Nuclear Pro-IL-16 Regulation of T Cell Proliferation: p27 KIP1-Dependent G0/G1 Arrest Mediated by Inhibition of Skp2 Transcription

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Nuclear Pro-IL-16 Regulation of T Cell Proliferation: 
p27KIP1-Dependent G<sub>0</sub>/G<sub>1</sub> Arrest Mediated by Inhibition of Skp2 Transcription<sup>1</sup>

David M. Center, William W. Cruikshank, and Yujun Zhang<sup>2</sup>

The precursor for IL-16 (pro-IL-16) is a nuclear and cytoplasmic PDZ domain-containing protein. In this study we have found that pro-IL-16 is absent or mutated in four T lymphoblastic leukemia cell lines examined. Ectopic expression of pro-IL-16 in pro-IL-16-negative Jurkat cells blocks cell cycle progression from G<sub>0</sub>/G<sub>1</sub> to S phase associated with elevated levels of the cyclin-dependent kinase inhibitor p27<sup>KIP1</sup>. Pro-IL-16 decreases p27<sup>KIP1</sup> degradation by reducing transcription and subsequent expression of Skp2, a key component of the SCFSkp2 ubiquitin E3 ligase complex. Taken together, these findings identify pro-IL-16 as a novel regulator of Skp2 expression and p27<sup>KIP1</sup> levels and implicate a role for pro-IL-16 in T cell proliferation. The Journal of Immunology, 2004, 172: 1654–1660.

In T cells, IL-16 is synthesized as an 80-kDa precursor, pro-IL-16 (1). After processing by caspase 3, the 121-aa C terminus is cleaved and secreted (2) as the biologically active immunomodulatory cytokine that binds to CD4 (3). Pro-IL-16 is present in abundance in resting T cells in both the nucleus and the cytoplasm (4). After T cell activation, one of the most highly inhibited mRNA species is pro-IL-16 (5, 6). In addition, pro-IL-16 protein disappears after T cell activation and is undetectable while cells are actively dividing. The mechanism of loss or degradation of pro-IL-16 under these circumstances is not known. A number of observations have suggested that pro-IL-16 itself may have unique cellular functions distinct from its role as a precursor for the secreted cytokine. There is high interspecies homology among all pro-IL-16 proteins, and each contains multiple motifs associated with intermolecular interactions (i.e., three domains found in proteins PSD-95/DlgA/ZO-1 (PDZ domains) and one potential SH3 domain binding site) and nuclear transport (i.e., a functional dual phosphorylation-regulated Ccn nuclear localization motif) (7).

The present studies addressed a possible role for pro-IL-16 in the regulation of T cell proliferation for several reasons. Proteins that use Ccn motifs to regulate nuclear localization appear to be associated with cell cycle regulation. Transient nuclear expression of pro-IL-16 in COS cells results in arrest at G<sub>0</sub>/G<sub>1</sub>. Last, there is precedent for cytokine precursors performing nuclear functions associated with cell growth in that the precursor for IL-1<sub>6</sub> translocates to the nucleus where, in MC cells, it induces transformation resulting in tumor-forming cells (8).

Materials and Methods

Cell lines and Abs

All T cell leukemia cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM l-glutamine, 25 mM HEPEs, and 100 U/ml penicillin/streptomycin. Nylon wool-nonadherent human T cells were prepared by a modification of the method described by Julius et al. (9). mAb (14.1) to IL-16 has been described previously (10). Abs specific for p27<sup>KIP1</sup>, p21<sup>CIP1</sup>/p21<sup>WAF1</sup>, cyclin E, Skp2, Skp1, and Cul1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-tubulin Ab was obtained from Sigma-Aldrich (St. Louis, MO).

Creation of green fluorescent protein (GFP)pro-IL-16, Δ nuclear localization sequence (NLS) GFPpro-IL-16, and GFP-expressing cell lines

Tet-Off Jurkat cells (Clontech Laboratories, Palo Alto, CA) were transfected with the pRevTRE expression vector containing either the cDNA for GFP linked to the cDNA for full length pro-IL-16 (GFPpro-IL-16), a GFP-linked pro-IL-16 cDNA in which the nuclear localization sequence of pro-IL-16 has been mutated (ΔNLS GFPpro-IL-16), or a GFP cDNA linked to an exogenous nuclear localization sequence by electrotransformation. Selection of stable cell lines was initiated 48 h after transfection using 100 μg/ml neomycin, 200 μg/ml hygromycin, and 2 μg/ml of the tetracycline analog, doxycycline (DOX), in RPMI 1640 complete medium changed every 4 days. After 2 wk, living cells were separated from dead cells and cultured in 25-cm<sup>2</sup> T flasks.

Western blot analysis

Stably transfected Jurkat cells were harvested by centrifugation, washed twice with cold PBS, and lysed in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.5 mM EDTA, 0.5% Nonidet P-40, plus a mixture of protease
inhibitors (protein lysis buffer). Protein concentrations were determined by the Bradford assay, and equal amounts of protein were used for each experiment. Proteins were subjected to electrophoresis through a 12% SDS-PAGE gel, electrophoretically transferred to nitrocellulose membrane, and probed with various Abs as indicated in each experiment. The secondary Abs labeled with HRP (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1/5000. Protein bands were visualized by ECL (Pierce, Rockford, IL). Densitometry was used for quantifying Western blots with National Institutes of Health Image 1.63 software.

Cell fractionation
Stably transfected Jurkat cells were harvested by centrifugation and washed twice with cold PBS before incubation with buffer I (see below) on ice for 15 min. Twenty microfilters of 10% Nonidet P-40 was added to the 400-μl cell suspension and hand-mixed for 10 s, then centrifuged at 2000 rpm for 5 min at 4°C using a desk-top microcentrifuge to pellet the nuclei. The supernatants were further fractionated by centrifugation at 14,000 rpm for 20 min at 4°C. The supernatant fraction of this centrifugation was collected and classified as the cytoplasmic fraction. The nuclear pellet was washed twice with ice-cold PBS before adding 50 μl of buffer II (see below) to lyse the nuclei. After 15 min on ice, nuclear lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and this supernatant fraction was classified as nuclear. The protein concentration in each fraction was determined using Bio-Rad bovine γ-globulin as standard protein and Bio-Rad protein assay reagent (buffer I: 20 mM Tris-HCl, 0.5 mM DTT, 10 mM β-glycerophosphate, 0.2 mM EGTA, 5 mM MgCl₂, and 25% glycerol; buffer II: 10 mM Tris-HCl, 0.5 mM DTT, 10 mM β-glycerophosphate, 0.2 mM EGTA, 5 mM MgCl₂, 350 mM KCl, and 25% glycerol; both buffers contain protease inhibitors: aprotinin, chymostatin, antipain, and pepstatin at 10 μg/ml, and PMSF at 1 mM).

Cell cycle analysis
Five million stably transfected Jurkat cells were washed with PBS buffer and fixed in 35% ethanol at 4°C for 4 h. After fixation, cells were washed twice with PBS before resuspension in propidium iodide/RNase A solution (50 μg/ml propidium iodide and 100 μg/ml RNase A). Cells were incubated with propidium iodide at room temperature in the dark for 1 h. Stained cells were analyzed by flow cytometry for light-scattering properties and for DNA content with a FACScan flow cytometer (BD Biosciences, Mountain View, CA). The percentages of the cells in different stages of the cell cycle were calculated by CellQuest (BD Biosciences).

Northern analysis of p27KIP1, Skp2, and Skp1 in Tet-off Jurkat cells
Jurkat clones expressing pro-IL-16, ΔNLS pro-IL-16, and GFP were incubated with or without doxycycline for 48 h. Total RNA was isolated by the RNeasy Mini Kit (Qiagen, Valencia, CA) and separated by formaldehyde gel electrophoresis. RNA was blotted onto Hybond N membranes and prehybridized for 45 min at 68°C, then hybridized for 1 h using Quikhyb (Stratagene, La Jolla, CA). The hybridization probes were generated by random labeling with Prime-a-Gene Labeling System (Promega, Madison, WI) using [α-32P]dCTP and p27KIP1, or Skp2, or Skp1 cDNA template.

Nuclear run-on
Stable Jurkat cells were induced to express wild-type pro-IL-16 (W), ΔNLSpro-IL-16 (ΔNLS), or GFP alone for 48 h, then nuclei were isolated using the Nuclei EZ Prep (Sigma-Aldrich) nuclei isolation kit. Nuclear suspensions (200 μl) each were incubated with 0.5 mM each of CTP, ATP, and GTP (100 μl of 0.5 M Na-polyribonucleotides, Boehringer Mannheim, Indianapolis, IN), and 250 μCi of [γ-32P]UTP (3000 Ci/mmol, DuPont-NEN, Boston, MA) in 100 μl of buffer I (50 mM Tris-Cl, pH 7.6, 50 mM KCl, 10 mM MgCl₂). Total newly transcribed RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA) and separated by formaldehyde gel electrophoresis. RNA was blotted onto Hybond N membranes and prehybridized for 45 min at 68°C, then hybridized at 68°C for 2 h using Quikhyb (Stratagene, La Jolla, CA). The hybridization probes were generated by random labeling with Prime-a-Gene Labeling System (Promega, Madison, WI) using [α-32P]dCTP and p27KIP1, or Skp2, or Skp1 cDNA template.

Results

Analysis of pro-IL-16 expression in human T cell leukemia cell lines
Pro-IL-16 is one of the most highly down-regulated mRNA species after TCR activation (5, 6). It is highly expressed in resting T cells and is virtually undetectable during active T cell proliferation. Without constitutive mRNA present, pro-IL-16 protein disappears after degradation before the cells enter S phase, and nuclear protein remains undetectable during active T cell proliferation. Because of these observations in T cells and the fact that nuclear expression of pro-IL-16 in COS cells results in cell cycle arrest at G₂/M (4), we searched for T cell lines suitable for determining a possible causal relationship between pro-IL-16 expression and T cell proliferation.

We examined pro-IL-16 expression in four actively proliferating human T cell lymphoblastic leukemia cell lines (Fig. 1) obtained from American Type Culture Collection. Three of the lines, Jurkat, HUT78, and SUP-T1, had no detectable protein (Fig. 1A) by Western blot analysis or mRNA expression by RT-PCR (Fig. 1B). Because activated and dividing T cells also fail to express pro-IL-16 protein and mRNA, we performed Southern blot analysis to determine whether these observations were merely a consequence of active cell proliferation (Fig. 1C). By Southern blot analysis all three cell lines showed homogenous deletion of the pro-IL-16 gene. Thus, in these three cell lines, pro-IL-16 is absent due to activity of T cells and the fact that nuclear expression of pro-IL-16 remains undetectable during active T cell proliferation. Because of these observations in T cells and the fact that nuclear expression of pro-IL-16 in COS cells results in cell cycle arrest at G₂/M (4), we searched for T cell lines suitable for determining a possible causal relationship between pro-IL-16 expression and T cell proliferation.

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FIGURE 1. Analysis of pro-IL-16 expression in human T cell leukemia cell lines. A, Western blot analysis demonstrates that the expression of pro-IL-16 is undetectable in Jurkat, SUP-T1, and HUT78 cells and is similar in H9 cells compared with the expression of pro-IL-16 in normal human peripheral blood T cells. All the lanes were loaded with 80 μg of total cellular protein. B, Semiquantitative RT-PCR analysis using primers that identify the coding region of pro-IL-16 mRNA shows that pro-IL-16 cDNA is absent in Jurkat, SUP-T1, and HUT78 cells (upper panel). The bottom panel shows equal cDNA input for each RT-PCR reaction. C, Southern blot analysis using a probe hybridizing to the region containing all three pro-IL-16 PDZ domains shows the absence of the pro-IL-16 gene in Jurkat, SUP-T1, and HUT78 cells.
deletion of the gene, not as a consequence of active T cell proliferation. The circumstance in H9 cells was different. H9 cells express levels of pro-IL-16 comparable to those in normal T cells (Fig. 1A). However, subcellular fractionation reveals that it fails to localize to the nucleus (Fig. 2A). We sequenced the pro-IL-16 cDNA from H9 cells and found mutations in the nuclear localization motif (Fig. 2B) that presumably prevent translocation into the nucleus. The high frequency of chromosomal deletions and mutations of pro-IL-16 in T cell malignancies is consistent with its location on the long arm of chromosome 15, 15q26.3 (11). The long arm of human chromosome 15 is characterized by frequent translocations and deletions associated with malignancies (12, 13), including T cell malignancies (14–17).

The absence of pro-IL-16 in Jurkat, HUT 78, and SUP-T1 cells and the lack of nuclear localization in H9 cells suggested to us that the absence of nuclear pro-IL-16 might play a role in the uncontrolled cell growth of these cells. This hypothesis is consistent with the structure of pro-IL-16, as the relationship between PDZ domain-containing proteins and growth suppression has been established since the description of the motif (18). This contrasts the putative function of nuclear pro-IL-16, which has transforming properties (8).

Expression of pro-IL-16 in pro-IL-16-negative Jurkat cells leads to G\textsubscript{0}/G\textsubscript{1} cell cycle arrest

The identification of pro-IL-16-negative cell lines provided us with the necessary cellular reagent to determine whether there is a causal relationship between nuclear pro-IL-16 expression and suppression of the cell cycle. Without endogenous pro-IL-16 to confound our results, we transfected pro-IL-16-negative Jurkat cells with a vector containing pro-IL-16 cDNA. Repeated attempts produced few positive transfectants in the selection medium for as long as 6 wk after transfection. At the same time Jurkat cells transfected with control vectors stabilized in 2–3 wk and continued to grow at the pace of the parental cells (data not shown). These observations suggested that pro-IL-16 expression resulted in growth suppression in Jurkat cells as pro-IL-16 expressing cells failed to grow, whereas untransfected cells grew normally. To circumvent the negative effect on cell proliferation in pro-IL-16-transfected cells we cloned wild-type pro-IL-16 into pRevTRE vector (Clontech Laboratories), which contains a tetracycline-inducible promoter and carries hygromycin selection marker. To examine the importance of nuclear localization as implied by the natural mutation in H9 cells, a pro-IL-16 cDNA with mutations in the NLS was cloned into pRevTRE vector. To facilitate FACS cell cycle analysis we fused GFP to the N terminus of pro-IL-16. To control for the possibility that the GFP moiety fused with pro-IL-16 has an

FIGURE 2. Subcellular localization of pro-IL-16 in H9 cells. A, Western blot analysis of inducible pro-IL-16 expression. Forty-eight hours after removal of DOX from the culture medium, Jurkat cells induced to express pro-IL-16 (lane 2), ΔNLS pro-IL-16 (lane 4), or GFP (lane 6) were harvested, and lysates of the cells were prepared. Equal amounts of total protein were separated by 12% SDS-PAGE, followed by Western blot analysis using an anti-IL-16 mAb and Abs for GFP (Santa Cruz Biotechnology) and tubulin (Sigma-Aldrich). B, Cell fractionation. Nuclear and cytoplasmic fractions of Jurkat cells 48 h after removal of DOX demonstrate that wild-type pro-IL-16 and the NLS-directed GFP are present in both cytoplasm and nucleus, whereas the pro-IL-16 with a mutated NLS fails to localize to the nucleus. C, Confocal images of DOX-inducible Jurkat cells. Forty-eight hours after removal of DOX, live Jurkat cells were examined by confocal microscopy. Green fluorescent images from each stable cell line, W (a), ΔNLS (c), and GFP (e), were taken, followed by brightfield differential interference contrast images W (b), ΔNLS (d), and GFP (f). Note that the green fluorescence of wild-type GFP-pro-IL-16 and nuclear localized GFP is found both in the cytoplasm and nucleus (a and c), whereas there is only cytoplasmic localization of ΔNLS GFP-pro-IL-16 protein in e.

FIGURE 3. Expression of pro-IL-16 in pro-IL-16-negative Jurkat cells. A, Western blot analysis of inducible pro-IL-16 expression. Forty-eight hours after removal of DOX from the culture medium, Jurkat cells induced to express pro-IL-16 (lane 2), ΔNLS pro-IL-16 (lane 4), or GFP (lane 6) were harvested, and lysates of the cells were prepared. Equal amounts of total protein were separated by 12% SDS-PAGE, followed by Western blot analysis using an anti-IL-16 mAb and Abs for GFP (Santa Cruz Biotechnology) and tubulin (Sigma-Aldrich). B, Cell fractionation. Nuclear and cytoplasmic fractions of Jurkat cells 48 h after removal of DOX demonstrate that wild-type pro-IL-16 and the NLS-directed GFP are present in both cytoplasm and nucleus, whereas the pro-IL-16 with a mutated NLS fails to localize to the nucleus. C, Confocal images of DOX-inducible Jurkat cells. Forty-eight hours after removal of DOX, live Jurkat cells were examined by confocal microscopy. Green fluorescent images from each stable cell line, W (a), ΔNLS (c), and GFP (e), were taken, followed by brightfield differential interference contrast images W (b), ΔNLS (d), and GFP (f). Note that the green fluorescence of wild-type GFP-pro-IL-16 and nuclear localized GFP is found both in the cytoplasm and nucleus (a and c), whereas there is only cytoplasmic localization of ΔNLS GFP-pro-IL-16 protein in e.
effect on cell growth and to control for ectopic expression of nuclear proteins in general, we constructed a vector in which a nuclear localization sequence has been attached to the N terminus of GFP.

Using these expression constructs, we generated stable Jurkat cell lines expressing wild-type pro-IL-16, ΔNLSpro-IL-16, or GFP alone, upon removal of the tetracycline analog, doxycycline from the culture medium. To examine the expression levels of the corresponding proteins, we performed Western blot analysis with both anti-IL-16 Ab and anti-GFP Ab (Fig. 3A). Nuclear fractionation followed by Western blot analysis, shown in Fig. 3B, demonstrated the nuclear and cytoplasmic localization of both wild-type pro-

IL-16 and GFP with an NLS, whereas the protein encoded by the GFPΔNLSpro-IL-16 cDNA remained almost entirely in the cytoplasm. Furthermore, in all subsequent experiments we selected Jurkat cells in which amount of nuclear and cytoplasmic pro-IL-16 expression after 48 h of induction closely approximated the amount of nuclear and cytoplasmic pro-IL-16 observed in normal T cells (Fig. 1).

Having confirmed the integrity of our stably transfected cell lines, we examined the effect of pro-IL-16 expression on cell cycle progression. Forty-eight hours after removal of DOX from the culture medium, Jurkat cells expressing wild-type pro-IL-16, ΔNLSpro-IL-16, or GFP alone were harvested, stained with propidium iodide, and examined for cell cycle profile using FACS analysis. As shown in Fig. 4, expression of wild-type pro-IL-16 for 48 h led to an increase of greater than 60% in the G0/G1 phase cell population and a substantial reduction in the S phase population compared with that of the GFP control. The cell cycle arrest caused by pro-IL-16 expression also depended on its nuclear localization, as there was almost no effect on the cell cycle profile when pro-IL-16 was expressed only in the cytoplasm (Fig. 4). The minimal effect is probably the result of the expression of a small amount of translocation of pro-IL-16 into the nucleus.

Pro-IL-16 regulates the level of p27KIP1 by inhibition of Skp2 transcription

To investigate whether the observed G0/G1 cell cycle arrest by pro-IL-16 was associated with alterations in the levels of cyclin-dependent kinase (CDK) inhibitors we examined the levels of the CIP/KIP family CDK inhibitors p21CIP1/WAF1 and p27KIP1, which are important in governing cell cycle transition from G0/G1 into S phase, and cyclin E in Jurkat cells expressing wild-type pro-IL-16, its mutant derivative ΔNLSpro-IL-16, or GFP alone. As shown in

FIGURE 4. FACS analysis of cell cycle distribution. Forty-eight hours after removal of DOX from the culture medium, Jurkat cells were harvested, fixed in 35% ethanol for 4 h, then stained with propidium iodide (50 and 100 μg/ml RNase A) for 30 min. Cell cycle analysis was performed on FACS-gated, green fluorescent-positive cells. DNA content was measured by propidium iodide staining. The percentage of cells in G0/G1 phase (first peak), S phase (the plateau between two peaks), and G2/M phase (second peak) are indicated. The depicted experiments are representative of four similar independent experiments.
Fig. 5A, the expression of wild-type pro-IL-16 led to significant elevations of p27\textsuperscript{KIP1} levels, as revealed by Western blot analysis with anti-p27\textsuperscript{KIP1} Ab and densitometry quantitation analysis (Fig. 5B). The accumulation of p27\textsuperscript{KIP1} appeared dependent on the nuclear localization of pro-IL-16, because the expression of pro-IL-16 retained in the cytoplasm resulted in an insignificant increase in p27\textsuperscript{KIP1} levels (Fig. 5A). In parallel, we examined the levels of p21\textsuperscript{CIP1/WAF1} or cyclin E by Western blot analysis. There were no changes in p21\textsuperscript{CIP1/WAF1} or cyclin E protein levels in cells expressing pro-IL-16 under any condition (Fig. 5A).

Accumulation of p27\textsuperscript{KIP1} can be regulated either by protein degradation or mRNA expression. First, we investigated whether the observed increase in p27\textsuperscript{KIP1} protein levels by pro-IL-16 is due to enhanced transcription of the p27\textsuperscript{KIP1} gene. As shown in Fig. 6, Northern blot analysis indicated that pro-IL-16 did not appear to regulate p27\textsuperscript{KIP1} mRNA levels, suggesting that pro-IL-16 mediates accumulation of p27\textsuperscript{KIP1} at a post-transcriptional level. Characterization of p27\textsuperscript{KIP1} levels in the presence of cycloheximide revealed that p27\textsuperscript{KIP1} protein stability is increased in the cells expressing pro-IL-16 (Fig. 7). Using this assay, we determined a p27\textsuperscript{KIP1} half-life of 1 h in the control Jurkat cells, which was increased to >6 h in pro-IL-16-expressing cells (Fig. 7). The data suggest that p27\textsuperscript{KIP1} up-regulation in pro-IL-16-expressing cells is mediated by changes in protein stability.

The stability of p27\textsuperscript{KIP1} protein is determined by the SCF\textsuperscript{Skp2} ubiquitin E3 ligase complex. The complex member Skp2 is required for the ubiquitination and subsequent degradation of p27\textsuperscript{KIP1} both in vivo (19, 20) and in vitro (19, 21). In that regard, Skp2 expression is required for G\textsubscript{1}-S transition in both transformed cells and diploid fibroblasts (22). As induction of pro-IL-16 leads to decreased degradation of p27\textsuperscript{KIP1}, we examined the protein levels of the essential members of the SCF\textsuperscript{Skp2} complex, Skp1, Skp2, and Cul1. As shown in Fig. 8, Skp2 protein was markedly decreased in Jurkat cells expressing wild-type pro-IL-16, but not in cells expressing ΔNLSpro-IL-16 or nuclear GFP. In contrast, the levels of Skp1 and Cul1 remained unaffected by nuclear pro-IL-16 expression. Northern blot analysis further indicated that the mRNA levels of Skp2 were greatly reduced in nuclear pro-IL-16-expressing Jurkat cells (Fig. 9, A and B), and that pro-IL-16-induced Skp2 down-regulation at the protein level is reflected by quantitatively similar changes (~3 fold less) in steady state RNA levels (Figs. 8B and 9B). To examine whether the reduced mRNA level of Skp2 was due to reduced transcription, we performed a nuclear run-on experiment (Fig. 9, C and D). We found that the transcription rate of the Skp2 gene is reduced ~90% after the expression of pro-IL-16, indicating that the transcription of Skp2 is repressed in pro-IL-16-expressing Jurkat cells. These data suggest that pro-IL-16-mediated accumulation of p27\textsuperscript{KIP1} is probably caused by the specific inhibition of Skp2 transcription, which results in decreased Skp2 protein expression and subsequent decreased ubiquitination and degradation of p27\textsuperscript{KIP1}.

We also examined the level of the INK family CDK inhibitor, p19\textsuperscript{INK4d}, because p19\textsuperscript{INK4d} has also been demonstrated to change during the cell cycle in an ubiquitin/proteasome-dependent mechanism (23). P19\textsuperscript{INK4d} is highly expressed in these Jurkat cells, and its cellular expression level was not affected by the expression of pro-IL-16 (data not shown).

Overexpression of Skp2 in Jurkat cells expressing pro-IL-16 overcomes pro-IL-16-induced cell cycle arrest through down-regulation of the p27\textsuperscript{KIP1} protein level

To confirm that pro-IL-16-induced transcriptional inhibition of Skp2 is responsible for growth suppression of Jurkat cells, we constructed the retroviral vector pMSCVneo-Skp2 containing the full-length human Skp2 cDNA and transferred it into Jurkat cells.
transcribed RNA from each Jurkat cell line were hybridized to 1/H9262 fi immobilized on nitrocellulose.

increase in the S phase cell population compared with that of Jurkat cells expressing wild-type pro-IL-16. Successful transfer of the Skp2 gene in neomycin-resistant cells and an elevated level of Skp2 protein expression were con

gene in neomycin-resistant cells and an elevated level of Skp2 expressing wild-type pro-IL-16. Successful transfer of the Skp2 gene in neomycin-resistant cells and an elevated level of Skp2 protein expression were confirmed by Western blot analysis with anti-human Skp2 Ab (Fig. 10B, lane 3 of panel 2). The pMSCV-neo-Skp2-transduced, pro-IL-16-expressing Jurkat cells were examined for cell cycle progression. Flow cytometric analysis of propidium iodide-stained DNA content showed that the pMSCV-neo-Skp2-transduced, pro-IL-16-expressing Jurkat cells have a 50% reduction of the G0/G1 phase cell population, and a 35% increase in the S phase cell population compared with that of Jurkat cells expressing pro-IL-16 alone. To further determine whether Skp2 is a critical target for pro-IL-16 regulation of p27KIP1 levels, we tested whether overexpression of Skp2 in Jurkat cells is sufficient to decrease levels of p27KIP1 when pro-IL-16 is present. As shown in Fig. 10B, overexpression of Skp2 in Jurkat cells expressing pro-IL-16 (lane 2 of panel 3) greatly reduced the p27KIP1 protein level (lane 3 of panel 3) to an extent similar to that in cells expressing GFP alone (lane 1 of panel 3). These results further support the hypothesis that down-regulation of Skp2 levels by pro-IL-16 expression is mechanistically linked to the p27KIP1 accumulation in these Jurkat cells.

Discussion

Our results demonstrate that restoration of expression of cytoplasmic and nuclear pro-IL-16 (but not cytoplasmic expression alone) in pro-IL-16-negative Jurkat cells potently suppresses growth by inducing accumulation of cells in G0/G1. These observations are not due to a toxic effect of ectopic expression of pro-IL-16, as in almost all experiments the ectopic expression in Jurkat cells was less than the expression observed in resting T cells (data not shown). The effect of pro-IL-16 appears to be mediated by its ability to down-regulate Skp2 transcription and subsequent protein expression, resulting in a significant accumulation of the cell cycle kinase inhibitor p27KIP1. Inhibition of G1 transition to S phase is a well-described effect of elevated levels of p27KIP1.

We do not believe that these observations imply that the sole abnormality resulting in dysregulated growth in Jurkat, H9, HUT78, and SUP-T1 cells is the loss of (nuclear) pro-IL-16. There are multiple genetic abnormalities in these cells. Restoration of any of the mutated or absent cell cycle inhibitors might decrease uncontrolled cell growth. Along these lines, the loss of pro-IL-16...
alone would not be predicted to be sufficient to induce T cell malignancies. The major effect on the cell cycle of pro-IL-16 appears to be in regulation of protein levels of p27kip1. Mice lacking p27kip1, or made transgenic for overexpression of Skp2 in CD4+ cells do not exhibit spontaneous T cell malignancies (24, 25), and thus the single loss of pro-IL-16 would be unlikely to be sufficient to permit the generation of T cell malignancies.

We do believe that these studies provide evidence for a unique role for a cytokine precursor, which when present in the nucleus serves a function of inhibiting progression of the cell cycle. It does this by decreasing the expression of Skp2, a key component of the SCFSkp2 ubiquitin E3 ligase complex, which, in turn, decreases p27kip1 degradation, thus raising p27kip1 levels, permitting it to inhibit transition from G0/G1 to S phase. Of all other cytokine precursors, only pro-IL-1α has been demonstrated to have a potential nuclear function (8). In that regard, overexpression of the pro-piece of IL-1α in rat glomerular mesangial cells induces true transformation, permitting those cells to form tumors in mice (8).

Although there are no apparent structural similarities between pro-IL-1α and pro-IL-16, both are processed by members of the caspase family (26, 27), and both mature cytokines are secreted from the cell without secretory leader sequences.

The rapid down-regulation of pro-IL-16 mRNA (5, 6) and protein expression after T cell activation further imply a potential role for nuclear pro-IL-16 in T cell proliferation. In this case the loss of nuclear pro-IL-16 may be necessary to permit progression of normal T cells into S phase after Ag presentation.

Pro-IL-16 contains no consensus sequences or motifs that imply enzymatic activities of any kind, and there are no DNA binding motifs. Thus, any function must be indirect. Considering the presence of the PDZ domains, the most likely hypothesis is that pro-IL-16 provides scaffolding for a nuclear complex that may repress Skp2 transcription. Along with the PDZ domains, the presence of the dual phosphorylation-regulated nuclear localization motif also suggests that pro-IL-16 could facilitate nuclear transport of essential complex members that themselves may lack nuclear localization sequences and thus also act as a nuclear chaperone. Both these potential mechanisms are currently under investigation.

The current studies may provide an insight into one of the essential molecular mechanisms involved in the development of T cell malignancies in which deletions, mutations, and translocations of genes located on the long arm of chromosome 15 are present. Supportive evidence for this lies in the association of the nearest known gene to pro-IL-16, the Bloom syndrome locus (15q26.1), with acute myelocytic leukemia and lymphomas (16, 17) and the relationship of the chromosomal translocation t(q22;q21) (15, 17) to the development of acute promyelocytic leukemia (15). Thus, this region of 15q appears to contain many genes that are altered in hemopoietic malignancies. We now include pro-IL-16 among these genes.

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