Association of Proliferating Cell Nuclear Antigen With Cyclin-Dependent Kinases and Cyclins in Normal and Transformed Human T Lymphocytes

By Agota Szepesi, Erwin W. Gelfand, and Joseph J. Lucas

The proliferating cell nuclear antigen (PCNA) is an auxiliary protein of DNA polymerase δ and appears to be needed for both DNA synthesis and DNA repair. It is present in low amount in resting normal human T lymphocytes and, upon mitogenic stimulation with phorbol dibutyrate and ionomycin, begins to increase in mid-G1 phase, approximately 12 to 15 hours before entry into S phase. PCNA continues to increase in amount throughout the cell cycle and remains high in proliferating cultures. PCNA was extracted from activated normal T cells and from the transformed T-lymphotblastoid cell line Jurkat by a method that recovered approximately 98% of total cellular PCNA but yet retained its associations with other proteins. PCNA immunoprecipitates possessed H1 histone kinase activity, which increased in parallel with increasing cellular content of PCNA. Both the cdc2 and cdk2 kinases were found associated with PCNA in normal T cells, in amounts consistent with detected kinase activity. The results indicate that PCNA is not an inhibitory molecule of cdk/cyclin activity. Both normal and transformed T cells contained PCNA in association with cdk2, cdk4, cdk5, and cdk6, with the amount of PCNA associated with these molecules increasing in the order listed. Relatively high amounts of PCNA were also found associated with cyclins D2 and D3, the major cyclin partners of cdk6 in T cells. Though detected in normal cells, PCNA/cdc2 complexes were present in exceedingly low amount, if at all, in Jurkat cells. This cell line appeared to contain more of nearly all of the cdk and cyclin molecules analyzed, but there seemed to be little difference in the patterns of association of these molecules with PCNA in the cell line as compared with normal human T cells.

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.27 Cells were cultured in RPMI medium containing 10% (vol/vol) fetal calf serum (FCS) and 2 mmol/L L-glutamine. Jurkat cells28 were maintained in the same medium. As described in the text, T cells were activated by the addition of phorbol dibutyrate (PDB) (10 mmol/L) and ionomycin (0.5 mmol/L). PDB, obtained from Sigma (St Louis, MO), was dissolved at a concentration of 10−3 mol/L in dimethyl sulfoxide (DMSO). Ionomycin, obtained from Calbiochem (San Diego, CA) was prepared as a 5-mmol/L stock solution in DMSO.

Immunoblot analysis. Cells were washed with phosphate-buffered saline (PBS) and then lysed at 4°C in RIPA buffer containing 25 mmol/L Tris-HCl, pH 7.4, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate (SDS), 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 50 mg/mL aprotinin, 50 mmol/L leupeptin and 0.1 mmol/L sodium orthovanadate.29 After removal of cellular debris by centrifugation, lysates were prepared for electrophoresis and polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli.30 When immuno-

From the Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO.

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Address reprint requests to Joseph J. Lucas, PhD, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St, Denver, CO 80206.

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precipitates, prepared as described below, were to be analyzed by immunoblotting, they were resuspended in the Laemmli sample buffer\textsuperscript{32} and electrophoresis was performed as noted above. After electrophoretic transfer of proteins to nitrocellulose membranes (Hybond-ECL; Amersham, Arlington Heights, IL) and blocking of the filters with a solution containing bovine serum albumin,\textsuperscript{31} filters were incubated with the appropriate primary antibody for 2 hours at room temperature. Appropriate secondary antibodies (1:5,000 dilution of either hors eradish peroxidase–linked sheep antimmunoglobulin or donkey antirabbit immunoglobulin; Amersham) were then incubated with the membranes for 1 hour. The specific proteins were then detected using an enhanced chemiluminescence (ECL) method (Amersham).

For detection of the proteins analyzed, the following primary antibodies were used: cdk2, a mouse monoclonal antibody directed against a peptide comprising the C-terminal 15 amino acids of the protein (Zymed, San Francisco, CA); cdk2, a rabbit polyclonal antibody directed against a peptide comprising the C-terminal 12 amino acids of the protein (UBI, Lake Placid, NY); cdk4, a rabbit polyclonal antibody directed against a peptide comprising the C-terminal 16 amino acids of the protein (UBI); cdk5, a rabbit polyclonal antibody raised against a peptide corresponding to the carboxy-terminus region between amino acid residues 306 to 326 (Santa Cruz Biotech); cyclin E, the cyclin E amino acid residues 274 to 290 (Santa Cruz Biotech); cyclin D2 amino acid residues 277 to 292 (Pharmingen); cyclin D3, the cyclin D3 antibody used for immunoblotting was a rabbit polyclonal antibody raised against a peptide corresponding to the carboxy-terminus region between amino acid residues 284 to 291 (Santa Cruz Biotech, Santa Cruz, CA); cdk6, a rabbit polyclonal antibody raised against a peptide corresponding to the carboxy-terminus region between amino acid residues 306 to 326 (Santa Cruz Biotech); cyclin E, the mouse monoclonal antibody HE 12, prepared using recombinant cyclin E protein (Pharmingen, San Diego, CA); cyclin D2, a rabbit polyclonal antibody raised against a peptide corresponding to human cyclin D2 amino acid residues 274 to 290 (Santa Cruz Biotech); cyclin D3, the cyclin D3 antibody used for immunoblotting was a mouse monoclonal antibody purchased from Pharmingen; PCNA, either the mouse monoclonal antibody PC10 (Santa Cruz Biotech) or a PCNA-reactive human serum (a gift of Dr Yoshinari Takasaki, Tokyo, Japan); p53, the mouse monoclonal antibody DO-1, which is directed against the epitope comprising amino acid residues 37 to 45 and recognizes both wild-type and mutant human p53 proteins (Santa Cruz Biotech).

Immunoprecipitation of protein complexes. For isolation of protein complexes, a modification of the method of Howe et al\textsuperscript{32} was used. Cells were washed with cold 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, 20 \( \mu \)g/mL aprotinin, 10 \( \mu \)g/mL leupeptin, 100 \( \mu \)mol/L sodium orthovanadate, 50 mmol/L NaF, and 0.1% Nonidet-P40 (NP-40 lyss buffer). To increase the extraction of nuclear proteins, the lysate was repeatedly extruded through a 25-G needle. Cell lysates were clarified by centrifugation in a microfuge for 15 minutes at 4°C and then precleared with protein G-Sepharose beads (Zymed, San Francisco, CA) for 30 minutes. Immune complexes were then formed, for 2 hours at 4°C, with the same antibodies (with two exceptions) that were used above (for immunoblotting), at concentrations (generally 5 to 10 \( \mu \)g antibody per sample) determined to quantitatively immunoprecipitate the molecules of interest. For immunoprecipitation of cyclin D3, a rabbit polyclonal antibody (Santa Cruz Biotech) raised against a peptide corresponding to human cyclin D3 amino acid residues 277 to 292 was used; for cyclin E, the mouse monoclonal antibody HE 67 (Pharmingen) was used. After formation of the immune complexes, they were precipitated by addition of protein G-Sepharose beads. When the samples were to be analyzed by immunoblotting, they were washed five times with the NP-40 lysis buffer and then disrupted by heating for 5 minutes at 90°C in the Laemmli PAGE sample buffer. If the immunoprecipitates were to be used for determinations of histone kinase activity, they were processed as described in the next section.

Enzymatic assay for cdk activity. The complexes prepared by immunoprecipitation (using antibodies to either PCNA, cdk2 or cdk2) 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 \( \mu \)L/sample) containing 50 mmol/L Tris-HCl, pH 7.4, 10 mmol/L magnesium chloride, 1 mmol/L dithiothreitol, 80 \( \mu \)mol/L adenosine triphosphate (ATP), 10 \( \mu \)g histone H1 (Boehringer-Manheim, Indianapolis, IN), and 40 \( \mu \)Ci \( \gamma $\textsuperscript{32}P$)-ATP, and incubated at 30°C for 15 minutes.\textsuperscript{31} All reagents were in excess and the reactions were stopped by addition of 5× concentrated 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 12% PAGE. The proteins were then transferred electrophoretically to nitrocellulose membranes, a step that reduced background levels of radioactivity present throughout the gels. The radioactive histone H1 bands were visualized by autoradiography and quantitated by phosphorimager analysis.

RESULTS

Induction of PCNA accumulation in normal human T lymphocytes. Normal human T lymphocytes were isolated from peripheral blood and activated in vitro using the combination of phorbol dibutyrate (PDB) and ionomycin. As described previously, this combination of stimuli induces the most effective, rapid, and synchronous entry of T cells into and through the cell cycle. Entry into S phase is first detected at about 30 hours after treatment.\textsuperscript{29,32} The synthesis of PCNA is regulated during the first cell cycle of normal human T lymphocytes induced to enter the cell cycle. It is present in low amount in normal quiescent cells and it has been reported that mitogens induce a coordinate synthesis of this protein and DNA,\textsuperscript{24,34} although the induction of increased PCNA accumulation in normal T cells clearly precedes S-phase entry itself.\textsuperscript{35} As shown in Fig 1, the 36-kD PCNA protein is present in low amounts in the resting cell population (0 time). However, increased accumulation of the protein is apparent as early as 15 hours after activation, some 15 hours before S-phase entry. The PCNA protein continues to increase in amount throughout the cell cycle, achieving maximal levels in the proliferating cell cultures (50 to 72 hours).

Efficiency of extraction of PCNA and associated molecules. In the work described below, the association of PCNA with a variety of other molecules will be demonstrated using the technique of immunoprecipitation. To perform this analysis, it is necessary to lyse cells and extract the molecules of interest under conditions that will maintain protein/protein interactions existing in vivo. However, this entails the potential problem that specific pools of the molecules will not be effectively recovered. To assess the efficiency of extraction of PCNA and some of its associated molecules, the following analysis was performed. The conditions chosen for extract preparation\textsuperscript{32} were a modification of those used for preparation of enzymatically active complexes of the cdk2 and cdk2 cyclin-dependent kinases with their associated cyclins (ie, primarily cyclins A, B, and E) and were similar to those used by Xiong et al\textsuperscript{3,16} in their initial identification of PCNA/cdk associations. Cells were disrupted by a solution containing the nonionic detergent NP-40 and lysates were repeatedly extruded through a 25-G
Fig 1. Time course of accumulation of the p36 PCNA protein in normal human T lymphocytes. Cell extracts prepared from cells harvested at 0, 15, 20, 30, 50, and 72 hours (lanes 1 to 7, respectively) after activation with PDB/ionomycin (I) were analyzed for PCNA content by the technique of immunoblotting, as described in the Methods. For this experiment, an entire culture was harvested and processed at each time point. Therefore, the cell numbers at the 50- and 72-hour time points have increased, partially accounting for the continued increase in PCNA amount after completion of the first cell cycle. (M) Lane in which molecular weight marker proteins were run.

needle. The residual nuclear/chromatin pellet and debris were removed by centrifugation. This pellet was normally discarded, but for estimation of the efficiency of recovery of PCNA, cdk2 and cdc2, as shown in Fig 2, the pellet was further extracted with a hot solution containing SDS. The entire residual nuclear extract and 2% of the original NP-40 cellular extract were subjected to PAGE and immunoblot analysis for the PCNA protein. Duplicate samples were analyzed for the cdk2 and cdc2 proteins. As shown in Fig 2, the PCNA signal detected for the entire nuclear extract was lower than that given by the initial lysate, indicating that at least 98% of the cellular PCNA content was recovered in the initial extract. Similar results were observed for the cdk2 protein. The cdc2 protein seemed to be even more effectively extracted as the signal given after analysis of the nuclear pellet was even lower. The results shown in Fig 2 were obtained using cells harvested at 72 hours after stimulation, but similar results were obtained using cells harvested at other time points.

Detection of PCNA-associated H1 histone kinase activity. The cdc2 and cdk2 kinases use the H1 histone protein as an excellent substrate under the reaction conditions described in the Methods. Since both of these kinases have been reported to associate with PCNA in human fibroblasts, the ability of the proteins coimmunoprecipitated with PCNA from normal and transformed (Jurkat) human T cells to phosphorylate H1 histone in vitro was examined. As shown in Fig 3, PCNA immunoprecipitates prepared from normal human T-cell extracts showed a detectable kinase activity as early as 15 hours after activation. The increase in kinase activity with time paralleled the overall increase in cellular PCNA content (Fig 1). PCNA-associated H1 histone kinase activity was also easily detectable in Jurkat cell extracts prepared from proliferating cells. As shown in the lower portion of Fig 3, when this activity was normalized to differences in cell number and cellular protein content of the normal and transformed cells, as described in the legend to Fig 3, the activity for Jurkat cells was approximately fourfold higher than the normal cells.

Although the PCNA-associated H1 histone-kinase activity was easily and reproducibly detected in T cells, this activity was small compared with the total H1 histone kinase activity of growing T cells, as demonstrated by the results shown in Fig 4. Here the H1 histone kinase activity associated with cdc2, cdk2, and PCNA immunoprecipitates were directly compared. For this experiment, the same numbers and source (donor) of cells were used. The overall cdk2 activity in this 72-hour cell sample was approximately twice as high as the cdc2 content. The PCNA-associated activity was approximately 4% of the cdc2 value and 2% of the cdk2 activity. Although these PCNA-associated values are low, they do not preclude the possibility that they play a key role(s) in T-cell function. For comparison, it should be noted that the cyclin E/cdk2 kinase activity in normal T cells before S-phase entry is approximately 8% of the total cdk2 activity found in mid-S phase (Lucas et al, manuscript submitted); yet, it appears that this cyclin E–associated activity is essential for normal S-phase entry.

Recent reports have shown that the p21 protein, which appears to be an inhibitor of cdk kinase activity, is often
that the maximal levels detected in the gels (for normal and transformed cells) were similar. In the graphical presentation of the data, the activity associated with Jurkat cells was corrected for this different cell number and also for the slightly different protein contents of the cell types. That is, the figure obtained for Jurkat cells (lane 8) was multiplied by a factor of 4, to account for the fact that four times more cells were used in the analysis of T cells; then the figure was divided by 1.18 to account for the slightly higher protein content of Jurkat cells as compared with normal-growing (72-hour) T cells.

Fig 3. The H1 histone kinase activity of PCNA immunocomplexes prepared from normal human T lymphocytes and Jurkat cells. Normal human T cells were harvested at 0, 15, 30, 40, 50, and 72 hours (lanes 7 through 2, respectively) after activation with PDBI; logarathmically growing Jurkat cells were also harvested. Cell extracts were prepared using the NP-40 lysis buffer and PCNA immunoprecipitates were isolated and then assayed for H1 histone kinase activity as described in the Methods. Duplicate 72-hour and Jurkat cell samples were also processed using a nonimmune antibody (C lanes). After the kinase reactions were halted, proteins were processed for gel electrophoresis and radiolabeled histone substrate was detected by autoradiography, as shown at the top. Radioactivity associated with H1 histone was quantitated by phophorimager analysis and the results are presented graphically at the bottom. A lower number of Jurkat cells was used for analysis so that the maximal levels detected in the gels (for normal and transformed cells) were similar. In the graphical presentation of the data, the activity associated with Jurkat cells was corrected for this different cell number and also for the slightly different protein contents of the two cell types. That is, the figure obtained for Jurkat cells (lane 8) was multiplied by a factor of 4, to account for the fact that four times more cells were used in the analysis of T cells; then the figure was divided by 1.18 to account for the slightly higher protein content of Jurkat cells as compared with normal-growing (72-hour) T cells.

Present, in normal fibroblasts, in quaternary complexes containing p21, PCNA, a cyclin, and a cdk. However, although many factors, including association with inhibitory molecules, may contribute to the low relative level of activity in PCNA immunoprecipitates, it appears that the major cause is that only small fractions of the total cellular cdc2 and cdk2 protein contents are in fact associated with PCNA in normal human T cells. This is demonstrated by the results presented in Fig 5. The relative amount of cdc2 protein associated with PCNA was estimated by comparing the cdc2 present in a PCNA immunoprecipitate with that present in a cellular extract. As shown in Fig 5, the signal generated by analysis of cdc2 in the PCNA immunoprecipitate is less than that seen when 5% of an extract prepared from the same number of cells as used for the immunoprecipitation was analyzed. Assuming quantitative recovery of the coimmunoprecipitated cdc2, it can therefore be estimated that less than 5% of the cellular cdc2 is associated with PCNA. Similar analysis of the cyclin E protein, also shown in Fig 5, indicated that a higher percentage of this molecule (>5%) was associated with PCNA. The relative amount of cdk2 associated with PCNA was estimated by an alternative method. In this case, the cdk2 protein was immunoprecipitated using a specific anti-cdk2 antibody. Aliquots representing either 5 or 40 million T cells were analyzed (lanes 7 and 8, respectively). As shown in Fig 5, the amount of cdk2 directly isolated from 5 million cells was more than the amount of PCNA-associated cdk2 coimmunoprecipitated, using the anti-PCNA antibody, from 40 million cells (lane 9). Again, assuming efficient immunoprecipitation of the molecules, it can be concluded that less than 12.5% (5/40) of the cellular cdk2 content is in association with PCNA in the normal growing T cells. Since the low levels of PCNA-associated kinase activity correlate with low levels of the kinases themselves, it appears likely that PCNA does not play an inhibitory role in the multiprotein complexes in which it is found.

Association of PCNA with cyclin-dependent kinases and cyclins in normal human T cells. Since the amount of PCNA and its associated kinase activity were highest in proliferating cells, T cells harvested at 72 hours after activation were chosen for this analysis. The PCNA, cdc2, and cdk2 proteins were each immunoprecipitated using the conditions described above and excess antibodies specific for each protein. In addition, nonimmune mouse and rabbit antisera were used as controls for the cdc2 and cdk2 antibodies, respectively. The immunoprecipitates were then disrupted, subjected to PAGE, and analyzed for PCNA protein by immunoblotting. As shown in Fig 6, the PCNA immunoprecipitate contained a large quantity of the 36-kD immunoreactive protein, and no detectable signal was seen in the two control immunoprecipitates. The cdk2 immunoprecipitate contained an easily detectable amount of PCNA, and the cdc2 immunoprecipitate contained a substantially lower but still clearly detectable amount.

The analysis was then extended to include the other cdks,
PCNA-ASSOCIATED CDKS

with PDB/I. After lysis of the cells with NP-40 lysis solution, four aliquots, each containing material from human T cells. T cells were harvested at 72 hours after stimulation.

The immunoprecipitates were assayed for H1 histone kinase activity as described in the Methods and the legend to Fig 3. As in Fig 3, the autoradiogram is presented at the top and the graphical representation of the data, obtained by phosphoroimage analysis, is presented below.

Fig 4. Comparative analysis of the H1 histone kinase activity associated with PCNA, cdk2, and cdc2 immunoprecipitates isolated from human T cells. T cells were harvested at 72 hours after stimulation with PDB/I. After lysis of the cells with NP-40 lysis solution, four aliquots, each containing material from 8 x 106 cells, were subjected to immunoprecipitation (IP) using antibodies specific for either cdk2, cdc2, or PCNA or a control (C) nonimmune antibody (lanes 1 through 4, respectively). The immunoprecipitates were assayed for H1 histone kinase activity as described in the Methods and the legend to Fig 3. As in Fig 3, the autoradiogram is presented at the top and the graphical representation of the data, obtained by phosphoroimage analysis, is presented below.

Fig 5. Determination of the relative percentages of cdc2, cyclin E, and cdk2 associated with PCNA in normal human T cells. For analysis of cdc2 (lanes 1 through 3) and cyclin E (lanes 4 through 6), cell pellets of T cells at 72 hours after activation were lysed with NP-40 lysis buffer, as described in the Methods. Immunoprecipitation was performed using antibody specific for PCNA (lanes 1 and 4). The procedure was also performed using control (Cont IP) antibodies from a nonimmune animal (lanes 2 and 5). The entire immunoprecipitates and aliquots representing about 5% of the total cell extracts (WC ext) were analyzed by immunoblotting (IB) for the cdc2 (lanes 1 through 3) and cyclin E (lanes 4 through 6) proteins. For analysis of cdc2 (lanes 7 through 9), a 72-hour T-cell pellet was lysed using NP-40 lysis buffer. The cdk2 protein was not included in this analysis as this protein has been difficult to routinely detect in normal human T cells (Lucas et al, unpublished data). As shown in Fig 7, immunoprecipitates of all three of these cdk4, 5, and 6. The cdk3 protein was not included in this analysis as this protein has been difficult to routinely detect in normal human T cells (Lucas et al, unpublished data). As shown in Fig 7, immunoprecipitates of all three of these cdk also contain the PCNA protein. In summary, the amount of PCNA appeared to be present in highest amount in the cdk6 immunoprecipitate, followed by cdk5, cdk4, cdk2, and cdc2. By comparison with results presented elsewhere and in Fig 9, below, this amount appeared not to correlate with the relative amount of the cdk proteins. It is also shown that PCNA can be coprecipitated with certain cyclins, such as cyclins D2 and D3, which are the two major cyclin partners of the cdk6 kinase in human T cells (Lucas et al, manuscript submitted).

Association of PCNA with cyclin-dependent kinases and cyclins in Jurkat cells. The association of PCNA with the same cdk4 and cyclins examined in normal T cells was next examined in the transformed T-cell line Jurkat. In this analysis, rapidly growing log-phase cells were used for analysis. As shown in Fig 8, PCNA was detected in association with cdk2, 4, 5, and 6, with increasing amounts found in the same order as for normal T cells. It was also present in immunoprecipitates of cyclin D2 and D3, with somewhat less found associated with cyclin D3 in the cell line as compared with in normal cells. As for normal T cells, PCNA was most difficult to detect in association with the cdc2 protein. As shown in Fig 8, even at a very high exposure of the (ECL) immunoblot, PCNA is not easily detectable in the cdc2 immunoprecipitate (lane 10).

Since the amounts of PCNA associated with each molecule could depend on the relative amount of each protein
present in the transformed and normal cell types, the relative amounts of each of the proteins under study were compared by immunoblotting, as shown in Fig 9. Here, the same amount of protein from an extract prepared from either growing Jurkat or (72 hours) normal human T cells were analyzed by immunoblotting. It was found that all of the cdks (cdc2, and cdk2, 4, 5, and 6) examined were in higher relative amounts in the cell line; cyclin D2 was also in higher relative amount, whereas cyclins E and D3 appeared to be in lower relative amount in the cell line. However, when ordered according to their ease of detection, there was a remarkable similarity in cellular content of the cell-cycle regulatory molecules between the two types. The apparent relative excess of most of the cell-cycle-related proteins in the cell line may be in part due to the fact that the entire population of Jurkat cells is in a rapidly growing phase, whereas the 72-hour population of normal cells contains small fractions of cells that have not properly progressed through their first G1 phase or have been re-arrested in the cell cycle.

Since, as discussed below, the p53 protein appears to play an indirect role in negatively regulating cdk activity, this protein was also examined (Fig 9). Here, immunoblotting was performed using a monoclonal antibody that recognizes both wild-type and mutant p53. In the extract prepared from this sample of normal T cells, a doublet of immunoreactive proteins was detected. As we and others have noted previously, a portion of the human population is polymorphic for the p53 protein and displays such a protein doublet. No p53 protein was detected in the Jurkat cell line, which has previously been reported to have mutations in the p53 gene. The entire gel is shown to demonstrate that no discernible immunoreactive species was present.

DISCUSSION

The association of PCNA with the network of cdks and cyclins was first observed using human fibroblast cell types, including normal cell strains and transformed derivatives, which were shown to differ dramatically in their patterns of intermolecular associations. In the present report, this finding was extended to include normal human T lymphocytes induced to proliferate in vitro. However, our comparative analysis of normal and transformed T cells has, as yet, shown little difference between the two cell types, whether in the overall relative amounts of the proteins analyzed or in their patterns of association with PCNA.

One difference, an apparent lack of association of PCNA with cdc2 in the cell line, may be due to the fact that this is the most difficult of the complexes to detect in the lymphocyte system. Only small fractions of cdc2 and PCNA seem to be associated with each other, even in normal T cells. A second difference, an apparent increased relative specific enzyme activity in the cell line as compared with normal

![Fig 6. Association of PCNA with cdc2 and cdk2 in normal human T cells. T cells were harvested at 72 hours after PDB/I treatment and duplicate samples were lysed and subjected to immunoprecipitation with antibodies specific for cdc2 (lane 1), PCNA (lane 3), or cdk2 (lane 6) or with control nonimmune mouse (lane 2, C1) or rabbit (lane 4, C2) antibodies. The immunoprecipitates were analyzed for the PCNA protein by the technique of immunoblotting, as described in the Methods.](image1)

![Fig 7. Association of PCNA with cdks 2, 4, 5, and 6 and with cyclins D2 and D3 in normal human T cells. T cells were harvested at 72 hours after PDB/I treatment and duplicate samples were lysed and subjected to immunoprecipitation (IP) with antibodies specific for cdk2 (lane 3), cdk4 (lane 4), cdk5 (lane 5), cdk6 (lane 6), cyclin D2 (lane 7, CylD2), or cyclin D3 (lane 8, CylD3), or with control nonimmune rabbit antibodies (lane 2, Cont). The immunoprecipitates were analyzed for the PCNA protein by the technique of immunoblotting, as described in the Methods. For localization of the PCNA protein, a sample of whole cell extract (lane 1, ext.) was also analyzed by immunoblotting. The high intensity of the signal detected in this lane appears to be primarily responsible for the slight signal seen in control lane 2, as compared with the lack of signal generally found in control lanes in other experiments, as seen in Fig 6 and 8, for example. The positions of heavy chain (HC) and light chain (LC), contributed by the immunoprecipitating antibodies, are also indicated.](image2)
PCNA-ASSOCIATED CDKS IN HUMAN T LYMPHOCYTES

Fig 8. Examination of the association of PCNA with cdc2, cdk2, 4, 5, and 6 and with cyclins D2 and D3 in the Jurkat T-lymphoblastoid cell line. Growing Jurkat cells were harvested and duplicate samples were subjected to immunoprecipitation (IP) using antibodies specific for cdk2 (lane 2), cyclin D2 (lane 5, CyD2), cyclin D3 (lane 6, CyD3), cdk2 (lane 8), or cdk6 (lane 10). or with control nonimmune rabbit antibodies (lane 7, Cont). The immunoprecipitates were analyzed for the PCNA protein by the technique of immunoblotting. The positions of heavy chain (HC) and light chain (LC), contributed by the immunoprecipitating antibodies, are also indicated.

cells, may be due to the fact that the cell line contains a somewhat higher amount of some of the molecules (such as cdk2) likely to be responsible for the observed H1 histone kinase activity. Alternatively, considering our inability to clearly detect cdc2 in the PCNA immunoprecipitates prepared from Jurkat cells, this increased activity may reflect aberrant regulation of cdk2 activity in Jurkat cells, a possibility currently under investigation. This is of special interest as this cell line appears to lack the p53 protein, which has been recently shown to trans-activate a gene encoding a protein (p21 or the waf-1 gene product) that suppresses the activity of cyclin-dependent kinases. An inhibitory protein (p21) likely to be the waf-1 protein in PCNA/cdk/cyclin complexes has been described. The possible absence of this protein in Jurkat cells could therefore lead to aberrantly high levels of kinase activity in the complexes.

The relative fractions of all of the molecules (PCNA itself, cdk2, and cyclins) present in PCNA/cdk/cyclin complexes appear to be small. The H1 histone kinase activity associated with PCNA was also small, corresponding to the low levels of these proteins found in association with PCNA. A further contributing factor to the low levels of activity could also be the presence of inhibitory molecules, such as p21, although the presence of such factors was not described in the studies reported here. Definitive identification and analysis of these molecules awaits the preparation of appropriate immunologic reagents. As noted above, the low relative PCNA-associated kinase activity found does not preclude the possibility that it plays key role(s) in cellular function. To place into perspective the finding that generally less than 5% of the molecules and activities studied were present in PCNA-containing complexes, it is noted that the cyclin E/cdk2 activity of normal T cells before S-phase entry is less than 10% of maximal levels observed in mid- to late S phase (Lucas et al, manuscript submitted). Yet, it is likely that this cyclin E-associated activity is essential for S-phase entry. It will be of interest to determine now whether these PCNA-associated fractions of cdk and cyclins are sequestered within specific areas of the cell, where low overall amounts of the molecules could yet be present in high concentration. Furthermore, it should be emphasized that among all of the molecules examined, PCNA appeared to be in lowest amount in complexes with cdc2 and cdk2. Finally, it is possible that the conditions used for cell lysis and extract preparation, though maintaining cdk/cyclin associations and enzyme activity, may yet partially disrupt the interaction of PCNA with these molecules, thus leading to low estimates of the extent of interaction of PCNA with other proteins.

The data indicate that most PCNA was associated with the cdk4, 5, and 6 cyclin-dependent kinases. It will be necessary to determine the specific kinase activities of these enzymes when associated with PCNA. Conditions for directly assaying these molecules have only been developed recently, since completion of the studies described here. In a recent analysis of the possible functions of the cdk5, in which dominant negative mutants of each of the six cdkks were expressed in cells, clear cell-cycle regulatory roles for only the cdc2, cdk2, and cdk3 proteins were demonstrated. Expression of the cdk4, 5, and 6 mutants appeared to have no discernable effect on the cell lines used. Taken together with other extensive, but still indirect, evidence, it has been surmised that the closely related cdk4 and cdk6 proteins may play roles in cell-cycle entry and early progression and that cdk5 may be involved in or associated with the expression of certain differentiated phenotypes. Possible roles for PCNA in these processes remain to be determined. Also, as described here, PCNA was also in relatively high amount in association with cyclins D2 and D3, which are the major, if not only, cdk6 partners in normal T cells (Lucas et al, manuscript submitted). The normal cyclin partners for cdk5 in human T cells are unknown, although association of cdk5 in human fibroblasts with cyclins of the D family has been described.

Certain complexes of cdk and cyclins have been found to associate with other proteins as well, perhaps most notably with transcription factors such as E2F and the Rb protein (retinoblastoma susceptibility gene product) and their homologs and related molecules.
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![Diagram of cyclins and PCNA](image)

Fig 9. Comparative analysis of the relative contents of cdc2, cdk2, and Cdk4 with p53 in normal and transformed (Jurkat) lymphocytes. Cell extracts were isolated from either normal T cells at 72 hours after activation (T) or growing Jurkat cells (J) and adjusted to equal protein content by appropriate dilution. Duplicate samples were then assayed for the entire set of 10 proteins by the technique of immunoblotting, as described in the Methods. Cyclin, cyclin. For analysis of the p53 protein, the entire gel is shown to demonstrate the apparently complete lack of immunoreactive protein in the Jurkat cell line.

It has been proposed that active cdcks may play an indirect role in the regulation of gene transcription. By analogy, a role for these kinases in DNA replication could also be proposed. However, more incisive molecular analysis, such as the determination of the precise sites in the cdcks or cyclins required for PCNA binding and the preparation and expression of proteins mutated in these sites, will be required for an understanding of these possible roles. A recent report demonstrating binding of PCNA to cyclins D1 or D3 in the absence of other cellular factors suggests that PCNA association with cdcks may be through its binding to cyclins. Last, it is suggested that direct comparisons between PCNA-containing complexes isolated from various normal and transformed cell types, such as human fibroblasts and lymphocytes, will help to clarify the apparent differences described in the present report and previous studies.

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A Szepesi, EW Gelfand and JJ Lucas