the levels of p33cdk2 activity present at 20 hours were nearly the same in cells activated in the presence or absence of DFO. By 30 hours, however, an inhibitory effect of the drug became evident, and the dramatic 10-fold increase associated with S-phase entry and progression was nearly completely inhib-
It has been shown in several cell systems that the p33\textsuperscript{cdk2} kinase forms active complexes primarily with two cyclins, cyclin E and cyclin A,\textsuperscript{26,30,40,46,48,52,53,55} and that the cyclin E-associated activity precedes cyclin A-associated activity in the G1 phase of the cell cycle.\textsuperscript{26,30,51,52} The effects of DFO on cyclin E- and A-associated H1 histone kinase activities were, therefore, examined, as shown in Fig 8. Cyclin E-associated activity was first detected in mid-G1 phase (about 20 hours); a larger increase, although not as great as that seen for overall p33\textsuperscript{cdk2} activity, was seen after S-phase entry. As for initial overall p33\textsuperscript{cdk2} activity, the initial appearance of cyclin E-associated activity was refractory to DFO. Even at 30 hours after activation, inhibition of cyclin E-associated activity was modest; the level of cyclin E-associated activity detected in S phase was also only partially (about 40%) inhibited by DFO. In contrast, the appearance of cyclin A-associated activity (Fig 8) was completely inhibited by DFO, consistent with observations that the drug prevents the appearance of cyclin A protein. It is also shown that a low level of cyclin A-associated activity appears at about 30 hours after stimulation (in the absence of DFO) and increases more dramatically as cells enter S phase. Thus, p33\textsuperscript{cdk2} activity seen before about 30 hours appears to be due primarily to cyclin E/p33\textsuperscript{cdk2}, and its synthesis and activity are largely resistant to the effects of DFO. Beginning at about 30 hours, p33\textsuperscript{cdk2} activity is due in some part to cyclin A/p33\textsuperscript{cdk2}. As cyclin A synthesis is completely inhibited, this component of p33\textsuperscript{cdk2} activity does not appear and cell cycle progression is halted. However, the possibility that the modest decrease seen in G1 phase cyclin E-associated activity also plays some role in the DFO-induced arrest is not precluded by the present data.

Effects of DFO on accumulation of cyclin E and cyclin A mRNAs. To further analyze the level at which DFO exerted its inhibitory effect on cyclin A protein accumulation, the levels of cyclin A and cyclin E mRNAs were compared. As shown in Fig 9, cyclin E mRNA was easily detected at 24 and 48 hours after T-cell activation. As expected based on the observation that cyclin E protein accumulation proceeded fairly normally during this period (Fig 4), DFO had little effect on cyclin E mRNA levels. Cyclin A mRNA levels, which could also be readily detected at both 24 and 48 hours after activation, appeared only slightly depressed in

![Figure 5](image-url)

**Fig 5.** Effect of DFO on the accumulation, rate of synthesis, and rate of degradation of the p33\textsuperscript{cdk2} protein in activated human T cells. (A) Accumulation of the p33\textsuperscript{cdk2} protein in T cells in the presence and absence of DFO were compared. T cells were activated with PDB/I in the absence (A, lanes 1 and 3) or presence of DFO (A, lanes 2 and 4) or of DFO and excess iron (A, lane 5) and harvested at 30 (A, lanes 1 and 2) or 40 (A, lanes 3 through 5) hours after incubation. The p33\textsuperscript{cdk2} protein was isolated by immunoprecipitation. After resolution of proteins by gel electrophoresis, the p33\textsuperscript{cdk2} protein was visualized by immunoblotting. (B) The rates of synthesis of the p33\textsuperscript{cdk2} protein in the presence and absence of DFO were compared. T cells were activated with PDB/I in the absence (B, lanes 1, 3, 4, and 6) or presence (B, lanes 2 and 5) of DFO. Cultures were metabolically labeled with \textsuperscript{35}S-methionine from 18 to 25 (B, lanes 1 through 3) or 26 to 33 (B, lanes 4 through 6) hours of incubation. The p33\textsuperscript{cdk2} protein was prepared by immunoprecipitation (B, lanes 2, 3, 5, and 6) with specific rabbit polyclonal antibody and detected by fluorography after resolution of protein by gel electrophoresis. Control radiolabeled samples were incubated with rabbit antiserum from a nonimmunized animal instead of the specific anti-p33\textsuperscript{cdk2} antiserum and were similarly processed (B, lanes 1 and 4) to facilitate identification of the p33\textsuperscript{cdk2} protein band, which is indicated in the figure. (C) The degradation of the p33\textsuperscript{cdk2} protein in the presence and absence of DFO were compared. T cells were activated with PDB/I in the absence (C, lanes 1 through 3) or presence (C, lanes 4 through 6) of DFO and metabolically labeled with \textsuperscript{35}S-methionine at 42 to 45 hours of incubation. Samples were harvested immediately after labeling (C, lanes 1 and 4) and at 2 (C, lanes 2 and 5) and 4 (C, lanes 3 and 6) hours after removal of the \textsuperscript{35}S-methionine from the cultures by extensive washing. The p33\textsuperscript{cdk2} protein, indicated in the figure, was isolated by immunoprecipitation and visualized by fluorography after resolution of proteins by gel electrophoresis, as described above. Protein extracts prepared from equal numbers of cells were loaded in the lanes.
Fig 6. Effect of DFO on p34cdc2- and p33
cdc2-associated H1 histone kinase activities in human T cells. T cells were activated with PDB/I either
in the presence or absence of DFO and harvested at 55 hours of incubation. The p34cdc2 and p33
cdc2 proteins were isolated by immunoprecipitation and assessed for H1 histone kinase activity, as described in Materials and Methods. The activity associated with immunoprecipitates prepared from DFO-treated cells is shown graphically, as compared with those prepared from the control activated cells. Background levels were determined by determining kinase activity associated with duplicate samples prepared without primary specific antibody. The insets show the radiolabeled H1 histone band, after gel electrophoresis and autoradiography, phosphorylated by kinase prepared from control activated cells (lanes 1) or from cells stimulated in the presence of DFO (lanes 2). Lanes 3 show the background levels of radioactivity caused by performing the reaction with samples prepared from cells activated in the absence of DFO, but processed without addition of the specific anti-p34cdc2 or anti-p33
cdc2 antibodies. The values given by these samples were subtracted from the experimental data.

DISCUSSION
Recent analysis of the cell cycle of mammalian cells has shown that major cell cycle transitions are regulated at least in part by a family of serine-protein kinases, called cyclin-dependent kinases, and the cyclin molecules with which they interact. One member of this family, p34cdc2 (or p34
\( ^{\text{cdk2}} \)), complexed with cyclins of the B family, plays a role in regulating entry into mitosis. Much evidence suggests that a second member of the kinase family, p33cdc2, complexed with cyclins of the A and/or E families, regulates earlier, important cell cycle regulatory events: commitment to cell division, occurring in late G1 phase (at a putative start or restriction point), the G1/S phase transition, and, perhaps, even passage through S phase. However, the fact that no in vivo substrates for these cdk/cyclin complexes have been definitively identified (although several have been proposed), combined with the apparent promiscuity of the enzyme in combining with cyclins of many families including not only A and E cyclins, but also members of the D family, have hampered convincing demonstration of the actual functions of the p33
\( ^{\text{cdk2}} \) kinase. Furthermore, the discovery of numerous other members of the cyclin-dependent kinase and cyclin families in animal cells suggests a possible level of complexity beyond simple models that describe the cell cycle in terms of a single linear chain of events in which major transitions are performed by single kinase/cyclin complexes. Even the model in which the p34
\( ^{\text{cdk2}} \) kinase regulates two major transitions in yeast is complicated by the recent identification of a multitude of different cyclins in yeast, each of which shows its own particular cell cycle-dependent pattern of synthesis and association with the p34
\( ^{\text{cdk2}} \) or p33
\( ^{\text{cdk2}} \) protein.

In the present study, the effects of DFO on proliferation of normal human T cells were defined in terms of the molecules involved in G1-phase progression and S-phase entry. Inhibition of cell cycle progression with DFO arrested the cells at a point in G1 phase before the appearance of cyclin A. However, the G1 phase-associated synthesis of cyclin E and p33
\( ^{\text{cdk2}} \) and their assembly into a complex with enzymatic activity proceeded almost normally. These results suggest that the p33
\( ^{\text{cdk2}} \)/cyclin A complex is needed for completion of the G1 phase of the cell cycle. However, the possibilities that the early cyclin E-associated activity produced in the presence of DFO, although little affected, is still insufficient for completion of G1-phase or that still other, as yet unidentified, cell cycle regulatory events are also inhibited have not been excluded. For example, whether or not DFO has an effect on the synthesis and/or activities of low-molecular-
weight proteins such as p21 and p27, which can inhibit the activity of cyclin-dependent kinases, remains to be determined. As the synthesis of DNA is blocked, because of inhibition of ribonucleotide reductase, many events associated with normal progression through S phase are also blocked. These events include the complete S phase-associated increases in (1) cyclin E and p33^{cdk2} protein levels, (2) cyclin E-associated kinase activity, and (3) complete hyperphosphorylation of the p110^{cdk2} protein. As shown in Figs 4, 7, and 8, all of these events are dampened. It is suggested, however, that these events are subsequent or secondary to the late G1-phase block described here and in previous reports. As events associated with S-phase progression, including the dramatic increase in p33^{cdk2}/cyclin E activity, were depressed by DFO, a role for this latter complex in S phase is blocked. These events include the complete inhibition of cyclin A protein in DFO-arrested cells affects the forms of the E2F transcription factor that are present. E2F, which can be found in complexes with cyclin A and cyclin E, appears to be needed in a free form for activation of transcription of genes necessary for S phase (reviewed by Ne~ins). Gel shift analysis has yielded the expected result that DFO inhibits the appearance of the cyclin A-containing form of E2F complex (Terada et al, unpublished results, February 1994). However, the effects of this on the relative amounts of other forms of E2F and on transcription of E2F-regulated genes require further study.

Since our initial reports on the inhibition of accumulation of p34^{cdk2} in normal T cells and neuroblastoma cells, the effects of another iron chelator, mimosine, on H1 histone kinases has been described. In contrast with results presented here, it was noted that the addition of mimosine to human fibroblasts entering the cell cycle resulted in essentially complete inhibition of total cdk2 activity. However, it should be noted that, whereas our studies used the minimal dose (10 mmol/L) of DFO needed for effective inhibition of S-phase entry, the latter study used a concentration of mimosine of 300 mmol/L. Determination of whether or not the observed differences seen are due to different concentrations of agents, to different cell types, or to actual differences in initiated before S-phase entry in normal human T cells. Initiation of its synthesis is independent of S-phase entry, and it is accumulated to aberrantly high levels in cells that are blocked at the G1/S interface by treatment with aphidicolin. We have, therefore, begun to examine events leading to production of cyclin A, and the effects of DFO on these events. Surprisingly, it was found that levels of cyclin A mRNA were little affected by the presence of DFO. Because the rate of synthesis of the p33^{cdk2} protein (Fig 5) and the kinetics of accumulation of cyclin E protein (Fig 4) are proceeding nearly normally during DFO arrest, when no detectable cyclin A protein is accumulated (Fig 4), the possibility arises that DFO is preferentially inhibiting the translation of certain mRNAs. Roles for iron in the translation of proteins have been demonstrated and discussed previously. Alternatively, DFO may be inhibiting the appearance of cyclin A mRNA in the cytoplasm. A possible role for iron in mRNA transport has also been recently noted.

Lastly, it is possible that the stability of any cyclin A protein that is synthesized is greatly reduced in the presence of the drug. All of these possibilities are amenable to direct experimental analysis, as is now in progress. Finally, it should be noted that synthesis of the p34^{cdk2} protein is also prevented by treatment with DFO. However, since this protein is not activated as a kinase until near the G2/M-phase border in the first cycle of normal T cells induced to enter the cell cycle, it is unlikely that inhibition of its synthesis is responsible for the arrest in late G1 phase observed here.

Now that the block to cyclin A production in DFO-arrested T cells has been identified, more incisive molecular analysis of the phenomenon, both in normal T cells and in cultured cell lines, will be possible. Such studies will include determination of the mechanism by which cyclin A absence affects subsequent events in T-cell cycle progression. To this end, we have begun an examination of how the absence of cyclin A protein in DFO-arrested cells affects the forms of the E2F transcription factor that are present. E2F, which can be found in complexes with cyclin A and cyclin E, appears to be needed in a free form for activation of transcription of genes necessary for S phase (reviewed by Ne~ins). Gel shift analysis has yielded the expected result that DFO inhibits the appearance of the cyclin A-containing form of E2F complex (Terada et al, unpublished results, February 1994). However, the effects of this on the relative amounts of other forms of E2F and on transcription of E2F-regulated genes require further study.

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the mechanisms of action of the drugs will likely require direct comparisons of the two systems. However, despite the outcome of such studies, it is important, in view of the current and potential clinical usefulness of DFO, to determine the mechanism(s) of action of the drug not only in normal human T cells, but also in other normal and transformed cell types. Finally, it is noteworthy that the effects described in the present report were all observed using a dose of 10 μmol/L, a concentration that can be achieved in humans in clinical applications, as noted above. As reported by Ajchenbaum et al., treatment of T cells with 100 μmol/L DFO resulted in dramatic inhibitory effects on expression of several cell cycle regulatory molecules, including cdk2 and, to a lesser extent, cyclin E. However, it is likely that this excess of chelating agent has other more nonspecific and toxic effects on sensitive cells. Consistent with the findings of Ajchenbaum et al., we had previously demonstrated that 100 μmol/L DFO, but not 10 μmol/L DFO, will cause an almost complete inhibition of the accumulation and phosphorylation of the p110 tumor suppressor protein.

Several recent studies suggest that cell cycle models developed solely from analysis of continuously growing, transformed cell lines may not adequately reflect mechanisms involved in normal cell growth. This possibility was identified in several earlier studies, that showed that the relative levels of certain molecules, such as some cdk and cyclins, varied greatly among various cell lines and did not reflect true levels in normal cells of origin. More recently, it was noted that certain complexes, comprised of cdk, cyclins, proliferating cell nuclear antigen (a component of DNA polymerase δ; PCNA), and other small molecules, were altered in composition or completely disrupted in normal fibroblastic cells after virus-induced transformation. Furthermore, our recent comparison of a growth-controlled B-lymphoblastoid cell line with normal lymphocytes raised the possibility that the mechanisms regulating the orderly activation of appropriate cdk5 was fundamentally altered in the cell line.

In brief, it appeared that both the cdc2- and cdk2-encoded kinases were activated to a high level before S-phase entry in the first cycle of cells exiting a resting (G0) state. Synthesis of the p34cdc4 protein also appeared to be aberrantly regulated in the cell line. Collectively, these observations, combined with a growing body of evidence showing abnormal cyclin production in a variety of tumors and tumor-derived cells, suggest the possibility that agents such as DFO that target pathways involved in the synthesis and/or regulation of these molecules might show useful, differential effects on normal and transformed cells, a possibility now being explored in further studies.
As noted above, drugs that can exert profound antiproliferative effects, such as DFO, are used in many clinical situations. Often, these agents inhibit cell growth at particular points in the cell cycle, and attempts have been made to define the molecular basis for such inhibitory effects. Emerging concepts and precise knowledge of the identities and mechanisms of action of the molecules regulating major cell cycle transitions should permit identification of the targets of such drugs and, as a result, their more efficacious use in clinical applications.

ACKNOWLEDGMENT

We are grateful to Drs Giulio Draetta, Steven Elledge, Steven Hanks, Tony Hunter, Matthew Meyerson, David Morgan, and Steven Reed for providing reagents (plasmids and antibodies) and suggestions concerning their use during the course of this work. We also thank Drs Paul Seligman, Jaime Modiano, and Richard Franklin for helpful discussions and the personnel of the Children's Hospital Blood Donor Center (Denver, Co) for providing us with human blood.

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JJ Lucas, A Szepesi, J Domenico, K Takase, A Tordai, N Terada and EW Gelfand