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IL-7 promotes T cell proliferation through destabilization of $p27^{Kip1}$

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Interleukin (IL)-7 is required for survival and homeostatic proliferation of T lymphocytes. The survival effect of IL-7 is primarily through regulation of Bcl-2 family members; however, the proliferative mechanism is unclear. It has not been determined whether the IL-7 receptor actually delivers a proliferative signal or whether, by promoting survival, proliferation results from signals other than the IL-7 receptor. We show that in an IL-7–dependent T cell line, cells protected from apoptosis nevertheless underwent cell cycle arrest after IL-7 withdrawal. This arrest was accompanied by up-regulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ through a posttranslational mechanism. Overexpression of $p27^{Kip1}$ induced G1 arrest in the presence of IL-7, whereas knockdown of $p27^{Kip1}$ by small interfering RNA promoted S phase entry after IL-7 withdrawal. CD4 or CD8 T cells transferred into IL-7–deficient hosts underwent G1 arrest, whereas 27^{Kip1}-deficient T cells underwent proliferation. We observed that IL-7 withdrawal activated protein kinase C (PKC)θ and that inhibition of PKCθ with a pharmacological inhibitor completely blocked the rise of $p27^{Kip1}$ and rescued cells from G1 arrest. The conventional pathway to breakdown of $p27^{Kip1}$ is mediated by S phase kinase-associated protein 2; however, our evidence suggests that PKCθ acts via a distinct, unknown pathway inducing G1 arrest after IL-7 withdrawal from T cells. Hence, IL-7 maintains T cell proliferation through a novel pathway of $p27^{Kip1}$ regulation.

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Abbreviations used: CDK, cyclin-dependent kinase; CKI, CDK inhibitor; Cks1, CDK subunit 1; KPC, Kip1 ubiquitination-promoting complex; PI, propidium iodide; PKC, protein kinase C; siRNA, small interfering RNA; Skp2, S phase kinase-associated protein 2.

The size of the peripheral T cell pool is tightly controlled through homeostatic mechanisms that regulate cell survival and proliferation. The cytokine IL-7, a product of nonlymphoid cells in lymphoid tissues, is one of the required stimuli for both survival and proliferation of most of the major subsets of peripheral T cells (1–7). Together with IL-7, weak signals from the TCR-recognizing self-peptide/MHC are required for survival and proliferation of naive CD4 and CD8 cells (1, 8, 9). Survival and proliferation of memory CD8 cells depends on IL-15 and IL-7 (2, 3). Memory CD4 cells become acutely dependent on IL-7 for homeostatic proliferation when TCR signaling is abolished (4).

Survival of T cells has been largely attributed to IL-7 regulation of the balance of proapoptotic versus antiapoptotic members of the Bcl-2 family. Thus, IL-7 protects T cells from death through the induction of antiapoptotic proteins Bcl-2 (10) and Mcl-1 (11), and inhibition of proapoptotic proteins Bax (12), Bad (13), and Bim (14). The proliferative mechanism of T cells in response to IL-7 has not been studied extensively. It has not been determined whether the IL-7 receptor delivers a proliferation signal per se, or alternatively whether the IL-7 effect is to maintain survival, permitting other signals to induce cell division.

As characterized in other cell types, proliferation depends on the activity of a series of protein complexes composed of cyclins and cyclin-dependent kinases (CDKs; reference 15). CDK activity is regulated through phosphorylation-dephosphorylation of the kinase subunit and in large part through inhibition by CDK inhibitors (CKIs; references 15 and 16). CKIs can be divided into two classes: inhibitors of CDK4 proteins (p16, p15, p18, and p19) and inhibitors of the Cip/Kip family ($p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$). $p21^{Cip1}$ and $p27^{Kip1}$ are able to constrain a broad spectrum of CDKs (16) and are expressed in peripheral T cells (17, 18). Mice lacking $p27^{Kip1}$ display gigantism with disproportionately enlarged lymphoid organs as a result of increased cellularity (19–21),

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suggesting that p27^{Kip1} could be an inhibitor of homeostatic proliferation of T cells. Peripheral T cells from p27^{Kip1}-transgenic mice show a dramatically reduced ability to proliferate in response to mitogenic stimulation (22). The role of p21^{Cip1} in T lymphocytes is less clear, with some evidence suggesting that it promotes T lymphocyte apoptosis mediated by Fas or protects activated/memory T cells from apoptosis (23).

p27^{Kip1} plays a pivotal role in the control of cell cycle G1 to S phase transition by inhibiting the activities of G1 cyclins/CDKs. In response to stimulation by growth factors, levels of p27^{Kip1} dramatically decrease, which appears to be a critical mechanism by which growth factors are capable of inducing cell cycle progression. IL-3 repressed p27^{Kip1} transcription in a murine pro–B cell line, Ba/F3 [24], as did IL-2 in CTLL cells (25). However, in most types of normal or transformed cells, p27^{Kip1} is regulated posttranslationally through ubiquitination and proteosomal degradation (26). Phosphorylation of p27^{Kip1} at threonine 187 (T187) by CDK2–cyclin E complexes is thought to initiate the major pathway for p27^{Kip1} protein degradation (27, 28). Several studies indicate that S phase kinase-associated protein 2 (Skp2), an F-box protein, functions as the receptor component of an SCF ubiquitin ligase complex, binding to p27^{Kip1} in conjunction with CDK subunit 1 (Cks1) only when T187 of p27^{Kip1} is phosphorylated. This results in the ubiquitination and degradation of p27^{Kip1} [(29–31)].

IL-7 has been previously reported to down-regulate p27^{Kip1} in T cell acute lymphoblastic leukemia cells, and this was proposed to promote clonal expansion of these transformed cells (32, 33). To understand how IL-7 affects cell cycle in nontransformed T cells, we evaluated the role of p27^{Kip1} in proliferation in an IL-7–dependent thymocyte line and in peripheral T cells in vivo, and we observed two pathways, Skp2- and protein kinase C (PKC)θ-dependent, by which IL-7 receptor regulates p27^{Kip1} degradation.

RESULTS

Bcl-2 cannot replace the requirement for IL-7 in promoting cell proliferation

IL-7 promotes survival by maintaining a favorable balance of Bcl-2 family members in which antiapoptotic proteins protect from proapoptotic proteins. One of the actions of IL-7 is to induce the synthesis of the antiapoptotic protein Bcl-2 (10), and transgenic expression of *bcl-2* has been shown to partially overcome the requirement for IL-7 for thymopoiesis (34). Although thymopoiesis requires both cell survival and proliferation, it was possible that IL-7 signaling did not induce proliferation directly but blocked apoptosis, thereby permitting other proliferative signals to drive the expansion of T cells. Because the D1 thymocyte line (35) responds to IL-7 by survival and proliferation, we examined to what extent overexpression of *bcl-2* could replace the IL-7 signal by stably transfecting D1 cells with a *bcl-2* retroviral expression vector. As shown in Fig. 1, withdrawal of IL-7 for 24 h from untransfected D1 cells induced both G1 arrest and ~30% of cells died. However, *bcl-2*–transfected D1 cells could survive indefinitely without IL-7 but nevertheless underwent G1 arrest. Restimulation of *bcl-2*–transfected cells with IL-7 restored a normal rate of proliferation (not depicted). Thus, Bcl-2 could replace the survival function of IL-7 but failed to replace the proliferative function, indicating that IL-7 induces a proliferation pathway that is distinct from the antiapoptotic pathway.

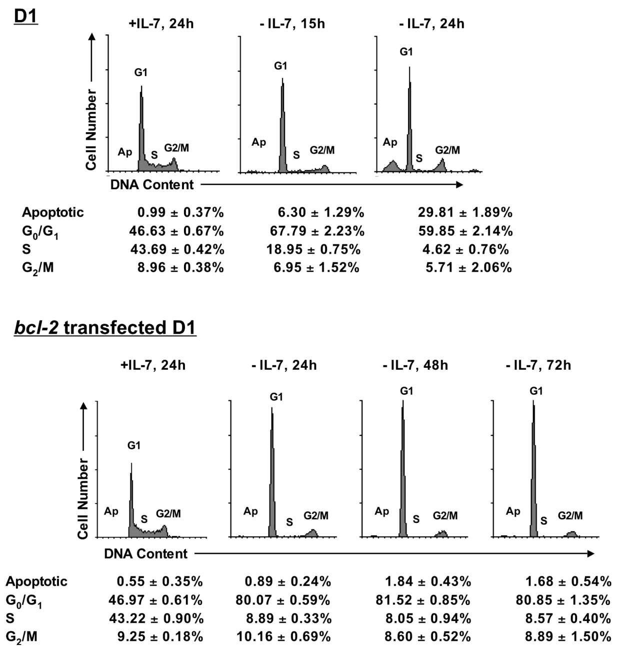

Figure 1. Proliferative effect of IL-7 is independent of survival effect. D1 or *bcl-2*–transfected D1 cells were cultured in the presence or absence of IL-7 for different time points. Cell cycle and survival were analyzed by PI staining after flow cytometric assay. G1 peak, S phase, G2/M phase, and apoptotic populations are indicated. Ap, apoptosis. Percentage of cells in different populations was calculated by ModFit LT software. The results are representative of three independent experiments.

IL-7 down-regulates p27^{Kip1} via posttranslational regulation

Entry into S phase can be inhibited by the CKI p27^{Kip1}, and it has been shown that IL-3 and IL-2 block p27^{Kip1} gene expression (24, 25). It was reported that in IL-7–responsive T cell acute lymphoblastic leukemia cells, both survival and cell division correlated with low levels of p27^{Kip1} protein (33). Therefore, we examined whether IL-7 influenced the expression of p27^{Kip1} in D1 cells or LN T cells. Western blotting analysis revealed that after IL-7 withdrawal from D1 cells, p27^{Kip1} protein levels rose dramatically, whereas levels of another CKI, p16, remained unchanged (Fig. 2 A). p21^{Cip1}, another CKI, was undetectable in D1 cells (not depicted). p27^{Kip1} protein levels also increased in primary cultures of T cells after 24 h of IL-7 deprivation. Both p16 and p21^{Cip1} expressions were not affected by IL-7 withdrawal in LN cells (Fig. 2 B). Overexpression of *bcl-2* in D1 cells altered neither cell cycle status (Fig. 1) nor p27^{Kip1} expression (Fig. 2 C). To determine whether IL-7 regulated p27^{Kip1} at the transcriptional level, we performed Northern blotting. Withdrawal of

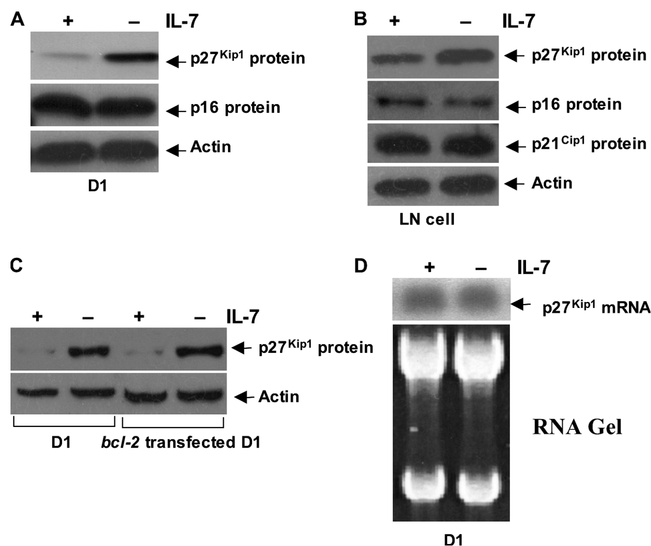

Figure 2. IL-7 down-regulates p27^{Kip1} posttranslationally. (A) D1 cells were cultured with (+) or without (−) IL-7 for 12 h. Cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies against p27^{Kip1}, p16, and β-actin. (B) LN T cells were isolated and grown with or without IL-7 for 24 h. Protein levels were measured by immunoblotting using antibodies against p27^{Kip1}, p16, p21^{Cip1}, and β-actin. (C) Cell lysates were prepared from D1 or *bcl-2*–transfected D1 cells cultured with or without IL-7 for 12 h. p27^{Kip1} protein level was measured by immunoblotting. (D) D1 cells were cultured with (+) or without (−) IL-7 for 12 h. Total RNAs were isolated and 20 μg total RNA was loaded to agarose gel (bottom). The RNA blot was probed with ^{32}P-labeled p27^{Kip1} cDNA fragment (top).

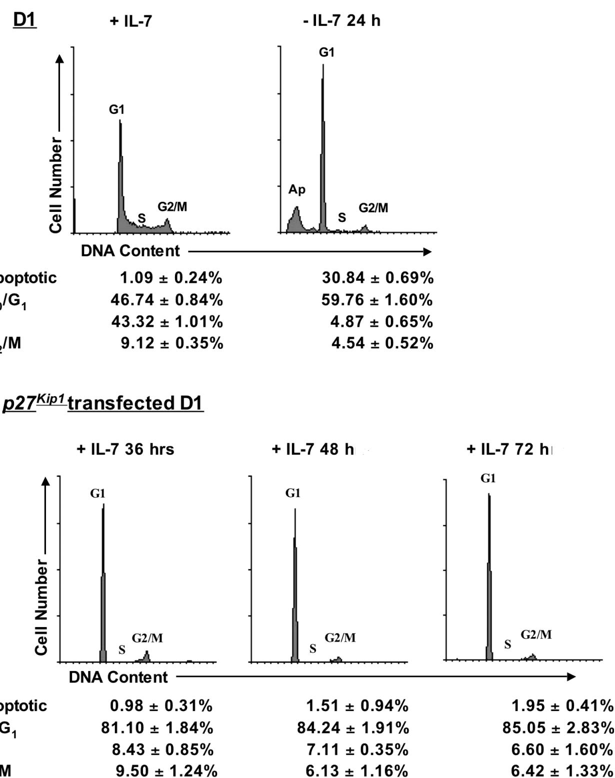

Figure 3. p27^{Kip1} causes G1 arrest without effect on survival in D1 cells. D1 cells were transfected with *p27^{Kip1}* and cultured with IL-7 for certain time points. Cell cycle and survival were analyzed by PI staining after flow cytometric analysis. G1 peak, S phase, G2/M phase, and apoptotic populations are indicated. Ap, apoptosis. Percentage of cells in different populations was calculated by ModFit LT software. The results are representative of three independent experiments.

IL-7 from D1 cells did not increase the mRNA levels of p27^{kip1} (Fig. 2 D). Therefore, unlike IL-3 or IL-2, IL-7 down-regulated p27^{Kip1} at a posttranslational level rather than at the transcriptional level.

p27^{Kip1} induces G1 arrest in D1 cells

To determine if p27^{Kip1} could contribute to G1 arrest after IL-7 withdrawal, D1 cells were transfected with p27^{Kip1} in a retroviral expression vector expressing GFP as a selective marker. 20 h later, GFP$^+$ cells were sorted and placed in culture with IL-7. D1 cells or p27^{Kip1}-transfected D1 cells were stained with propidium iodide (PI) and cell cycle status was analyzed by flow cytometry. As shown in Fig. 3, in the presence of IL-7, overexpression of p27^{Kip1} in D1 cells induced complete G1 arrest 36 h after transfection and no apoptosis was observed. To determine whether p27^{Kip1} was required for G1 arrest after IL-7 withdrawal, we introduced a small interfering RNA (siRNA), pMIG-hU6-sip27^{Kip1} (sip27^{Kip1}), to knockdown expression of endogenous p27^{kip1} in D1 cells. The expression of p27^{Kip1} was strongly inhibited by sip27^{Kip1} at 15 h after IL-7 deprivation, whereas the control-scrambled siRNA showed no effect (Fig. 4, top). PI staining analysis showed that cell cycle progression into S phase after IL-7 withdrawal was also enhanced by sip27^{Kip1}. After 15 h of IL-7 deprivation, the number of cells bearing sip27^{Kip1} in S phase increased to 28.55%, as compared with 18.36% of that in control cells (Fig. 4, bottom). There was no difference in cell survival between D1 with sip27^{Kip1} and D1 with siRNA control (Fig. 4, bottom). These data implicate the up-regulation of p27^{Kip1} as an important mediator of G1 arrest after IL-7 withdrawal.

p27^{Kip1} deficiency partially restores T cell proliferation in IL-7–deficient hosts

Having shown that in the D1 thymic cell line p27^{Kip1} plays an important role in cell cycle arrest after IL-7 withdrawal, we tested this model using peripheral T cells in vivo. After transfer of T cells into a host depleted of lymphocytes, the survival and expansion of most subsets requires IL-7 (1, 3). This is termed the "homeostatic" role of IL-7, distinct from the thymopoietic role, enabling us to evaluate the role of p27^{Kip1} in IL-7 signaling in normal T cells. LN cells from p27^{Kip1} KO or C57BL/6 (WT) mice were labeled with CFSE and transferred into sublethally irradiated Rag$^{-/-}$ or IL-7$^{-/-}$/Rag$^{-/-}$ recipient mice. 6 d after transfer, donor cell homeostatic proliferation profiles were analyzed by staining with anti-CD4 or anti-CD8 followed by flow cytometry. The recoveries of CFSE$^+$ donor cells per mouse were as follows: $9.8 \pm 2.8 \times 10^4$

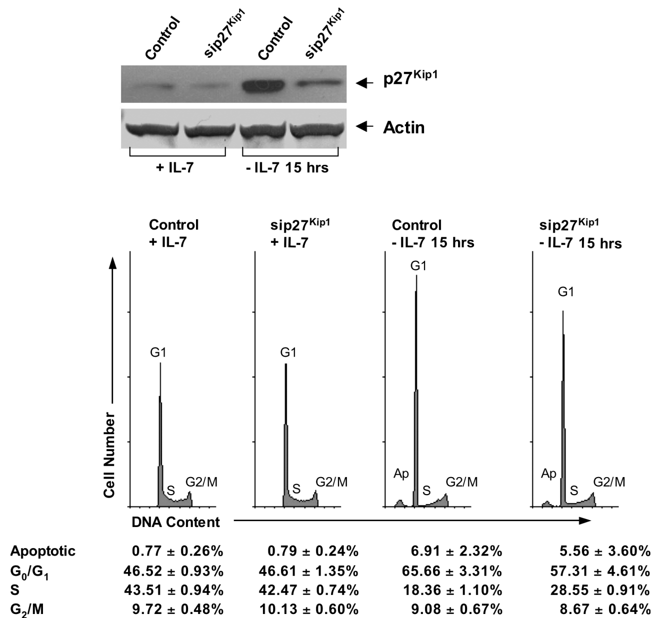

Figure 4. siRNA knockdown of p27^{Kip1} expression protects from cell cycle arrest induced by IL-7 deprivation in vitro. D1 cells transfected with either pMIG-hU6-sip27^{Kip1} (sip27^{Kip1}, small interfering p27^{Kip1}) or pMIG-hU6-NC (Control, scrambled siRNA control) were kept in IL-7 or without IL-7 for 15 h. Total cell extracts were subjected to immunoblotting with antibodies against p27^{Kip1} and β-actin (top). Cell cycles were determined by PI staining (bottom). Percentage of cells in different populations was calculated by ModFit LT software. A representative experiment of three performed is shown.

(p27^{Kip1} KO into Rag$^{-/-}$ host), 9.0 ± 1.2 × 10^4 (WT into Rag$^{-/-}$ host), 4.1 ± 1 × 10^4 (p27^{Kip1} KO into IL-7$^{-/-}$/Rag$^{-/-}$ host), and 3.1 ± 0.9 × 10^4 (WT into IL-7$^{-/-}$/Rag$^{-/-}$ host). These recoveries of CFSE$^+$ cells are in the range of 2–5% of cells transferred into IL-7–competent hosts, comparable to similar published studies.

As shown in Fig. 5, the proliferation of CD8 cells was faster (79.6% of WT and 80.4% of p27^{Kip1} KO cells underwent six divisions) compared with CD4 cells (52.2% of WT and 53.8% of p27^{Kip1} KO cells underwent three to four divisions; Fig. 5, A and B). In agreement with previous studies, homeostatic survival and proliferation of peripheral T cells was dramatically reduced in IL-7$^{-/-}$/Rag$^{-/-}$ mice (20.3% of CD4 and 38.9% of CD8 cells had divided), as compared with that in Rag$^{-/-}$ hosts (52.2% of CD4 and 79.6% of CD8 cells had divided; Fig. 5, B and D). Because cell death eventually occurs in the absence of IL-7, we chose an early time point (6 d) at which cell cycle arrest occurred in many cells before they died. About 20.3% of WT CD4 cells underwent just one division, and 38.9% of WT CD8 cells underwent two divisions in IL-7$^{-/-}$/Rag$^{-/-}$ hosts (Fig. 5 D). p27^{Kip1} deficiency enhanced both CD4 and CD8 cell proliferation in the absence of IL-7. In comparison with WT cells in IL-7$^{-/-}$/Rag$^{-/-}$ hosts, 42.9% of p27^{Kip1}-deficient CD4 cells underwent two divisions, and 53.4% of p27^{kip1}-deficient CD8 cells underwent four divisions (Fig. 5, C and D). p27^{Kip1} deficiency did not completely replace the IL-7 signal (42.9 vs. 52.2% of proliferated CD4 and 53.4 vs. 79.6% of proliferated CD8; Fig. 5, B and C) because it would not be expected to protect from apoptosis. Hence, p27^{Kip1} is a key negative cell cycle regulator controlled by IL-7 in T cells.

IL-7–induced p27^{Kip1} degradation mediated by Skp2-dependent and Skp2-independent/PKC-dependent pathways

We then investigated the molecular mechanism of IL-7–induced p27^{kip1} degradation. Phosphorylation of p27^{kip1} at threonine 187 (T187) or serine 10 (S10) has been reported to affect its stability (36, 37). We examined whether p27^{Kip1} phosphorylation is regulated by IL-7 in D1 cells. D1 cells were deprived of IL-7 for various times, and as shown in Fig. 6 A, p27^{Kip1} protein levels began to rise within 4 h and continually increased up to 12 h after IL-7 withdrawal. The increase in p27^{Kip1} correlated with its phosphorylation at T187, whereas its phosphorylation at S10 remained unchanged (Fig. 6 A).

In the course of a previous study of cell death pathways, we had made the fortuitous observation that PKC inhibitors could affect cell cycle. Several studies have shown that PKCδ or PKCα could inhibit cell proliferation concomitant with increasing p27^{Kip1} protein levels in some cell types (38–40). Therefore, we assayed the impact of PKC inhibitors on p27^{Kip1} expression in D1 cells and observed that a PKC inhibitor, Gö6850 (inhibitor of classic and novel PKCs), blocked p27^{Kip1} up-regulation and decreased phosphorylation at T187 after IL-7 withdrawal, whereas the classic PKC inhibitor Gö6976 did not (Fig. 6 B). These data suggest that one of the novel PKCs is activated after IL-7 withdrawal and induces the accumulation of p27^{Kip1}.

The phosphorylation of T187 rose in parallel to the rise in p27^{Kip1} protein, which was surprising because phospho-T187 was previously implicated in destabilizing the protein (28, 36). To examine whether T187 phosphorylation destabilized p27^{Kip1} protein in D1 cells, we generated p27^{Kip1} mutants. Replacing T187 with alanine (T187A) would prohibit phosphorylation, whereas replacing T187 with arginine (T187D) would mimic phosphorylation. FLAG-tagged p27^{Kip1} constructs (with GFP as a selective marker) were transfected into D1 cells, and 48 h later cell lysates were subjected to immunoblotting analysis with anti-FLAG and anti-GFP. The T187A mutant was higher than the WT protein level, suggesting that phosphorylation did in fact destabilize p27^{Kip1}. Confirming this hypothesis, mimicking phosphorylation in the T187D mutant reduced p27^{Kip1} levels. As a control, p27^{Kip1} S10A-FLAG was detected at the same level as the WT, and equal GFP expression reflected the same transfection efficiency (Fig. 6 C). These data suggest that phosphorylation of T187 promotes p27^{Kip1} degradation, confirming previous studies. However, after IL-7 withdrawal, another mechanism (through a novel PKC) has a dominant stabilizing effect, resulting in a rise of both phosphorylated and nonphosphorylated p27^{Kip1}.

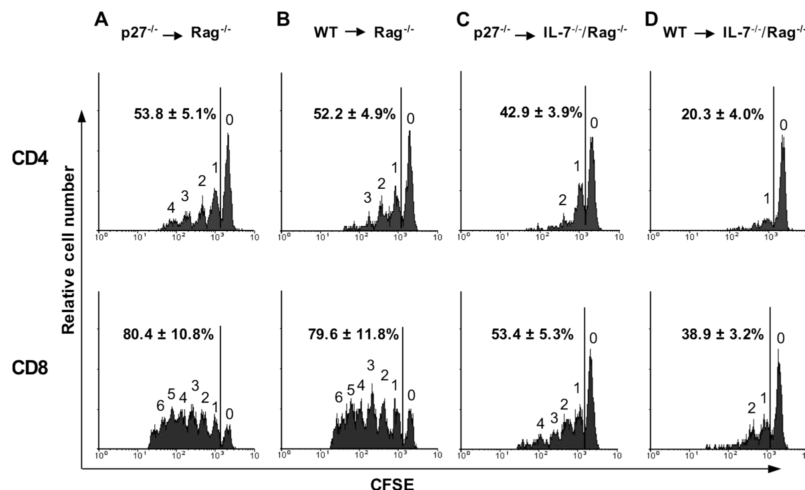

Figure 5. The loss of p27^{Kip1} compensates the loss of IL-7–promoting T cell proliferation in vivo. Lymphocytes were isolated from LNs from p27$^{-/-}$ or WT mice and labeled by CFSE, and then transferred intravenously into irradiated IL-7$^{-/-}$/Rag$^{-/-}$ or Rag$^{-/-}$ mice. 6 d later, spleens and LNs were removed from recipient mice and lymphocytes were prepared. Donor T lymphocytes were analyzed for CFSE intensity by gating on CD4^{+} and CD8^{+} cells using flow cytometry. Numbers over the peaks indicate the number of cell divisions. Vertical line represents the gate used for calculating the percentage of cells that had divided, which was calculated using WinMDI software (reference 3). Data are representative of two experiments.

T187 phosphorylation-dependent p27^{Kip1} degradation had been shown to require Skp2 and Cks1 (29, 30), and Skp2 and Cks1 expression is growth regulated and controlled by growth factors (41). Therefore, we examined the expression of Skp2 and Cks1 and observed that both Skp2 and Cks1 protein levels dropped after 12 h of IL-7 deprivation in D1 cells, suggesting that their decline could at least contribute to some of the accumulation of p27^{Kip1} at later time points. However, the PKC inhibitor Gö6850 did not prevent the decline of Skp2 and Cks1 (Fig. 6 D), although it blocked p27^{Kip1} accumulation (Fig. 6 B). Thus, the PKC pathway of p27^{Kip1} protein accumulation after IL-7 withdrawal in T cells is not through degradation of Skp2 and Cks1. Collectively, our findings indicate that two distinct mechanisms mediate

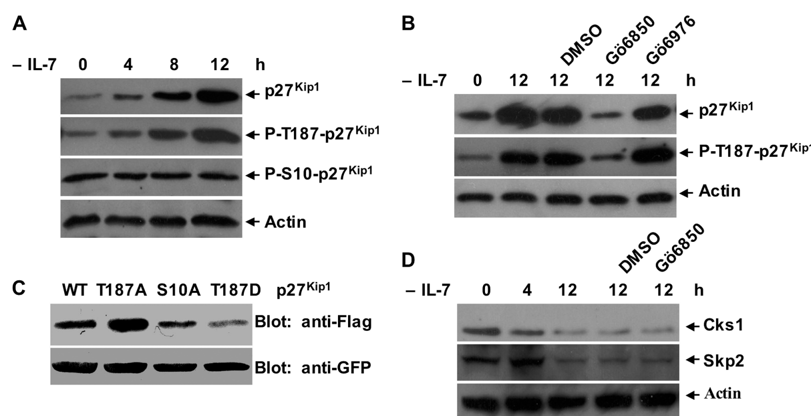

Figure 6. Inhibition of PKC after IL-7 withdrawal blocks p27^{Kip1} induction through a Skp2 and T187 phosphorylation–independent pathway. (A) Protein levels of p27^{Kip1} and phospho-p27^{Kip1} were measured in D1 cells. Cells were deprived of IL-7 for various time points and cell lysates were resolved by SDS-PAGE. Immunoblotting was performed with antibodies against p27^{Kip1}, phospho-p27^{Kip1} (T187 or S10), and β-actin. (B) The effect of PKC inhibitor on p27^{Kip1} stability was determined. IL-7 was withdrawn from D1 cells for 12 h and 5 μM of the PKC inhibitor Gö6850 was added at the time of IL-7 withdrawal. Protein levels were measured by immunoblotting using antibodies against p27^{Kip1}, phospho-p27^{Kip1} (T187), and β-actin. 0.1% of DMSO was included as a negative control. (C) p27^{Kip1} stability affected by phosphorylation was analyzed in D1 cells overexpressing the FLAG-tagged p27^{Kip1} WT, single mutant T187A, S10A, or T187D, respectively. Extrogenous protein levels were detected by immunoblotting using antibody against FLAG. The GFP expression level was also measured as transfection efficiency control. (D) Skp2 and Cks1 expressions were measured in D1 cells. Cell lysates were prepared from cells deprived of IL-7 for 4 and 12 h, or cells cultured without IL-7 for 12 h and 5 μM of the PKC inhibitor Gö6850. Immunoblotting was analyzed by specific antibodies against Skp2, Cks1, and β-actin. 0.1% of DMSO was included as a negative control.

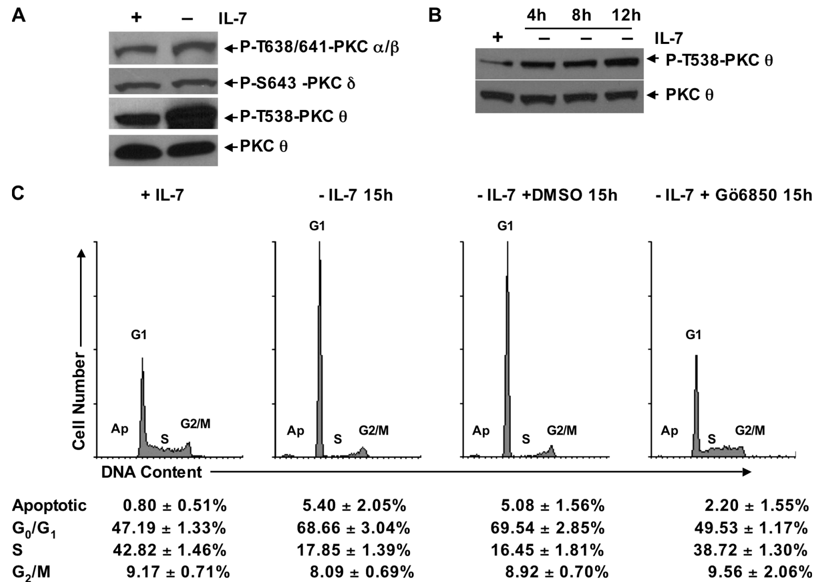

Figure 7. PKCθ is activated and inhibition of PKCθ prevents cell cycle G1 arrest after IL-7 withdrawal. (A) PKC activation was analyzed in D1 cells of IL-7 withdrawal for 12 h. Cell lysates were resolved by SDS-PAGE and immunoblotted using a phospho-PKC sample kit. Total PKCθ was measured as a loading control. (B) PKCθ activation was determined in D1 cells after IL-7 deprivation. Cell lysates were made from cells deprived of IL-7 for 4, 8, and 12 h, and PKCθ activation was assayed by immunoblotting using an antibody specific for PKCθ phosphorylation at threonine 538. (C) D1 cells were cultured without IL-7 and treated with 5 μM Gö6850 for 15 h. Cell cycle was analyzed by PI staining and the percentage of cells in different populations was calculated by ModFit LT software. Results were pooled from three experiments.

IL-7–induced p27Kip1 degradation in T cells: (a) Skp2-/T187 phosphorylation-dependent pathway and (b) a pathway blocked by a novel PKC.

IL-7 deprivation activates PKCθ, and inhibition of PKCθ prevents G1 arrest

Having observed the involvement of a novel PKC in p27Kip1 up-regulation after IL-7 withdrawal, we then assessed which PKC isozyme is activated by IL-7 withdrawal. D1 cells were cultured with or without IL-7, and as shown in Fig. 7 A, PKCα, PKCβ, PKCδ, and PKCθ were present in D1 cells, but only PKCθ activation rose after IL-7 withdrawal. PKCθ activation occurred within 4 h after IL-7 withdrawal and remained activated up to 12 h (Fig. 7 B). To determine if PKC played a key role in cell cycle arrest, D1 cells were treated with the PKC inhibitor Gö6850 after withdrawal of IL-7. By blocking PKC activation, G1 to S phase transition was restored in D1 cells after withdrawal of IL-7 for 12 h (Fig. 7 C). The classical PKC inhibitor Gö6976 had no effect (not depicted). Our findings suggest that IL-7 withdrawal activates PKCθ, which, through an unknown mechanism, stabilizes p27Kip1 protein that in turn induces G1 arrest.

DISCUSSION

T lymphocytes depend on IL-7 for survival and homeostatic proliferation. The survival function of IL-7 is largely through maintaining a favorable balance of Bcl-2 family members. Here we show that IL-7 stimulates proliferation independently of survival and evaluate the role of the CKI p27Kip1. We show that withdrawal of IL-7 induced G1 arrest and up-regulated p27Kip1. Overexpression of p27Kip1 induced G1 arrest in the presence of IL-7, and deletion of p27Kip1 partially replaced IL-7 in supporting T cell proliferation both in a cell line and in normal T cells in vivo. IL-7–induced p27Kip1 degradation involved two distinct mechanisms: the conventional Skp2-/T187 phosphorylation-dependent pathway and a Skp2-independent/PKCθ-dependent pathway that appeared to be the dominant mechanism. Fig. 8 illustrates one possible model that incorporates these hypotheses.

IL-7 induces synthesis of Bcl-2 both in pro–T cells (10, 42) and mature T cells (3, 43). Protection of T cells from death is the major function of Bcl-2, although other studies have shown an antiproliferative effect in T cells (44, 45). However, analysis of *bcl-2*–transgenic mice showed that Bcl-2 inhibited proliferation of T cell progenitors in response to signals from the pre-TCR but not the IL-7 receptor (45). We also observed that Bcl-2 prevented D1 cell death from the loss of IL-7 (Fig. 1) but did not inhibit proliferation in the presence of IL-7 (Fig. 1). Transgenic expression of Bcl- 2 in IL-7R$^{-/-}$ mice rescued T lymphopoiesis (34, 46), suggesting that survival but not proliferation is the major function of IL-7 in T cell development. However, IL-7 is also required for homeostatic proliferation of mature T cells, and a *bcl-2* transgene did not replace this IL-7 function (1). We have confirmed that a *bcl-2* transgene protected T cells from death after transfer into an IL-7$^{-/-}$ host but did not restore the capacity to proliferate (unpublished data). Thus, IL-7 receptor delivers a proliferative effect that is distinct from the survival effect involving Bcl-2 family members.

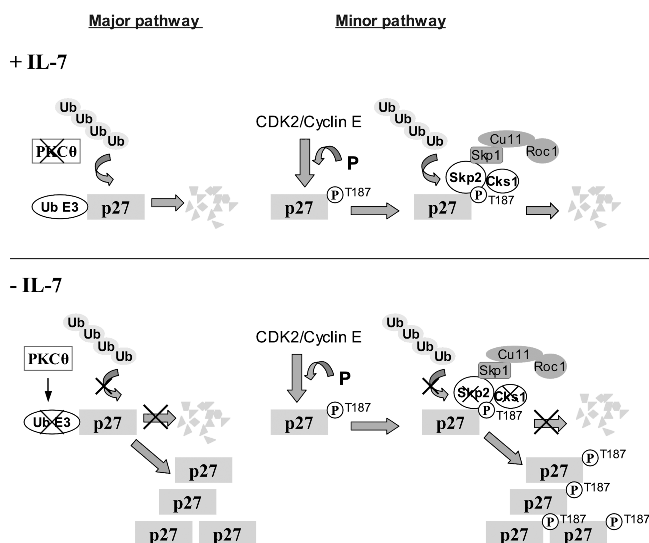

Figure 8. Model: IL-7 regulation of p27Kip1 degradation in T cells. Top: In the presence of IL-7, the major pathway to p27^{Kip1} degradation is shown on the left and involves an unknown ubiquitin E3 ligase complex, possibly KPC1 and KPC2. The minor pathway on the right is the conventional Skp1/Cks1 pathway and depends on phosphorylation of T187. Bottom: In the absence of IL-7, PKCθ is activated, inactivating the major p27^{Kip1} degradation pathway and resulting in p27^{Kip1} accumulation. The minor pathway of degradation is inactivated later and also contributes to p27^{Kip1} accumulation.

Several studies have suggested that p27^{Kip1} limits T cell proliferation because there are more cycling thymocytes and peripheral T cells in p27$^{Kip1-/-}$ mice (19–22). We examined several CKIs and found that p27^{Kip1} was down-regulated by IL-7 in D1 cells (Fig. 2 A) and primary T cells (Fig. 2 B). Regulation of p27^{Kip1} levels is primarily posttranslational in most cell types (27, 28, 47). Although there are also reports of transcriptional induction after cytokine withdrawal from hematopoietic cell lines (24, 25), we did not observe p27^{Kip1} mRNA induction by IL-7 withdrawal in D1 cells (Fig. 2 D). FoxO3 has been implicated in p27^{Kip1} gene induction by IL-2 or IL-3 withdrawal; however, we did not observe FoxO3 activation by IL-7 withdrawal (unpublished data), consistent with the observed posttranslational mechanism.

Because phosphorylation of p27^{Kip1} at T187 by CDK1 or CDK2 has been shown to induce p27^{Kip1} degradation in other cell types (29, 30, 36), it was unexpected that phosphorylation accompanied the increase in protein levels after IL-7 withdrawal (Fig. 6, A and B). We verified that phosphorylation of T187 can also destabilize p27^{Kip1} in the IL-7–dependent cell line used in our studies (Fig. 6 C). Perhaps the observed phosphorylated p27^{Kip1} is either on the way to degradation or is in an intracellular compartment lacking the machinery to degrade it, or that another stabilization mechanism protects it from degradation. As will be discussed, the major p27^{Kip1} degradation pathway regulated by IL-7 in these cells appears do be unrelated to T187 phosphorylation.

Embryonic fibroblasts from T187A p27^{Kip1} knockin mice retained some capacity to degrade p27^{Kip1} after serum starvation in embryonic fibroblasts (48). In CD4 T cells from these mice, although the ability of TCR signals to induce p27^{Kip1} breakdown was lost, high levels of IL-2 induced degradation of p27^{Kip1}, suggesting the existence of a T187-independent pathway. Thus, IL-7 could induce a T187 phosphorylation-independent pathway, eliminating p27^{Kip1} in cycling T cells.

Phosphorylation of p27^{Kip1} at T187 has been shown to be required for Skp2-mediated p27^{Kip1} proteolysis (29), which also requires Cks1 association with Skp2 (30, 31). However, analysis of p27^{Kip1} ubiquitination in lymphocytes from Skp2$^{-/-}$ revealed a second pathway that was independent of Skp2 and T187 phosphorylation (49). Both pathways may be operating in the IL-7 response. IL-7 withdrawal eventually decreased both Skp2 and Cks1 expression (Fig. 6 D), and T187 phosphorylation somewhat destabilized p27^{Kip1} in D1 cells (Fig. 6 C). However, a PKC inhibitor prevented p27^{Kip1} protein accumulation despite the decline in Skp2 and Cks1 after IL-7 withdrawal (Fig. 6, B and D), suggesting an independent pathway. The latter appears the more dominant pathway because the PKC inhibitor was sufficient to relieve cell cycle arrest in the absence of IL-7 (Fig. 7).

Among the PKC isoforms, we observed that PKCθ was activated after IL-7 withdrawal and inhibition of PKCθ activity prevented cell cycle arrest (Fig. 7). PKCθ is predominantly expressed in T cells and promotes T cell proliferation induced by TCR and CD28 engagement (50). Our findings in the IL-7 pathway suggest that PKCθ can also block T cell proliferation by stabilizing p27^{Kip1} protein via a Skp2-independent mechanism. Because p27^{Kip1} lacks a consensus PKCθ target site, we hypothesize a novel intermediate that stabilizes p27^{Kip1} (Fig. 8).

In the IL-7 pathway, one candidate for conjugating ubiquitin to p27^{Kip1} is a newly identified ubiquitin-conjugating complex, Kip1 ubiquitination-promoting complex (KPC)1 and KPC2 (51, 52). This complex has been shown to induce breakdown of p27^{Kip1} during G1 phase in embryonic fibroblasts. The mechanism for regulating KPC during cell cycle is different from Skp2/Cks1, which is ubiquitinated and degraded in S-G2 phase. KPC is located in the cytosol where its levels remain constant throughout the cell cycle. During G1 phase, p27^{Kip1} is exported from the nucleus and targeted for degradation by KPC in the cytosol. IL-7 stimulation could therefore induce nuclear export of p27^{Kip1}- and KPC-targeted degradation if it uses the mechanism of serum stimulation of embryonic fibroblasts. Alternatively, IL-7 stimulation could block nuclear import of human p27^{Kip1}, which can be phosphorylated in its nuclear localization sequence by AKT (53–55); however, murine p27^{Kip1} lacks this site.

Our evidence suggests that Skp2/Cks1 is not the major pathway from IL-7 receptor to p27^{Kip1} degradation. This is relevant to a recent report that Skp2/Cks1 induces degradation not only of p27^{Kip1}, but also of Rag2 (56), which is required for VDJ recombination in developing thymocytes. If IL-7 were to induce Skp2/Cks1, then Rag2 would degrade and interrupt

VDJ recombination, whereas the opposite is actually observed: IL-7 is required for VDJ recombination of the TCR-γ locus and facilitates rearrangement of other loci (57).

We recently reported that another proliferative pathway is regulated by IL-7 (58). The phosphatase Cdc25a removes an inhibitory phosphate from the active site of CDK2, and we observed that after IL-7 withdrawal, Cdc25a degrades downstream of a stress response. Thus, IL-7 stimulates CDK2 by two mechanisms: one by removing an inhibitory phosphate, and second, as reported here, by degrading an inhibitor, $p27^{Kip1}$. We have shown that both of these mechanisms are functionally important in normal T cells in that proliferation is induced, in the absence of IL-7, by introducing either a Cdc25a mutant that does not degrade or, as shown here, by eliminating $p27^{Kip1}$. These studies show that cell cycle regulation in lymphocytes can involve mechanisms that differ considerably from those in cell types previously studied, such as fibroblasts, and suggest that other proliferative stimuli in lymphocytes, in addition to IL-7, should be examined.

MATERIALS AND METHODS

Mice. $p27^{Kip1}$-deficient ($p27^{-/-}$) mice (21), originally on a background mix of 129 and C57BL/6, were backcrossed onto C57BL/6 and maintained by heterozygous breeding. $IL\text{-}7^{-/-}/Rag^{-/-}$ mice were obtained from R. Murray (EOS Biotechnology, San Francisco, CA). $Rag^{-/-}$ mice were purchased from The Jackson Laboratory. Both $IL\text{-}7^{-/-}/Rag^{-/-}$ and $Rag^{-/-}$ mice are maintained by homozygous breeding at the National Cancer Institute (NCI), Frederick Cancer Research and Development Center animal facility. NCI-Frederick Animal Care and Use Committee approved all experiments on mice.

Cell lines. The IL-7–dependent thymocyte cell line D1 was generated from p53 KO mice (35). D1 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine, 1% penicillin-streptomycin, 50 μM β-mercaptoethanol (Invitrogen), and 50 μg/ml murine recombinant IL-7 (PeproTech). Transfection used the retrovirus packaging cell line phoenix-Eco, maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin. The PKC inhibitors Gö6850 and Gö6976 were purchased from CalBiochem.

DNA constructs and retroviral infection. The retroviral vector pMIG containing EGFP as marker and human *bcl-2* retroviral expression vector was described previously (13). Mouse $p27^{Kip1}$ cDNA construct pRC-$p27^{Kip1}$ was provided by P. Coffer (University Medical Center, Utrecht, Netherlands; reference 24). The full-length $p27^{Kip1}$ WT, mutant $p27^{kip1}$ T187A, T187D, and S10A cDNAs with FLAG epitope tags in the COOH terminus were amplified by PCR from pRC-$p27^{Kip1}$ and cloned into the retroviral vector pMIG.

Individual retroviral constructs were transfected into the phoenix-Eco package cell line using Fugene-6 reagent (13). The retrovirus-containing supernatants were harvested after 48 h and loaded onto a RetroNectin (TaKaRa)-coated plate, and then D1 cells were added and infected overnight. GFP^+ cells were sorted and analyzed.

Antibodies and immunoblotting. Rabbit anti–$p27^{Kip1}$, phospho-PKC antibody sampler kit, and goat anti–mouse IgG and goat anti–rabbit IgG coupled to horseradish peroxidase were purchased from Cell Signaling Biotechnology. Rabbit anti–phospho-T187–$p27^{Kip1}$ was from CalBiochem. Mouse anti–PKCθ (E-7) and rabbit anti–p16 were obtained from Santa Cruz Biotechnology, Inc. Rabbit anti–phospho-S10–$p27^{Kip1}$, mouse anti–Skp2, and rabbit anti–Cks1 were from Zymed Laboratories. Mouse anti–FLAG and mouse anti–GFP were from Stratagene. Mouse anti–p21 was purchased from BD Biosciences. 5×10^6 cells were lysed in Triton X-100 lysis buffer supplemented with protease inhibitor cocktails (Roche). 50 μg of protein lysates was resolved by SDS-PAGE on 12% Tris-Glycine gels (Invitrogen) and transferred to nitrocellulose membranes. Blots were probed with specific primary antibodies, followed by the appropriate secondary antibodies conjugated to horseradish peroxidase and then visualized by chemiluminescence. The chemiluminescent Western detection kit was purchased from Roche.

Retrovirus-mediated siRNA. The DNA nucleotides encoding mouse $p27^{Kip1}$ siRNA (GenBank accession no. U09968, nucleotide 175–195) were ligated into pSilence 2.1-U6 hygro (Ambion) under the expression of the human U6 promoter according to the manufacturer's protocol. The DNA fragment containing human U6/$p27^{Kip1}$ siRNA was amplified by PCR and ligated into the SalI site of the retroviral vector pMIG to generate pMIG-hU6-sip27^{Kip1}. D1 cells were infected twice with pMIG-hU6-sip27^{Kip1} retrovirus and GFP^+ cells were sorted after 24 h of infection. Western blotting and PI staining were performed to analyze $p27^{Kip1}$ protein expression and cell cycle.

Cell cycle analysis. Cell cycle was determined by PI staining as described previously (35). In brief, cells were placed in detergent buffer containing 50 μg/ml RNase A (Roche) at a concentration of 1–2 × 10^6 cells/ml, and then mixed with an equal volume of PI (50 μg/ml; Sigma-Aldrich) and incubated at room temperature in the dark for 1 h. DNA contents were assayed by flow cytometry. Data were analyzed using ModFit LT software.

CFSE labeling and adoptive transfer of T cells. LNs from $p27^{Kip1}$ KO or WT mice were homogenized in RPMI 1640 containing 5% FBS and filtered through a 100-μm mesh nylon screen (BD Falcon). Red blood cells were removed with treatment by ACK lysing buffer (BioSource). LN cells were resuspended in PBS containing 5% FBS and warmed to 37°C, and then incubated for 10 min with 5 μM CFSE (Invitrogen) followed by two washes with PBS. 2–5 × 10^6 CFSE-labeled cells were suspended in PBS and adoptively transferred into $IL\text{-}7^{-/-}/Rag^{-/-}$ or $Rag^{-/-}$ recipient mice by intravenous injection. The recipient mice received whole body γ-irradiation (600 Rd) at least 3 h before the injection. 6 d later, the host mice were killed and LN and spleen cells were stained with PE-Cy5–conjugated anti-CD4 (BD Biosciences) or PE-Cy5–conjugated anti-CD8 (BD Biosciences). The intensity of CFSE on donor cells was analyzed by gating on either $CD4^+$ or $CD8^+$ on a FACScan flow cytometer.

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