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Identification of Multiple Cell Cycle Regulatory Functions of $p57^{Kip2}$ in Human T Lymphocytes[1]

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The specific functions of $p57^{Kip2}$ in lymphocytes have not yet been fully elucidated. In this study, it is shown that $p57^{Kip2}$, which is a member of the Cip/Kip family of cyclin-dependent kinase inhibitors, is present in the nuclei of normal resting (G_0) T cells from peripheral blood and in the nuclei of the T cell-derived Jurkat cell line. Activation through the TCR results in rapid transport of cytoplasmic cyclin-dependent kinase 6 (*cdk*6) to nuclei, where it associates with cyclin D and $p57^{Kip2}$ in active enzyme complexes. Using purified recombinant proteins, it was shown in vitro that addition of $p57^{Kip2}$ protein to a mixture of cyclin D2 and *cdk*6 enhanced the association of the latter two proteins and resulted in phosphorylation of $p57^{Kip2}$. To probe further the function of $p57^{Kip2}$, Jurkat cells stably transfected with a plasmid encoding $p57^{Kip2}$ under control of an inducible (tetracycline) promoter were made. Induction of $p57^{Kip2}$ resulted in increased association of *cdk*6 with cyclin D3, without receptor-mediated T cell stimulation. The overall amounts of *cdk*6 and cyclin D3, and also of *cdk*4 and cyclin E, remained unchanged. Most notably, increased $p57^{Kip2}$ levels resulted in marked inhibition of both cyclin E- and cyclin A-associated *cdk*2 kinase activities and a decrease in cyclin A amounts. Therefore, although facilitating activation of *cdk*6, the ultimate outcome of $p57^{Kip2}$ induction was a decrease in DNA synthesis and cell proliferation. The results indicate that $p57^{Kip2}$ 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_1 phase are important targets in the induction and progression of malignancy (1–3). During early G_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)[3] (RB1) are primary regulators of the G_0/G_1 transition and G_1 progression. Their activities as growth suppressors are modulated by multiple phosphorylations, performed mostly by the G_1 phase cdks, cyclin-D-dependent kinases (cdk4 and cdk6) and cyclin E/cdk2 (7). Activity of the cyclin D/cdks is primarily regulated by cyclin-dependent kinase inhibitors (CDKIs) of the Ink4 family ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, $p19^{Ink4d}$), whereas cyclinE/cdk2 is regulated by Cip/Kip family CDKIs, which include $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (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 $p18^{Ink4c}$ have enlarged lymphoid organs, and T cells derived from them are hyperproliferative after TCR stimulation (10, 11); in contrast, T cells derived from $p19^{Ink4d}$-deficient animals appear to proliferate normally (10). The other Ink4 CDKIs, $p16^{Ink4a}$ and $p15^{Ink4b}$, may be involved in T cell senescence (12). The $p27^{Kip1}$ 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 $p27^{Kip1}$ protein, along with $p21^{Cip1}$, may also play a role in the induction of T cell anergy (19, 20). The last CDKI, $p57^{Kip2}$, 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, $p21^{Cip1}$ appears to be an inhibitor of rho-kinase (32), whereas $p57^{Kip2}$ can regulate the cellular localization of LIM kinase, a kinase involved in actin filament dynamics (33). The involvement of $p27^{Kip1}$ 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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[3] Abbreviations used in this paper: pRb, retinoblastoma susceptibility gene protein; *cdk*, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; Tet, tetracycline.

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In addition to its larger size and more complex structure, the p57^{Kip2} protein has other features distinguishing it from the p21^{Cip1} and p27^{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^{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^{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^{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^{Kip2} in various cell lines of B and T cell-derived malignancies appears variable, some having high levels of and others no detectable p57^{Kip2} protein. Of interest, some lines not producing p57^{Kip2} were found to have methylated p57^{Kip2} genes; treatment of some of these lines with 5-aza-2′-deoxycytidine resulted in demethylation of the gene and expression of the p57^{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^{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^{Kip2} protein is present in high amount 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^{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^{Kip2} in events occurring later in the cell cycle is also demonstrated. Overexpression of p57^{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^{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 (*tet*)-inducible expression of p57^{Kip2} were constructed by cotransfection of the cells with pTRE-p57^{Kip2} and pTK-Hy plasmids (in a 10:1 ratio). After 36 h of growth, hygromycin was added at a concentration of 300 μg/ml. Fresh medium with hygromycin was added after each 4 days in culture. By ~2 wk in culture, only drug-resistant cells survived. They were isolated and evaluated for doxycycline inducibility of p57^{Kip2} expression, as described in the text. For experiments using activated cells, primary T cells were incubated with PHA (10 μg/ml), and Jurkat cells were stimulated with PHA (10 μg/ml) and the mAb to CD28, clone 9.3 (1000-fold dilution of ascites fluid; kindly supplied by Bristol-Myers Squibb, Seattle, WA). Metabolic labeling of cellular proteins was performed by incubating cells for 9 h in methionine-free medium containing dialyzed FCS and 150 μCi/ml [^{35}S]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 (5×10^6) were incubated in medium containing [^{3}H]thymidine for 6-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^{Kip2}, which was used at a 1/500 dilution. For detection of specific proteins, an ECL method was used, as described previously (50).

Immunoprecipitation

Immunoprecipitation was performed, as described previously (50). In brief, whole cells or isolated nuclei were incubated in an immunoprecipitation buffer, which contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% (v/v) Tween 20, 10 μg/ml leupeptin, 20 μg/ml aprotinin, 0.1 mM PMSF, 10 mM β-glycerophosphate, and 0.1 mM orthovanadate. They were then sonicated for 15 s at 4°C. After preclearing using protein G-coupled Sepharose beads (Zymed Laboratories, 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^{Kip2}, cyclin D2, and cdk6 were subcloned into the pBAC transfer plasmid (Novagen/EMD Biosciences, Madison, WI). Recombinant baculoviruses were isolated by cotransfection of Sf9 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$_6$ tags at their N-terminal ends, thus permitting isolation by Ni^{2+}-chelating affinity column 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^{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 $MgCl_2$, 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^{Rb}) as substrate.

Results

The p57^{Kip2} CDKI is present in T cells

Normal human T cells isolated from peripheral blood are in a resting (G_0) state. Stimulation through the TCR activates several signal transduction cascades that result in cytokine production and cell growth and division. As shown in Fig. 1*A*, the CDKI p57^{Kip2} was present in resting T cells and exhibited little change in overall amount throughout cell cycle progression after stimulation with

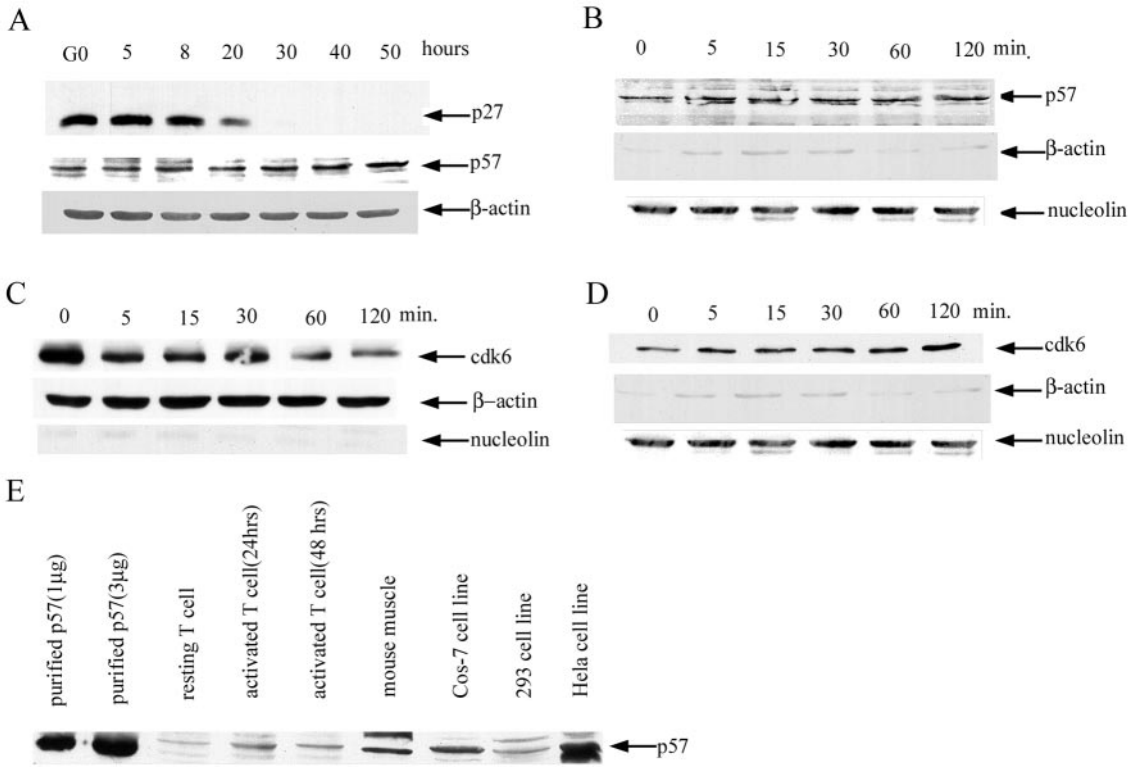

FIGURE 1. Detection of the $p57^{Kip2}$ protein in normal and transformed (Jurkat) T cells. *A*, Normal human T cells were stimulated with PHA, and at the times indicated the cellular contents of $p27^{Kip1}$ and $p57^{Kip2}$ were evaluated by immunoblotting. The level of β-actin is shown as a control 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 *cdk*6, 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.

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. 1*A* 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. 1*B*, 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. 1*C*) and nuclear (Fig. 1*D*) 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 β-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. 1*E*, 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. 1*E*, all of the lanes were loaded with the same overall amount of cell protein (10 μg), except for the first two lanes, in which two different amounts (1 and 3 μg) 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. 2*A*) and cdk6 and cyclin D3 in Jurkat cell nuclei (Fig. 2*B*). As noted previously, cyclin D2, although detected in Jurkat cells, does not associate with cdk6 (50, 51); in normal T

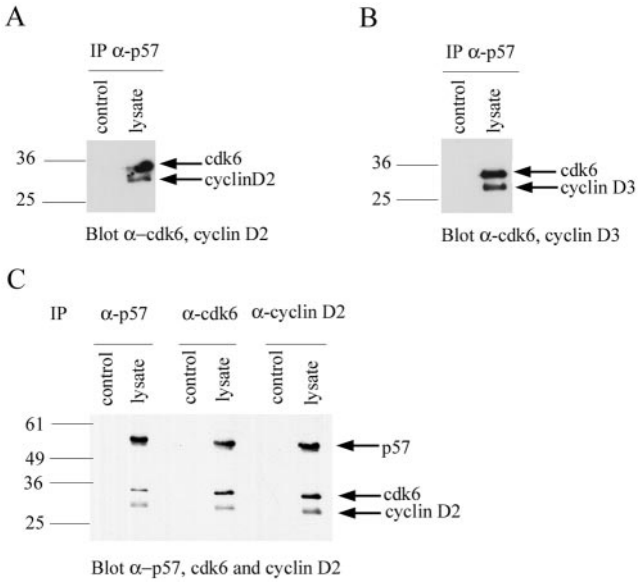

FIGURE 2. The p57^{Kip2} protein forms a complex with cyclin D and *cdk*6 in the nuclei of T cells. *A*, The p57^{Kip2} protein was immunoprecipitated (using a goat anti-p57 Ab) from a nuclear lysate prepared from normal T cells stimulated for 12 h with PHA. The immunoprecipitated proteins were resolved by gel electrophoresis and probed for the presence of *cdk*6 and cyclin D2 using a mixture of specific Abs (rabbit) to the two proteins. For the control lane, the analysis was performed without cell lysate and shows that the bands seen in the sample lane are not due to proteins present in the immunoprecipitation reagents. *B*, The p57^{Kip2} protein was immunoprecipitated (using a goat anti-p57 Ab) from a nuclear lysate prepared from Jurkat cells stimulated for 30 min with PHA and anti-CD28. The immunoprecipitated proteins were resolved by gel electrophoresis and probed for the presence of *cdk*6 and cyclin D3 using a mixture of specific Abs (rabbit) to the two proteins. For the control lane, the analysis was performed without cell lysate. *C*, Normal T cells were stimulated with PHA for 12 h. Three equal aliquots of the cell lysate were subjected to immunoprecipitation using Abs (to p57^{Kip2}, *cdk*6, or cyclin D2) chemically coupled to beads. Eluted protein complexes were resolved by gel electrophoresis and probed for the presence of p57^{Kip2}, *cdk*6, and cyclin D2 using a mixture of specific Abs to the three 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).

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/p57^{Kip2} complex was also confirmed directly in experiments in which complexes were isolated using Abs chemically linked to a matrix. This permitted identification of p57^{Kip2}, without interference by IgG H chain present in the immunoprecipitates. 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 p57^{Kip2}, cdk6, or cyclin D2. The complexes were isolated and probed, by immunoblot analysis, for their contents of p57^{Kip2}, cdk6, and cyclin D2. As shown in Fig. 2*C*, the amount of p57^{Kip2} was similar in all three samples, suggesting that most of the p57^{Kip2} 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 p57^{Kip2}. Because whole cell lysates were used in this analysis, it is likely that some cdk6 and/or cyclin D2 not associated with p57^{Kip2} were in the cytoplasm. Analysis of immunoprecipitates isolated from cells metabolically labeled with [^{35}S]methionine also supported the conclusion that a large fraction of each molecule existed in a trimolecular p57^{Kip2}/cdk6/cyclin D2 complex (data not shown).

Enzymatic activity of p57^{Kip2}/cdk6/cyclin D complexes

The association and activity of p57^{Kip2}, 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. 3*A*, p57^{Kip2} 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 p57^{Kip2} was seen in *lane 3*. In *lane 4*, p57^{Kip2} 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 p57^{Kip2} was again seen. These results indicate that the cdk6/cyclin D2 complex can phosphorylate p57^{Kip2}. To investigate whether or not the trimolecular p57^{Kip2}/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 p57^{Kip2} or cdk6, and used in in vitro kinase assays with added (truncated) recombinant pRb as substrate. As shown in Fig. 3*B*, the p57^{Kip2} (*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. 3*C*, immunoprecipitates prepared using Abs to

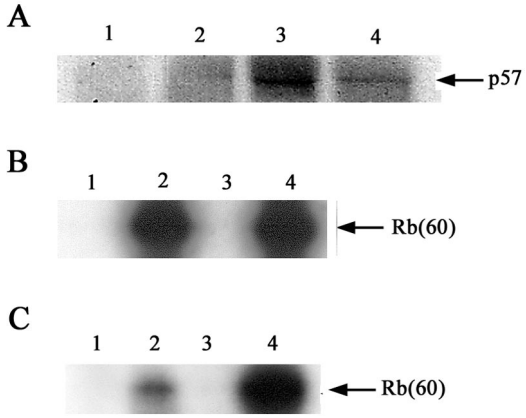

FIGURE 3. Enzymatic activity is associated with p57^{Kip2}-containing protein complexes. *A*, Purified recombinant *cdk*6 and cyclin D2 were assembled into a protein complex, and the complex was isolated by immunoprecipitation of *cdk*6. The complex was able to phosphorylate purified recombinant p57^{Kip2} added in an in vitro kinase assay (*lane 3*). Neither cyclin D2 (*lane 1*) nor *cdk*6 (*lane 2*) alone could phosphorylate p57^{Kip2}. As shown in *lane 4*, a *cdk*6 immunoprecipitate from normal human T cells (stimulated for 12 h with PHA) also could phosphorylate added recombinant p57^{Kip2} in an in vitro kinase assay. *B*, Nuclear extracts were prepared from normal human T cells stimulated for 12 h with PHA. Immunoprecipitates prepared using Ab to p57^{Kip2} (*lane 2*) and *cdk*6 (*lane 4*) could both phosphorylate added recombinant pRb protein, to a similar extent, in an in vitro kinase assay. For *lanes 1* and *3*, the analyses were performed without added nuclear lysates. *C*, Nuclear extracts were prepared from Jurkat cells stimulated for 30 min with PHA and anti-CD28. Immunoprecipitates prepared using Ab to p57^{Kip2} (*lane 2*) or *cdk*6 (*lane 4*) could both phosphorylate added recombinant pRb protein in an in vitro kinase assay. Control immunoprecipitates using the two antisera without added extract showed no activity (*lanes 1* and *3*).

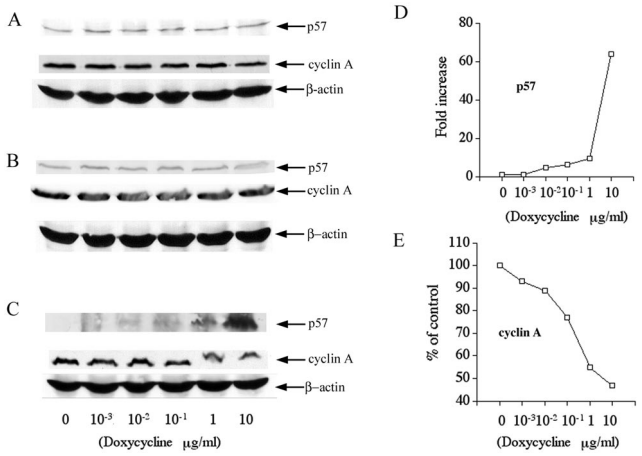

FIGURE 4. Effects of induced overexpression of p57^{Kip2} on the cellular contents of cell cycle regulatory molecules. Cultures of Jurkat cells (*A*), Tet-On Jurkat cells (*B*), and Tet-On p57^{Kip2} Jurkat cells (*C*) were treated for 20 h with various doses of doxycycline, and then assessed by immunoblot analysis for levels of p57^{Kip2}, cyclin A, and β-actin. Changes in p57^{Kip2} and cyclin A levels in Tet-On p57^{Kip2} Jurkat cells were quantitated and are presented in *D* and *E*, respectively.

p57^{Kip2} (*lane 2*) or cdk6 (*lane 4*) each showed phosphorylation of pRb in the in vitro assay, although the cdk6 sample was more effective, suggesting that in Jurkat cells, a portion of the cdk6 resided in complexes not containing p57^{Kip2}. The Jurkat cell line is known to have a very large amount of cdk6, in great excess to that seen in normal T cells (50, 51).

Construction and characterization of Jurkat T cells with Tet-inducible p57^{Kip2}

To further investigate the function of p57^{Kip2} in T cells, we constructed a Jurkat cell line stably transfected with a plasmid encoding p57^{Kip2} under the control of a Tet promoter (see *Materials and Methods*). As shown in Fig. 4*C*, addition of doxycycline to these cells induced a dose-dependent increase in the cellular content of p57^{Kip2}. The p57^{Kip2} levels were quantitated and are shown graphically in Fig. 4*D*. No changes in the levels of several other cell cycle regulatory molecules, including cdk4, cdk6, cdk2, cyclin D3, or cyclin E, were seen (data not shown). A significant decrease in cyclin A level (Fig. 4, *C* and *E*) was seen with increasing doses of doxycycline, which, as discussed further below, may be at least partially responsible for the inhibitory effects on cell growth that p57^{Kip2} exerts. Doxycycline treatment of the parental Jurkat cells (Fig. 4*A*) or Jurkat cells stably transfected with the Tet-promoter plasmid without the p57^{Kip2} sequence (Fig. 4*B*) induced no significant changes in p57^{Kip2} or cyclin A levels. Likewise, no changes in the levels of the other cell cycle regulatory molecules analyzed (cdk4, cdk6, cdk2, cyclin D3, and cyclin E) were seen in these two cell lines (data not shown).

Promotion of cdk*6/cyclin D association by p57^{Kip2}*

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 p57^{Kip2} in the association of cdk6 and cyclin D in T cells was therefore examined using two approaches. As shown in Fig. 5*A*, induction of p57^{Kip2} 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 cyclin D-associated 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 p57^{Kip2}). As suggested above, because Jurkat cells contain a high level of cdk6, cyclin D3 may be in limiting amounts as compared with cdk6 and p57^{Kip2} under these conditions. That p57^{Kip2} 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. 5*B*, addition of increasing amounts of p57^{Kip2} 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.

Inhibition of cdk*2 activities and cell proliferation by p57^{Kip2}*

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 p57^{Kip2} 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 (49), activities were determined using immunoprecipitates

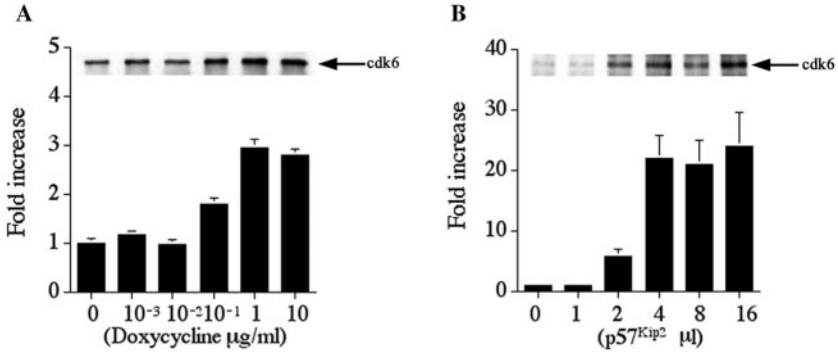

FIGURE 5. The p57^{Kip2} protein promotes the association of cyclin D and *cdk*6. *A*, Cultures of the Tet-On p57^{Kip2} Jurkat cell line were treated for 20 h with various doses of doxycycline, and cyclin D3 was prepared from cell lysates by immunoprecipitation. The amounts of *cdk*6 associated with cyclin D3 were assessed by immunoblot analysis; a representative example is shown. Amounts of *cdk*6 were also quantitated, as described in *Materials and Methods*, and the results from duplicate experiments are presented in graphical form. *B*, Purified recombinant *cdk*6, cyclin D2, and various amounts of p57^{Kip2}, indicated under the figure, were mixed together. After incubation in an assembly buffer, cyclin D2 was immunoprecipitated, and the amounts of *cdk*6 associated with cyclin D2 were evaluated by immunoblot analysis. A representative immunoblot is shown. Amounts of *cdk*6 were also quantitated, as described in *Materials and Methods*, and results from duplicate experiments are presented in graphical form.

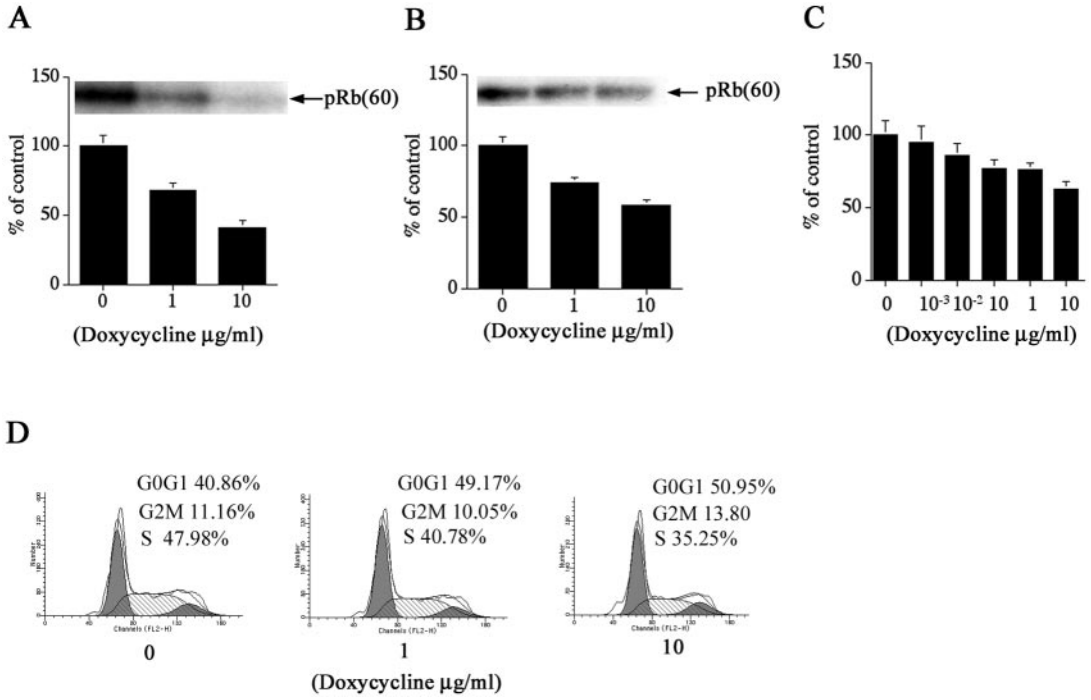

FIGURE 6. Induced overexpression of $p57^{Kip2}$ inhibits cyclin E- and cyclin A-associated kinase activities and DNA synthesis in Tet-On $p57^{Kip2}$ Jurkat cells. Cells were induced with doxycycline, and the kinase activities associated with immunoprecipitates of cyclin E (*A*) and cyclin A (*B*) were determined using an in vitro kinase assay. Representative autoradiograms are shown. The cyclin-associated kinase activities were also quantitated, as described in *Materials and Methods*, and results from duplicate experiments are presented in graphical form. For *C*, cultures of Tet-On $p57^{Kip2}$ Jurkat cells were incubated with various doses of doxycycline for 20 h and then labeled for 6 h with [^{3}H]thymidine. The amount of radiolabeled thymidine incorporated into DNA was determined, as described in *Materials and Methods*. *D*, Flow cytometric analysis of DNA content of Tet-On $p57^{Kip2}$ Jurkat cells incubated with 0, 1, or 10 μg/ml doxycycline for 20 h is shown.

prepared using either cyclin E (Fig. 6*A*) or cyclin A (Fig. 6*B*) 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 $p57^{Kip2}$.

Finally, to assess the effect of elevated $p57^{Kip2}$ levels on biological function, the ability of the cells to replicate DNA was determined. In the experiments shown in Fig. 6, $p57^{Kip2}$ levels were elevated by incubation of cells with doxycycline for 20 h, and DNA synthesis was then assessed by labeling with [^{3}H]thymidine (Fig. 6*C*). A progressive decrease in DNA synthesis with increased levels of $p57^{Kip2}$ was observed, indicating that $p57^{Kip2}$ had an inhibitory effect on cell cycle progression, perhaps by slowing S phase entry. Analysis of the cells by flow cytometry (Fig. 6*D*) after staining with propidium iodide showed that doxycycline treatment induced a progressive decrease in the fraction of cells in S phase, with a corresponding accumulation in G_1 phase. Flow cytometric analysis after staining for annexin V showed no increase in staining with increasing dose of doxycycline, suggesting that increased $p57^{Kip2}$ 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 $p57^{Kip2}$. 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. Doxycyline 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 $p57^{Kip2}$ 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 $p57^{Kip2}$ 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 $p57^{Kip2}$ loss on lymphocyte growth and activity requires knowledge of its normal functions. In this study, it was shown that $p57^{Kip2}$ 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 G_1 phase. This is in dramatic contrast to $p27^{Kip1}$, which shows a dramatic decrease in amount after T cell activation. In Jurkat cells, $p57^{Kip2}$ 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, $p57^{Kip2}$ 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 $p57^{Kip2}$, suggesting perhaps that in T cells at least, $p57^{Kip2}$ 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 $p57^{Kip2}$, 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 $p57^{Kip2}$ 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 $p27^{Kip1}$ can block cyclin E-dependent *trans* activation of cyclin A gene transcription (57). Thus, inhibition of cyclin A-associated kinase activity by $p57^{Kip2}$ 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 $p57^{Kip2}$ 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 $p57^{Kip2}$ 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 functions of pRb are reversed by successive phosphorylations, performed first by cyclin D-associated kinases (cdk4/cdk6) and then by cyclin E/cdk2 (58–62). The pRb protein has numerous sites that can be phosphorylated by cyclin-dependent kinases; in this model, it has remained unclear whether or not the intermediate forms of phosphorylated pRb have distinctive functions. It has been proposed that cyclin D-associated kinases convert pRb to a form that partially relieves its growth-suppressing functions, permitting a low level of expression of the E2F transcription factor-regulated cyclin E gene (63). Activation of cyclin E/cdk2 results in additional phosphorylation of pRb and release of activating E2F factors, resulting in transcription of genes expressing proteins needed for S phase entry and progression, including cyclin A (2, 63). According to this model, loss of $p57^{Kip2}$ would be expected to hinder cell cycle progression, because it promotes cyclin D/cdk6 assembly and is needed for full activation of the cyclin D-associated kinases that perform the initial phosphorylations of pRb.

However, an alternative model of pRb family protein function suggests that cyclin D-associated kinases and cyclin E/cdk2 in fact have opposing roles in regulating Rb family proteins. As suggested by Ezhevsky et al. (58, 59), pRb in resting (G_0) cells, such as normal T cells, is in an unphosphorylated, essentially inert form. Maintenance of cells in the resting (G_0) state is dependent upon the growth-suppressing activity of p130(RB2) rather than pRb (61, 64–67). After cell cycle entry (accompanied by phosphorylation of p130), initial phosphorylations of pRb by cyclin D-associated kinases (cdk4 and/or cdk6) convert it to the active growth-suppressing (hypophosphorylated) form that sequesters the transcription-activating members of the E2F family. During this period of inhibition of further cell cycle progression, in early G_1 phase, the cell can increase in mass, an aspect of this model consistent with genetic analysis of *Drosophila* showing 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 G_1 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 $p57^{Kip2}$ expression in tumor cells might have an especially potent effect on stimulating cell cycle progression. Loss of $p57^{Kip2}$ would inhibit activation of cyclin D-associated kinases needed to form the hypophosphorylated, i.e., growth-suppressing, form of pRb in early G_1 phase. Perhaps more importantly, $p57^{Kip2}$ would not be present to restrain the growth-stimulatory functions of cyclin E- and cyclin A-cdk2 complexes in late G_1 and S phases. The multiple roles of $p57^{Kip2}$ in T cell cycle progression and the impact of $p57^{Kip2}$ loss during tumorigenesis are summarized in Fig. 7.

Results presented above also indicate that cyclin D/cdk6 complexes can phosphorylate $p57^{Kip2}$. 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 $p27^{Kip1}$, thereby targeting it for ubiquitin-mediated degradation (72, 73). Because $p57^{Kip2}$ levels remain fairly constant throughout the cell cycle, in contrast to $p27^{Kip1}$ (13, 14), it seems unlikely that the function of the observed cyclin D/cdk6-mediated phosphorylation of $p57^{Kip2}$ is to promote its rapid degradation, although the stabilities of the differentially phosphorylated species of $p57^{Kip2}$ are yet to be determined experimentally. In this regard, it has recently been reported (74) that phosphorylation in the C-terminal region of $p57^{Kip2}$, which is homologous to the degradation-targeting site in $p27^{Kip1}$, does not

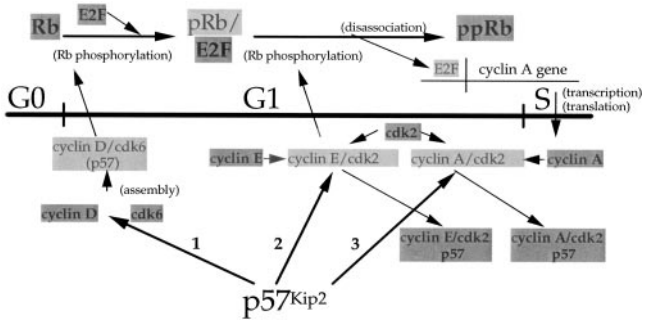

FIGURE 7. Multiple functions of $p57^{Kip2}$ in cell cycle progression in T lymphocytes. The $p57^{Kip2}$ protein has at least three functions in T cells: 1) $p57^{Kip2}$ promotes the assembly of cyclin D and *cdk*6, which, according to this model, phosphorylates Rb to form the hypophosphorylated form of Rb, which sequesters activating E2F (E2F1,2,3) family members and prevents them from activating transcription of E2F-responsive genes. Loss of $p57^{Kip2}$ in tumor cells would prevent formation of the growth-suppressing form of Rb. 2) $p57^{Kip2}$ inhibits the activity of *cdk*2/cyclin E. Loss of $p57^{Kip2}$ would result in continued activity of *cdk*2/cyclin E when its activity should be suppressed and thereby promotes completion of G_1 phase and entry into S phase. 3) $p57^{Kip2}$ inhibits the activity of *cdk*2/cyclin A. Loss of $p57^{Kip2}$ would result in continued activity of *cdk*2/cyclin A when its activity should be suppressed and thereby promotes entry into and passage through S phase. Loss of $p57^{Kip2}$ might also enhance *cdk*2/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/*cdk*2 (through loss of $p57^{Kip2}$) would promote disassociation 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/*cdk*2 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 p57^{Kip2} proteolysis, although p57^{Kip2} does appear to contain a destabilizing element located in its N-terminal region. A final noteworthy observation reported in this work is the finding that elevation of p57^{Kip2} 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 p57^{Kip2} 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 p57^{Kip2}.

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