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Identification of Multiple Cell Cycle Regulatory Functions of p57\textsuperscript{Kip2} in Human T Lymphocytes\textsuperscript{1}

Guiming Li, Joanne Domenico, Joseph J. Lucas, and Erwin W. Gelfand\textsuperscript{2}

The specific functions of p57\textsuperscript{Kip2} in lymphocytes have not yet been fully elucidated. In this study, it is shown that p57\textsuperscript{Kip2}, which is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, is present in the nuclei of normal resting (G\textsubscript{0}) T cells from peripheral blood and in the nuclei of the T cell-deriving Jurkat cell line. Activation through the TCR results in rapid transport of cytoplasmic cyclin-dependent kinase 6 (cdk6) to nuclei, where it associates with cyclin D and p57\textsuperscript{Kip2} in active enzyme complexes. Using purified recombinant proteins, it was shown in vitro that addition of p57\textsuperscript{Kip2} protein to a mixture of cyclin D2 and cdk6 enhanced the association of the latter two proteins and resulted in phosphorylation of p57\textsuperscript{Kip2}. To probe further the function of p57\textsuperscript{Kip2}, Jurkat cells stably transfected with a plasmid encoding p57\textsuperscript{Kip2} under control of an inducible (tetracycline) promoter were made. Induction of p57\textsuperscript{Kip2} resulted in increased association of cdk6 with cyclin D3, without receptor-mediated T cell stimulation. The overall amounts of cdk6 and cyclin D3, and also of cdk4 and cyclin E, remained unchanged. Most notably, increased p57\textsuperscript{Kip2} levels resulted in marked inhibition of both cyclin E- and cyclin A-associated cdk2 kinase activities and a decrease in cyclin A amounts. Therefore, although facilitating activation of cdk6, the ultimate outcome of p57\textsuperscript{Kip2} induction was a decrease in DNA synthesis and cell proliferation. The results indicate that p57\textsuperscript{Kip2} is involved in the regulation of several aspects of the T cell cycle. The Journal of Immunology, 2004, 173: 2383–2391.

Molecules that regulate entry into and passage through G\textsubscript{1} phase are important targets in the induction and progression of malignancy (1–3). During early G\textsubscript{1} phase, cells respond to their environment and can be induced to commit to the process of cell division (4, 5). For T cells, such inductive stimuli include interaction of specific Ags with the TCR, followed by cytokine stimulation (reviewed in Ref. 6). The Rb family proteins p130 (RB2) and retinoblastoma susceptibility gene protein (pRb)\textsuperscript{3} (RB1) are primary regulators of the G\textsubscript{1}/G\textsubscript{0} transition and G\textsubscript{1} progression. Their activities as growth suppressors are modulated by multiple phosphorylations, performed mostly by the G\textsubscript{1} phase cdks, cyclin-D-dependent kinases (cdk4 and cdk6) and cyclin E/cdk2 (7). Activity of the cyclin D/cdk4 is primarily regulated by cyclin-dependent kinase inhibitors (CDKIs) of the Ink4 family (p16\textsuperscript{ink4a}, p15\textsuperscript{ink4b}, p18\textsuperscript{ink4c}, p19\textsuperscript{ink4d}), whereas cyclin E/cdk2 is regulated by Cip/Kip family CDKIs, which include p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2} (7–9).

Accumulating evidence suggests that members of both families of CDKIs play important roles in the regulation of T cell proliferation, differentiation, and function. Mice lacking p18\textsuperscript{ink4c} have enlarged lymphoid organs, and T cells derived from them are hyperproliferative after TCR stimulation (10, 11); in contrast, T cells derived from p19\textsuperscript{ink4d}-deficient animals appear to proliferate normally (10). The other Ink4 CDKIs, p16\textsuperscript{ink4a} and p15\textsuperscript{ink4b}, may be involved in T cell senescence (12). The p27\textsuperscript{Kip1} CDKI is present in high amount in resting T cells and decreases in amount after stimulation with mitogens and cytokines (13, 14). Evidence suggests that this down-regulation plays a key role in the development, proliferation, and immune responses of T cells (15–18). The p27\textsuperscript{Kip1} protein, along with p21\textsuperscript{Cip1}, may also play a role in the induction of T cell anergy (19, 20). The last CDKI, p57\textsuperscript{Kip2}, has been little studied in lymphocytes, as it was thought not to be significantly expressed in lymphoid organs (21). Recent reports (22–24) and data presented in this work show that it is in fact present in normal human T cells and in some T cell-derived cell lines.

In addition to being primary negative regulators of cdk2, Cip/Kip CDKIs also interact with cdk4 and cdk6 (25). Although they do not inhibit the activity of the latter kinases (at least when present in cyclin/cdk/CDKI trimeric complexes), the cyclin D-cdk4/CDKI and cyclin D-cdk6/CDKI complexes nonetheless may be functional in regulating cell cycle progression (26, 27). It appears that cyclin D-cdk4/cdk6 complexes sequester Cip/Kip CDKIs, preventing them from interacting with and inhibiting the activity of cdk2. When present at sufficiently high levels, Ink4 CDKIs bind to cdk4 and cdk6, freeing Cip/Kip proteins to bind to and inhibit cdk2 when its activity is not needed in the cell (7, 26, 28). Cip/Kip CDKIs may also have other functions, having been proposed to be necessary for promoting accumulation of D-type cyclins in the nucleus and to be assembly factors essential for the association of cyclin Ds with cdk4 and cdk6 (26, 28, 29). Possible cytoplasmic functions for Cip/Kip CDKIs have also been proposed (30, 31). For example, p27\textsuperscript{Kip1} appears to be an inhibitor of rho-kinase (32), whereas p57\textsuperscript{Kip2} can regulate the cellular localization of LIM kinase, a kinase involved in actin filament dynamics (33). The involvement of p27\textsuperscript{Kip1} in cell migration and actin dynamics, at least partially through regulation of the RhoA pathway, has also been described (34–36).

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\textsuperscript{3}Abbreviations used in this paper: pRb, retinoblastoma susceptibility gene protein; cdk, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Tet, tetracycline.
In addition to its larger size and more complex structure, the p57\textsuperscript{Kip2} protein has other features distinguishing it from the p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} members of the Cip/Kip family. It shows complex patterns of expression during development, being present mostly in terminally differentiated cells in skeletal muscle, brain, heart, lungs, and eye (21, 37). Its expression is regulated by the p73, and not by the p53 member of the p53 tumor suppressor family (38). Its gene is present at chromosome 11p15.5 (21), a region associated with sporadic cancers of breast, liver, and bladder (39–41), and with Beckwith-Wiedemann syndrome (42), although its role in this latter disorder, if any, is unclear (21, 43, 44). The p57\textsuperscript{Kip2} gene is an imprinted gene, but the mechanism by which the maternal allele in humans is preferentially expressed is unclear, although it probably does not involve methylation, as it does in the mouse (43, 45, 46).

Interest in the function of p57\textsuperscript{Kip2} in lymphocytes has been recently stimulated by several findings of potential clinical importance, including relatively high frequencies of aberrant methylation in the promoter region of the p57\textsuperscript{Kip2} gene in primary diffuse large B cell lymphoma (54.9%), in follicular lymphoma (44.0%), and in acute lymphocytic leukemia, in both newly diagnosed (50%) and relapsed (52%) patients (23, 24). Expression of p57\textsuperscript{Kip2} in various cell lines of B and T cell-derived malignancies appears variable, some having high levels of and others no detectable p57\textsuperscript{Kip2} protein. Of interest, some lines not producing p57\textsuperscript{Kip2} were found to have methylated p57\textsuperscript{Kip2} genes; treatment of some of these lines with 5-aza-2'-deoxycytidine resulted in demethylation of the gene and expression of the p57\textsuperscript{Kip2} protein (22–24). In some cell lines at least, silencing of the gene appeared to involve not only regional methylation, but also histone deacetylation (22). The promoter region of the p57\textsuperscript{Kip2} gene appeared to have little or no methylation of CpG islands in normal human cells (22, 23).

In the present study, we show that the p57\textsuperscript{Kip2} protein is present in high amounts in normal peripheral blood T lymphocytes. It is a nuclear protein that binds to, but does not inhibit the activity of cyclin D/cdk6. It is proposed that p57\textsuperscript{Kip2} promotes the association of cyclin D and cdk6 and, as such, may play a role in T cell cycle entry. A role for p57\textsuperscript{Kip2} in events occurring later in the cell cycle is also demonstrated. Overexpression of p57\textsuperscript{Kip2} leads to decreased amounts of cyclin A and reduced cyclin A- and cyclin E-associated cdk2 activities. These results are considered in light of current models of cell cycle regulation and observations that loss of p57\textsuperscript{Kip2} expression may be a common and important occurrence in some lymphoid cell malignancies.

Materials and Methods

Cell culture

Primary human T lymphocytes were isolated from human peripheral blood, as described previously (47). Jurkat (E6-1), COS-7, 293, and Hela cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 medium containing 10% (v/v) FCS. Jurkat Tet-On cells were obtained from BD Clontech (Palo Alto, CA). It and derivative cell lines were cultured in RPMI 1640 medium containing 10% (v/v) Tet system-approved FCS, 2 mM l-glutamine, and 10 mM HEPES. Jurkat Tet-On cells with tetracycline (tetracycline, San Francisco, CA), immune complexes were isolated by incubation with specific primary Abs, followed by addition of protein G-coupled Sepharose beads. Immunoprecipitates were washed five times with immunoprecipitation buffer and then resuspended in electrophoresis sample buffer (51), heated at 95°C for 5 min, and then used for analysis. In some experiments, immunoprecipitates were prepared using Abs that had been chemically linked to a matrix, using a Profound Immunoprecipitation Kit (Pierce, Rockford, IL) with the method supplied by the manufacturer.

Protein expression in insect cells

The cDNA sequences for p57\textsuperscript{Kip2}, cyclin D2, and cdk6 were subcloned into the bPAC transfer plasmid (Novagen/EMD Biosciences, Madison, WI). Recombinant baculoviruses were isolated by cotransfection of S9 insect cells (Invitrogen Life Technologies, Carlsbad, CA) with the respective transfer plasmids and baculovirus, using protocols recommended by the supplier. Proteins were isolated from High Five insect cells (Invitrogen Life Technologies) infected with recombinant baculoviruses. The recombinant proteins had poly-His tags, which were used for affinity chromatography. His-tagged proteins were eluted from columns using 100 mM imidazole. Identity and purity of proteins were ascertained by gel electrophoresis and immunoblot analysis.

Assembly and phosphorylation of recombinant proteins

Interactions of purified, recombinant cdk6, cyclin D2, and p57\textsuperscript{Kip2} were evaluated, as described in the text. Assembly of protein complexes was performed at 30°C, for 30 min, unless specified otherwise, in a buffer containing 80 mM sodium glycerophosphate (pH 7.3), 15 mM MgCl\textsubscript{2}, 20 mM EGTA, 2 mM DTT, 10 μg/ml leupeptin, 0.1 mM sodium orthovanadate, and 3 mg/ml BSA (26). Immunoprecipitation of specific components from the assembly reactions and their evaluation using immunoblot analysis or in vitro kinase assays were performed using techniques described in the appropriate sections of Materials and Methods.

Kinase assays

The cdk6 and cyclin A- and cyclin E-associated kinase activities were performed, as described in detail in previous publications (49, 50, 52, 53), using immunoprecipitates prepared using specific Abs to cdk6, cyclin A, or cyclin E, and a recombinant truncated pRb protein (p60\textsuperscript{Rb}) as substrate.

Results

The p57\textsuperscript{Kip2} CDKI is present in T cells

Normal human T cells isolated from peripheral blood are in a G\textsubscript{0}/G\textsubscript{1} state. Stimulation through the TCR activates several signal transduction cascades that result in cytokine production and cell growth and division. As shown in Fig. 1A, the CDKI p57\textsuperscript{Kip2} was present in resting T cells and exhibited little change in overall amount throughout cell cycle progression after stimulation with [35S]methionine (sp. act. 1175 Ci/mmol; DuPont-New England Nuclear, Wilmington, DE). Labeling of DNA was performed, as described previously (48). In the experiments described in this work, cells (5 × 10\textsuperscript{6}) were incubated in medium containing [3H]thymidine for 8–12 h periods. Flow cytometric analysis of DNA content was performed, as described previously (49). Annexin V staining was performed using an ApoTarget annexin V FITC apoptosis kit (BioSource International, Camarillo, CA), used as directed by the manufacturer.

Immunoblot analysis

Whole cell lysates and nuclear extracts were prepared, and immunoblot analysis was performed, as described previously (50). Primary Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). They were used at a dilution of 1/1000, except for Ab to p57\textsuperscript{Kip2}, which was used at a 1/500 dilution. For detection of specific proteins, an ECL method was used, as described previously (50).
PHA. Some change in amount over that seen in resting cells was apparent between 20 and 30 h after stimulation; as noted further below, the degree of this change varied among samples of purified T cells isolated from various individuals. That the cells used in Fig. 1A were efficiently activated and entered the cell cycle is shown by the dramatic loss of p27 Kip1, a change that is necessary for activation of cdk2 and entry into S phase of the cell cycle (13, 14). The p57 Kip2 protein was also examined in the Jurkat T cell-derived tumor line, a rapidly proliferating transformed cell type that, after stimulation, will synthesize the cytokine IL-2. As shown in Fig. 1B, Jurkat cells also contained p57 Kip2, and the amount of this protein remained unchanged in the nucleus after stimulation of the cells with PHA and anti-CD28, a treatment that induces maximal cytokine production in these cells. In this experiment, p57 Kip2 was examined in isolated nuclei, as little or no p57 Kip2 could be detected in the cytoplasm of Jurkat cells (or of normal T cells) at any point in the cell cycle (data not shown). During the 2-h time period studied in this experiment, cdk6 was translocated from the cytoplasm to the nucleus of the cells, as shown by analysis of cdk6 amounts in the cytoplasmic (Fig. 1C) and nuclear (Fig. 1D) fractions of the cells. As shown previously (50, 52), this nuclear translocation of cdk6 is associated with its activation as a kinase. The efficacy of the fractionation technique used is illustrated by the contents of /H9252-actin and nucleolin in the cytoplasmic and nuclear fractions, respectively.

In its initial description (21), p57 Kip2 was shown to be present in high amount in muscle tissue. As shown in Fig. 1E, the relative amount of p57 Kip2 in freshly isolated mouse muscle was compared with that seen in three primate cell lines and in primary human T cells. COS-7 cells contained an amount comparable to muscle tissue, whereas Hela cells contained a higher amount and 293 cells contained less. The preparation of T cells used in this experiment contained an amount of p57 Kip2 similar to 293 cells. At 24 h after activation, the amount increased ~4-fold over that seen in resting T cells and then decreased in amount. As noted above, variations in the degree of increase in p57 Kip2 levels after cell activation were seen among individuals. In the analysis shown in Fig. 1E, all of the lanes were loaded with the same overall amount of cell protein (10 /H9262 g), except for the first two lanes, in which two different amounts (1 and 3 /H9262 g) of purified recombinant p57 Kip2 were loaded as markers, to establish the efficacy of the immunoblot method in detecting p57 Kip2.

Association of p57 Kip2 with cdk6 and cyclin D

To determine whether p57 Kip2 plays a role in the regulation of cdk6, its possible association with this kinase and D-type cyclins was examined. Nuclear extracts were prepared from normal T cells (after 12-h treatment with PHA) or Jurkat cells (after 30-min stimulation with PHA and anti-CD28), and p57 Kip2 was isolated by immunoprecipitation. At these time points, cdk6 has reached its maximal levels of activity in the two systems (50, 52). Immunoblot analysis of the immunoprecipitated samples for other molecules showed that p57 Kip2 was associated with cdk6 and cyclin D2 in T cell nuclei (Fig. 2A) and cdk6 and cyclin D3 in Jurkat cell nuclei (Fig. 2B). As noted previously, cyclin D2, although detected in Jurkat cells, does not associate with cdk6 (50, 51); in normal T

![FIGURE 1. Detection of the p57 Kip2 protein in normal and transformed (Jurkat) T cells. A, Normal human T cells were stimulated with PHA, and at the times indicated the cellular contents of p27 Kip1 and p57 Kip2 were evaluated by immunoblotting. The level of β-actin is shown as a control for protein loading. B, Jurkat cells were stimulated with PHA and a mAb to CD28, and at the times indicated the nuclear contents of p57 Kip2, β-actin, and nucleolin were evaluated. At the same time points, both the cytoplasmic (C) and nuclear (D) contents of cdk6, β-actin, and nucleolin in Jurkat cells were examined by immunoblotting. E, The content of p57 Kip2 was assessed by immunoblotting in normal human resting T cells and in T cells from the same individual after stimulation for 24 or 48 h with PHA. The amounts of p57 Kip2 can be compared with those seen in normal primary mouse muscle tissue and three primate cell lines (COS-7, 293, and Hela cells). For each lane, 10 µg of total cellular protein was analyzed. For comparison, 1 and 3 µg of purified recombinant p57 Kip2 were run in the first two lanes, respectively.](http://www.jimmunol.org/DownloadedFrom)
cells, cyclin D2 is the first cyclin to show an increase in amount and association with cdk6 after TCR stimulation (52, 53).

The existence of the trimolecular cyclin D2/cdk6/p57Kip2 complex was also confirmed directly in experiments in which complexes were isolated using Abs chemically linked to a matrix. This permitted identification of p57Kip2, without interference by IgG H or L chains or contaminants. Equal aliquots of a cell lysate from normal human T cells at 12 h after activation were incubated with beads coupled to Abs specific for either p57Kip2, cdk6, or cyclin D2. The complexes were isolated and probed, by immunoblot analysis, for their contents of p57Kip2, cdk6, and cyclin D2. As shown in Fig. 2C, the amount of p57Kip2 was similar in all three samples, suggesting that most of the p57Kip2 present in the cells was in the trimolecular complex at this time after cell activation. The amounts of cdk6 and cyclin D2 varied somewhat among the samples, suggesting perhaps that some cdk6 and cyclin D2 existed either as individual molecules or in cdk6/cyclin D2 complexes not containing p57Kip2. Because whole cell lysates were used in this analysis, it is likely that some cdk6 and/or cyclin D2 not associated with p57Kip2 were in the cytoplasm. Analysis of immunoprecipitates isolated from cells metabolically labeled with [35S]methionine also supported the conclusion that a large fraction of each molecule existed in a trimolecular p57Kip2/cdk6/cyclin D2 complex (data not shown).

Enzymatic activity of p57Kip2/cdk6/cyclin D complexes

The association and activity of p57Kip2, cyclin D2, and cdk6 were further examined in vitro, using proteins isolated from an insect cell expression system (see Materials and Methods). In the experiment shown in Fig. 3A, p57Kip2 was added to insect cell lysates containing cyclin D2 (lane 1), cdk6 (lane 2), or cyclin D2 and cdk6 together (lane 3). After a 30-min period for protein assembly, an in vitro kinase assay (without any other added proteins) was performed. A band of phosphorylated p57Kip2 was seen in lane 3. In lane 4, p57Kip2 prepared from insect cells was used as a substrate with cyclin D2/cdk6, which was immunoprecipitated from activated normal human T cells. A phosphorylated protein with the molecular size of p57Kip2 was again seen. These results indicate that the cdk6/cyclin D2 complex can phosphorylate p57Kip2. To investigate whether or not the trimolecular p57Kip2/cdk6/cyclin D complex is enzymatically active with respect to other substrates, the following experiments were also performed. Nuclear extracts were prepared from normal T cells, immunoprecipitated with Abs to either p57Kip2 or cdk6, and used in an in vitro kinase assay with added (truncated) recombinant pRb as substrate. As shown in Fig. 3B, the p57Kip2 (lane 2) and cdk6 (lane 4) immunoprecipitates from normal T cells phosphorylated pRb to a similar extent. A similar experiment was performed using nuclear extracts from Jurkat cells stimulated for 30 min with PHA and anti-CD28. As shown in Fig. 3C, immunoprecipitates prepared using Abs to p57Kip2, cdk6, and cyclin D2 were immunoprecipitated using Abs (to p57Kip2, cdk6, or cyclin D2) chemically coupled to beads. Eluted protein complexes were resolved by gel electrophoresis and probed for the presence of p57Kip2, cdk6, and cyclin D2 using a mixture of specific Abs to the two proteins. For control lanes, mock samples were processed with the same coupled Ab preparations; the results show that the bands seen in lysate sample lanes are not due to components of the Ab preparations (e.g., IgG H or L chains or contaminants).
and the results from duplicate experiments are presented in graphical form. Cyclin D3 was prepared from cell lysates by immunoprecipitation. The amounts of cyclin D3 associated with cyclin D2 were evaluated by immunoblot analysis. A representative immunoblot is shown. Amounts of cyclin D3, cyclin A, and β-actin. Changes in p57Kip2 and cyclin A levels in Tet-On p57Kip2 Jurkat cells were quantitated and are presented in D, E, respectively.

FIGURE 4. Effects of induced overexpression of p57Kip2 on the cellular contents of cell cycle regulatory molecules. Cultures of Jurkat cells (A), Tet-On Jurkat cells (B), and Tet-On p57Kip2 Jurkat cells (C) were treated for 20 h with various doses of doxycycline, and then assessed by immunoblot analysis for levels of p57Kip2, cyclin A, and β-actin. Changes in p57Kip2 and cyclin A levels in Tet-On p57Kip2 Jurkat cells were quantitated and are presented in D, E, respectively.

As noted above, a primary function of CDKIs of the Cip/Kip family is inhibition of cdk2 kinase activity. The effect of increased levels of p57Kip2 on kinase activity in Jurkat cells was therefore assessed, as shown in Fig. 6. Because immunoprecipitates of cdk2 from T cells contain a mixture of both cdk2/cyclin A and cdk2/cyclin E, activities were determined using immunoprecipitates from T cells (data not shown).

Inhibition of cdk2 activities and cell proliferation by p57Kip2

As noted above, a primary function of CDKIs of the Cip/Kip family is inhibition of cdk2 kinase activity. The effect of increased levels of p57Kip2 on kinase activity in Jurkat cells was therefore assessed, as shown in Fig. 6. Because immunoprecipitates of cdk2 from T cells contain a mixture of both cdk2/cyclin A and cdk2/cyclin E, activities were determined using immunoprecipitates

FIGURE 5. Promotion of cdk6/cyclin D association by p57Kip2

It has been proposed that CDKIs of the Cip/Kip family can promote the assembly of cdk/cyclin D complexes (26, 28). The role of p57Kip2 in the association of cdk6 and cyclin D in T cells was therefore examined using two approaches. As shown in Fig. 5A, induction of p57Kip2 in Jurkat cells resulted in an increased level of cdk6 associated with cyclin D3. In this experiment, cells were either untreated or treated with increasing amounts of doxycycline, and cdk6 was examined in immunoprecipitates that were prepared using an Ab to cyclin D3. Higher levels of cdk6 were seen in cells treated with the three highest amounts of the inducing drug, although the amount of cyclin D3 associated with cdk6 did not show a clear linear increase with increasing drug concentration (and amount of p57Kip2).

As suggested above, because Jurkat cells contain a high level of cdk6, cyclin D3 may be inhibited at limiting amounts as compared with cdk6 and p57Kip2 under these conditions. That p57Kip2 promoted the assembly of cdk6 and cyclin D2 was also ascertained using proteins produced in the insect cell system described above. As shown in Fig. 5B, addition of increasing amounts of p57Kip2 to a mixture of cdk6 and cyclin D2 (in an assembly buffer) resulted in increased association of cdk6 with cyclin D2. In this experiment, cyclin D2 was immunoprecipitated after a 30-min incubation of the recombinant proteins (to permit complex formation), and cdk6 was then detected by immunoblot analysis.

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FIGURE 5. The p57Kip2 protein promotes the association of cyclin D and cdk6. A, Cultures of the Tet-On p57Kip2 Jurkat cell line were treated for 20 h with various doses of doxycycline, and cyclin D3 was prepared from cell lysates by immunoprecipitation. The amounts of cdk6 associated with cyclin D3 were assessed by immunoblot analysis; a representative example is shown. Amounts of cdk6 were also quantitated, as described in Materials and Methods, and results from duplicate experiments are presented in graphical form. B, Purified recombinant cdk6, cyclin D2, and various amounts of p57Kip2, indicated under the figure, were mixed together. After incubation in an assembly buffer, cyclin D2 was immunoprecipitated, and the amounts of cdk6 associated with cyclin D2 were evaluated by immunoblot analysis. A representative immunoblot is shown. Amounts of cdk6 were also quantitated, as described in Materials and Methods, and results from duplicate experiments are presented in graphical form.
prepared using either cyclin E (Fig. 6A) or cyclin A (Fig. 6B) and recombinant pRb as substrate. At the time point assessed (20 h after induction with doxycycline), significant decreases in both cyclin A- and cyclin E-associated cdk2 activity were seen after induction of p57Kip2.

Finally, to assess the effect of elevated p57Kip2 levels on biological function, the ability of the cells to replicate DNA was determined. In the experiments shown in Fig. 6, p57Kip2 levels were elevated by incubation of cells with doxycycline for 20 h, and DNA synthesis was then assessed by labeling with [3H]thymidine (Fig. 6C). A progressive decrease in DNA synthesis with increased levels of p57Kip2 was observed, indicating that p57Kip2 had an inhibitory effect on cell cycle progression, perhaps by slowing S phase entry. Analysis of the cells by flow cytometry (Fig. 6D) after tuning with propidium iodide showed that doxycycline treatment induced a progressive decrease in the fraction of cells in S phase, with a corresponding accumulation in G1 phase. Flow cytometric analysis after staining for annexin V showed no increase in staining with increasing dose of doxycycline, suggesting that increased p57Kip2 levels did not induce apoptosis, at least not by 20 h after treatment (data not shown). Finally, determination of cell numbers in cultures treated with various doses of doxycycline confirmed a decreased growth rate after induction of p57Kip2. By 3 days after incubation, cell numbers in cultures incubated at the three lowest doses of doxycycline (10⁻³, 10⁻², and 10⁻¹ μg/ml) were reduced by ~20%, compared with untreated cells. Doxycycline at a concentration of 1 μg/ml caused a 45% inhibition of proliferation; 10 μg/ml prevented proliferation almost completely. Doxycycline had no significant effect on proliferation of control Jurkat cells transfected with the empty Tet-On vector, except at the highest dose used (10 μg/ml). Taken together, the results presented in this work demonstrate that the ultimate effect of p57Kip2 on cell proliferation is a negative one, despite its function in promoting cdk6/cyclin D assembly.

Discussion
Progression through the cell cycle involves passage through a series of highly regulated steps, disruption of which can result in malignant cell growth. Recently, a new focus has centered on the p57Kip2 protein, whose expression appears to be suppressed in a high fraction of several types of lymphocyte-derived tumors (22–24). Understanding the full impact of p57Kip2 loss on lymphocyte growth and activity requires knowledge of its normal functions. In this study, it was shown that p57Kip2 is a nuclear protein present throughout the T cell cycle. In normal T cells, it remains fairly constant in amount, with some increase in late G1 phase. This is in dramatic contrast to p27Kip1, which shows a dramatic decrease in amount after T cell activation. In Jurkat cells, p57Kip2 is also a nuclear protein and remains constant in amount during the initial period after stimulation through the TCR and CD28, when cdk6 is translocated to the nucleus and activated as a kinase. The fact that it does not show substantial changes in amount suggests that its activity may be modulated primarily by phosphorylation and/or by differential associations with other molecules. As noted above, p57Kip2 can in fact be phosphorylated by its cdk6/cyclin D partners. Analysis of the functions of these changes is in progress. Also of interest is the predominantly nuclear localization of p57Kip2, suggesting perhaps that in T cells at least, p57Kip2 may not be involved in recently described cytoplasmic functions of the Cip/Kip proteins, such as actin dynamics and cell migration (30–36). Alternatively, low levels of cytoplasmic p57Kip2, not detected.
by the methods used in this study, might mediate such functions in T cells.

Results presented in this study suggest several points at which p57Kip2 may function in control of the T cell cycle: in facilitating cyclin D/cdk6 assembly, in modulating cyclin E- and cyclin A-associated kinase activities, and in regulating cyclin A production. The latter three phenomena, down-regulation of cyclin E- and cyclin A-associated kinases and of cyclin A protein levels, clearly fit within current paradigms of cell cycle control, which suggest that the primary function of Cip/Kip CDKIs is inhibition of the activity of cdk2, among whose functions are inactivation of the growth-suppressing activity of pRb. Activation of cyclin E/cdk2 is required for full phosphorylation of pRb and release of the transcription-activating members of the E2F family. These transcription factors are essential for activation of genes coding for a variety of proteins needed for S phase entry and progression, among which are cyclin A (reviewed in Refs. 7, 54–56). It has been shown previously, for example, that p27Kip1 can block cyclin E-dependent trans activation of cyclin A gene transcription (57). Thus, inhibition of cyclin A-associated kinase activity by p57Kip2 in T cells most likely occurs by two mechanisms: through direct inhibition of cyclin A/cdk2 complexes, and indirectly by preventing full activation of transcription of the cyclin A gene, which requires cyclin E/cdk2. Loss of p57Kip2 in tumor cells, leading to inappropriate activity of cdk2, elevated cyclin A levels, and cyclin A-associated kinase activity, might therefore be expected to have a dramatic stimulatory effect on cell growth. Understanding how p57Kip2 loss would affect the cell cycle through cdk6/cyclin D is less clear. A widely accepted model of cell cycle control through pRb proposes that the growth-suppressing activity of pRb is mediated through the hyperphosphorylated form, that cdk4 regulates cell size rather than cell cycle progression itself (68, 69). Activation of cyclin E/cdk2 results in phosphorylation of pRb (to the hyperphosphorylated form), completion of G1 phase, and S phase entry. Consistent with this model are results showing that enforced overexpression of cdk6 in mouse 3T3 fibroblasts or human breast epithelial cells markedly decreased the growth rate of cells, whereas a dominant-negative form of cdk6 had no such effect (70, 71).

When considered in light of this modified model of growth control in which cyclin D-associated kinases and cyclin E/cdk2 have different and opposing roles in pRb family protein regulation, loss of p57Kip2 expression in tumor cells might have an especially potent effect on stimulating cell cycle progression. Loss of p57Kip2 would inhibit activation of cyclin D-associated kinases needed to form the hypophosphorylated, i.e., growth-suppressing, form of pRb in early G1 phase. Perhaps more importantly, p57Kip2 would not be present to restrain the growth-stimulatory functions of cyclin E- and cyclin A/cdk2 complexes in late G1 and S phases. The multiple roles of p57Kip2 in T cell cycle progression and the impact of p57Kip2 loss during tumorigenesis are summarized in Fig. 7.

Results presented above also indicate that cyclin D/cdk6 complexes can phosphorylate p57Kip2. As noted, the identity of the phosphorylated residue(s) and the functional consequence of this modification are currently under study. It has been reported that cyclin E/cdk2 can phosphorylate p27Kip1, thereby targeting it for ubiquitin-mediated degradation (72, 73). Because p57Kip2 levels remain fairly constant throughout the cell cycle, in contrast to p27Kip1 (13, 14), it seems unlikely that the function of the observed cyclin D/cdk6-mediated phosphorylation of p57Kip2 is to promote its rapid degradation, although the stabilities of the differentially phosphorylated species of p57Kip2 are yet to be determined experimentally. In this regard, it has recently been reported (74) that phosphorylation in the C-terminal region of p57Kip2, which is homologous to the degradation-targeting site in p27Kip1, does not inhibit activity of p57Kip2 in T lymphocytes. The p57Kip2 protein has at least three functions in T cells: 1) p57Kip2 promotes the assembly of cyclin D and cdk6, which, according to this model, phosphorylates Rb to form the hypophosphorylated form of Rb, which sequesters activating E2F (E2F1,2,3) family members and prevents them from activating transcription of E2F-responsive genes. Loss of p57Kip2 in tumor cells would prevent formation of the growth-suppressing form of Rb. 2) p57Kip2 inhibits the activity of cdk2/cyclin E. Loss of p57Kip2 would result in continued activity of cdk2/cyclin E when its activity should be suppressed and thereby promotes completion of G1 phase and entry into S phase. 3) p57Kip2 inhibits the activity of cdk2/cyclin A. Loss of p57Kip2 would result in continued activity of cdk2/cyclin A when its activity should be suppressed and thereby promotes entry into and passage through S phase. Loss of p57Kip2 might also enhance cdk2/cyclin A activity indirectly by increasing the levels of cyclin A protein. The cyclin A gene is an E2F-responsive gene; enhanced activity of cyclin E/cdk2 (through loss of p57Kip2) would promote dissociation of pRb and E2F, enhancing transcription of E2F-responsive genes such as the cyclin A gene. Because the cyclin E gene itself is also regulated by E2F, cyclin E/cdk2 activity may be similarly enhanced. Molecules highlighted within light gray boxes are the active forms of the molecules; those in dark gray boxes are inactive.
result in p57Kip2 proteolysis, although p57Kip2 does appear to contain a destabilizing element located in its N-terminal region. A final noteworthy observation reported in this work is that elevation of p57Kip2 levels in Jurkat cells promoted assembly of cyclin D3 and cdk6 in the absence of any surface receptor-mediated stimulation. It has been shown previously that stimulation of Jurkat cells through CD3 and CD28 causes a rapid nuclear translocation and activation of cdk6, followed by IL-2 synthesis (50). Nuclear translocation and assembly of cdk6 with cyclin D2 also occur soon after activation of normal peripheral blood T cells (50, 52, 75). If this cdk6 mobilization is a prerequisite for full activation of T cells, then loss of expression of p57Kip2 might have effects not only on cell proliferation, but also on T-cell-mediated immune responses. These results suggest the need for further analysis of immune responses in patient-derived lymphocytes that have lost expression of p57Kip2.

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cells prior to events characteristic of the middle to late G1 phase of the cycle. J. Cell. Physiol. 154:7.