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Cell Cycle-Dependent Regulation of FLIP Levels and Susceptibility to Fas-Mediated Apoptosis

Alicia Algeciras-Schimnich,* Thomas S. Griffith,‡ David H. Lynch,‡ and Carlos V. Paya†*

Activation-induced cell death of peripheral T cells results from the interaction between Fas and Fas ligand. Resting peripheral T cells are resistant to Fas-induced apoptosis and become susceptible only after their activation. We have investigated the molecular mechanism mediating the sensitization of resting peripheral T cells to Fas-mediated apoptosis following TCR stimulation. TCR activation decreases the steady state protein levels of FLIP (FLICE-like inhibitory protein), an inhibitor of the Fas signaling pathway. Reconstitution of intracellular FLIP levels by the addition of a soluble HIV transactivator protein-FLIP chimera completely restores resistance to Fas-mediated apoptosis in TCR primary T cells. Inhibition of IL-2 production by cyclosporin A, or inhibition of IL-2 signaling by rapamycin or anti-IL-2 neutralizing Abs prevents the decrease in FLIP levels and confers resistance to Fas-mediated apoptosis following T cell activation. Using cell cycle-blocking agents, we demonstrate that activated T cells arrested in G1 phase contain high levels of FLIP protein, whereas activated T cells arrested in S phase have decreased FLIP protein levels. These findings link regulation of FLIP protein levels with cell cycle progression and provide an explanation for the increase in TCR-induced apoptosis observed during the S phase of the cell cycle. The Journal of Immunology, 1999, 162: 5205–5211.

Apoptosis is a mechanism of cell death that is fundamental in the control of cellular homeostasis in most multicellular organisms. In the immune system, a form of lymphocyte apoptosis called activation-induced cell death (AICD) plays a critical role in the termination of the immune response and in the induction of peripheral T cell tolerance to self Ags (1, 2). A major mechanism controlling AICD relies on the interaction of the Fas receptor with its ligand, FasL (1–4). Fas is constitutively expressed in resting T cells and can be further up-regulated following cell activation (5). The membrane-bound FasL is not constitutively present in resting T cells but can be induced following TCR stimulation (6–8). The relevance of this molecular mechanism in controlling peripheral T cell homeostasis is highlighted by the fact that mutations in Fas or FasL result in deficient AICD responses, and hence in autoimmune and lymphoproliferative-like diseases (9–13). The molecular mechanism by which Fas/FasL interactions control AICD of peripheral T cells is poorly understood. Induction of FasL, following T cell activation is not sufficient to induce T cell apoptosis as Fas expressing resting T cells are resistant to Fas stimulation (14). Therefore, it has been postulated that T cell activation, in addition to inducing de novo synthesis of FasL, results in the induction of a susceptibility state to Fas-mediated apoptosis. One possibility is that IL-2 production, secondary to T cell activation, induces susceptibility to AICD mediated by Fas/FasL interactions. In fact, Fas-mediated apoptosis is potentiated by IL-2 (15), and T cells from IL-2 and IL-2R α-chain knockout mice are resistant to AICD (16–18).

Fas receptor activation first requires its trimerization by FasL. The trimerized receptor binds the adaptor protein FADD through interaction of the death domain present in these two proteins (19, 20). FADD, in turn, recruits the caspase domain containing protein caspase-8 (FLICE/MACH-1) (21, 22). Caspase-8 is then activated, leading to the activation of a cascade of cysteine proteases or caspases that results in cell death by apoptosis (23). This chain of events can be inhibited by FLIP, a FLICE-like inhibitor protein. FLIP was first described as a viral product that inhibited Fas- and TNF-mediated apoptosis (24, 25) and later was described to be present in mammalian cells (26–29). It is now believed that FLIP competitively inhibits binding of caspase-8 to the Fas receptor complex, thus shutting off the downstream Fas signaling pathway. High levels of FLIP have been suggested to correlate with resistance to Fas-mediated apoptosis in naive peripheral T cells and in melanoma tumors (26). A recent study using T cell clones from an IL-2 knockout mice showed that IL-2 is necessary to decrease FLIP levels by suppressing gene transcription (30). However, the exact molecular mechanism by which T cell activation and, more specifically, IL-2 signaling regulate FLIP protein levels needs to be fully characterized.

In this study we have investigated whether the sensitization of T cells to Fas-mediated apoptosis following TCR stimulation is mediated by the down-regulation of inhibitors of the Fas signaling pathway such as FLIP. Using primary human peripheral CD3⁺ T cells, we demonstrate that T cell activation by TCR decreases the steady state protein levels of FLIP. Inhibition of IL-2 production by cyclosporin A or inhibition of IL-2 signaling by rapamycin or anti-IL-2-neutralizing Abs prevents the down-regulation of FLIP protein levels and confers resistance to Fas-mediated apoptosis to TCR-activated T cells. We demonstrate that the decrease in FLIP levels observed following IL-2 stimulation correlates with IL-2-induced cell cycle progression, identifying the S phase as the one with decreased FLIP protein levels. These findings link regulation of FLIP protein levels with cell cycle progression and provide an

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2 Abbreviations used in this paper: AICD, activation-induced cell death; FasL, Fas ligand; FADD, Fas-associated death domain; FLICE, FLICE-like IL-1 converting enzyme (ICE); FLIP, FLICE-like inhibitory protein; CsA, cyclosporin A; HA-TAT-FLIP, hemagglutinin-tagged HIV transactivator-FLIP.
explanation for the increase in TCR-induced apoptosis observed during the S phase of the cell cycle.

Materials and Methods

Cells and culture conditions

To isolate CD3 T cells, PBMC from healthy donors were isolated from buffy coats by density gradient centrifugation (Ficoll-Hypaque, Pharmacia LKB Biotechnology, Piscataway, NJ). PBMCs were then depleted of monocytes by two cycles of plastic adherence, and CD3 T cells were purified by neuraminidase-treated SRBC rosetting. The remaining cell population was repeatedly found to be 98% CD3 T cells as determined by flow cytometric analysis. CD3 T cells used in the various experiments were maintained in RPMI 1640 supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, and antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin) (BioWhittaker, Walkersville, MD) at 2 × 10^6 cells/ml in 24-well plates.

Abs, reagents, and plasmids

The apoptosis-inducing anti-Fas cross-linking mAbs (clone CH-11, IgM) was purchased from Upstate Biotechnology (Lake Placid, NY) and was used at 500 ng/ml. The anti-IL-2-neutralizing Ab was obtained from R&D Systems (Minneapolis, MN), and anti-IL-6 Abs (1010) were a gift fromimmunex (Seattle, WA). The pharmacological inhibitors were cyclosporin A (CsA), rapamycin, aphidicolin, and mimosine were purchased from Calbiochem (La Jolla, CA). The anti-human FLIP antiserum was generated by injecting rats with a peptide spanning amino acids 2–26 of human FLIP (SAEVHQQVEALTDDEKMEFLHRCD2) (26, 31). The anti-FLICE Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

CD3 T cell cross-linking

Purified CD3 T cells were incubated for 5 min at 4°C with the mAb anti-CD3 (OKT3, American Type Culture Collection, Manassas, VA) and anti-CD28 (Becton Dickinson, Mountain View, CA), or with an isotype-matched control mouse IgG2a (Sigma, St. Louis, MO) at a concentration of 5 µg of Ab/2 × 10^6 cells/ml. Ab-bound cells were then washed and cross-linked by incubation in 24-well Nunclon plates (Sigma) that had been previously coated with goat anti-mouse IgG (Bender, Vienna, Austria) and incubated for the indicated times at 37°C. Goat anti-mouse Ab pre-coating was performed using 20 µg of the Ab preparation in 200 µl of 0.05 M carbonate buffer/well for 2 h at 37°C, and cells were washed twice with 10% RPMI 1640.

For T cell activation in the presence of inhibitors, CsA was added to the cells (200 nM) 1 h before CD3 cross-linking. Rapamycin (100 nM), anti-IL-2 (30 µg/ml), and anti-IL-6 (30 µg/ml) neutralizing Abs were added at the time of cross-linking. The inhibitors were present during the length of T cell activation. For FLIP rescue experiments, HA-TAT-FLIP fusion protein was added to cell cultures 1 h before anti-Fas stimulation.

For cell cycle synchronization, CD3 T cells were CD3 cross-linked in the presence of CsA for 20 h, after which cells were extensively washed and incubated with recombinant human IL-2 (200 U/ml; Chats, Emeryville, CA) in the absence or the presence of the indicated cell cycle blocker for the indicated time period. Cell cycle blockers were used at 5 µg/ml aphidicolin and 300 µM mimosine.

Cell death induction and analysis

To determine Fas-mediated apoptosis, CD3 cross-linked T cells were treated with anti-Fas cross-linking IgM Ab (CH-11) for 24 h during the indicated incubation times. Flow cytometric analysis for apoptosis was performed by propidium iodide staining. Briefly, cells were harvested, and washed in 1 ml of PBS. Cells were resuspended in saponin buffer (20 mM HEPES (pH 7.4), 120 mM NaCl, and 60 µg/ml saponin) containing 50 µg/ml RNase A and 20 µg/ml propidium iodide, incubated for 1 h at 37°C in the dark, and analyzed immediately on a FACScan flow cytometer (Becton Dickinson). The results were calculated using CellQuest software (Becton Dickinson), and cell death was determined by gating in the subdiploid population.

The percent cell death using trypan blue dye exclusion was calculated as follows: (total number of blue cells)/(100 × total number of cells). Results from cultures from triplicate cells were used to calculate the mean and SD.

T cell proliferation assays

Thymidine incorporation was measured after 24, 48, and 72 h of stimulation. Cells (1 × 10^6) were pulsed by the addition of 1.25 µCi of [methyl-3H]thymidine (5.0 Ci/mmol; Amersham, Arlington Heights, IL) for 8 h. Cells were harvested, and thymidine incorporation was measured on a Matrix 96 direct Betaplate counter (Packard, Meriden, CT). Data are expressed as the mean counts per min of triplicate wells.

Cell extraction and Western immunoblotting

Total cellular protein extracts were obtained by washing the cells twice in PBS and resuspending the cells in lysis buffer (1× PBS, pH 7.4, containing 0.5% Nonidet P-40, 0.5 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, and 2 µg/ml pepstatin). Cells were kept on ice for 10 min and were centrifuged at 12,000 × g for 15 min. The amount of cellular protein present in the clarified supernatant was calculated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

For Western blotting, equal amounts of cellular protein (15 or 25 µg) for each sample were loaded and separated on 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) using standard procedures. Blots were hybridized with anti-FLIP rat serum, followed by rabbit anti-rat IgG (HRP conjugated; Amersham) and anti-FLICE Abs followed by HRP-linked protein A. Anti-actin polyclonal Ab (Sigma) was used as the control for equal protein loading.

TAT fusions protein purification

The human FLIP cDNA (obtained from Dr. Jurg Tschopp, Epalinges, Switzerland) was cloned into the Xhol-NcoI site of pHA-TAT vector, which contains the C-terminal mini transduction domain from the HIV TAT protein (32, 33). HA-TAT-FLIP fusion protein was transformed into BL-21 cells and expressed in exponentially growing BL-21 cells cultured in selection medium by a 2-h treatment with isopropyl β-D-thiogalactoside (IPTG). The fusion protein was purified by sonication in 10 ml of 8 M urea, 100 mM NaCl, and 20 mM HEPES (pH 8.0). Lysates were clarified by centrifugation, and the supernatant was loaded onto a 3-ml Ni-NTA column (Qiagen, Chatsworth, CA) in the above buffer containing 10 mM imidazole. HA-TAT-FLIP fusion protein was eluted by increasing concentration of imidazole followed by dialysis. Protein purity was analyzed by Coomassie blue staining. Intracellular levels of HA-TAT-FLIP were detected by Western blot analysis using anti-HA Abs. HA-TAT-FLIP fusion protein internalized in a concentration-dependent first-order equilibrium reaction of reaching maximum intracellular concentrations in 30 min (33). The TAT-E7 plasmid was provided by Dr. Steven Dowdy and purified as described above.

Cell sorting

For cell cycle analysis of FLIP levels, CD3XL T cells were washed with PBS and resuspended in PBS containing 10 µg/ml of Hoechst 33342 (Calbiochem, La Jolla, CA) for 1 h at 37°C. Cells were immediately analyzed using a FACStar (Becton Dickinson), gated, and sorted into G0/G1 and S phases based on Hoechst 33342 fluorescence and DNA content.

Densitometry analysis

Immunoreactive bands on Western blots were analyzed using ADBIS software (San Diego, CA). Background levels were subtracted from each sample. FLIP and FLICE protein levels were normalized to actin levels for each sample. The protein levels of the control (IgGXL) were arbitrarily set at 1.0, and the change in protein levels was calculated as the protein levels in the experimental samples (CD3XL and CD3XL/CD28XL) divided by the protein levels in the control sample (IgGXL).

Results

T cell activation and FLIP protein levels

Previous reports demonstrated that T cell activation is a necessary step to render resting lymphocytes susceptible to Fas-mediated apoptosis (reviewed in Refs. 34 and 35). To study the T cell activation-mediated events that result in susceptibility to Fas-induced apoptosis, we used human primary CD3 T cells. First, we asked whether TCR activation results in down-regulation of inhibitors of the Fas signaling pathway, such as FLIP. Resting primary peripheral CD3 T cells were stimulated with IgG isoform, anti-CD3, or the combination of anti-CD3 and anti-CD28 Abs for different time periods, after which cells were lysed, and FLIP protein levels were analyzed by immunoblotting. In parallel, the effect of CD3 or CD3 and CD28 cross-linking was verified by analyzing lymphocyte proliferation as measured by [3H]thymidine incorporation during the different incubation periods (data not shown). As shown
stimulated T cells, suggesting that CD3-mediated T cell activation is sufficient to cause a reduction of FLIP protein levels in the absence of CD28 coactivation.

We next determined whether the decreased FLIP protein levels observed following T cell activation correlated with susceptibility to Fas-mediated apoptosis. CD3+ T cells were activated for 24 or 72 h with anti-CD3 Abs (alone or in combination with anti-CD28 Abs) or IgG isotype Abs, followed by the addition of an anti-Fas IgM agonist Ab for 20 h. The highest degree of susceptibility to Fas-mediated apoptosis was observed after 72 h of CD3 activation (Fig. 1C), a time period that correlated with the marked decrease in FLIP protein levels (Fig. 1, A and B). Similar results were observed when anti-CD3 and anti-CD28 Abs were combined, confirming that CD3-mediated T cell activation alone is sufficient both to cause a decrease in FLIP levels and to render the cell susceptible to Fas-mediated apoptosis.

To show that FLIP degradation is the event necessary for induction of Fas-mediated AIDC, we aimed at expressing exogenous FLIP in activated T cells. For this we used a novel transduction system of a full-length fusion protein containing HIV-Tat, which has previously been shown to be internalized into >99% of target cells (33). CD3+ T cells were activated for 72 h with anti-CD3 Abs and were treated with HA-tagged TAT-FLIP fusion protein or an irrelevant fusion protein (TAT-E7) for 1 h followed by the addition of anti-Fas Ab for 20 h. As shown in Fig. 2, addition of HA-TAT-FLIP, but not the TAT-E7 fusion protein, to CD3+-activated T cells reverses the susceptibility to Fas-mediated apoptosis in a concentration-dependent manner without affecting viability in control mock-treated cells. These results indicate that, following T cell activation, decreased levels of FLIP are sufficient to induce a state of susceptibility to Fas-mediated apoptosis in peripheral human T lymphocytes.

**IL-2 production and signaling are required for decreased FLIP protein levels**

IL-2 is one of the prevalent cytokines produced following T cell activation. In addition, IL-2 has been shown to predispose mature T lymphocytes to apoptosis (15). IL-2 seems to play a critical role in the control of AIDC, since mice lacking IL-2R α-chain signaling subunit are defective in Fas-mediated apoptosis and have abnormal lymphocyte accumulation (16–18, 36). Based on this information, we have investigated the potential role of IL-2 in regulating FLIP protein levels following TCR activation in primary resting T cells. CD3+ T cells were activated with anti-CD3 Abs for 72 h in the presence or the absence of CsA, an inhibitor of the initial phase of TCR-mediated T cell activation and IL-2 production (37). Immunoblot analysis of cytosolic extracts demonstrated that the decreased levels of FLIP 72 h following T cell activation by anti-CD3 Abs, but not isotype control Abs, were inhibited by CsA (Fig. 3A), implying that events that ensue following T cell activation, such as IL-2 production, can alter intracellular levels of FLIP.

Since CsA blocks many signal transduction pathways triggered by TCR activation, including those leading to IL-2 production (37), we narrowed the role to IL-2 by asking whether its neutralization following T cell activation would affect intracellular levels of FLIP. CD3+ T cells were stimulated, or not, with anti-CD3 Abs in the presence of neutralizing IL-2- or IL-6-specific Abs for 72 h, after which the levels of FLIP were analyzed by immunoblotting. The presence of anti-IL-2-neutralizing Abs during the process of T cell activation partially abrogated the decrease in FLIP protein levels, whereas anti-IL-6 Abs did not have an effect on FLIP protein levels (Fig. 3B). These results support the requirement of IL-2 in the regulation of FLIP protein levels following T cell activation.
To further confirm that the modification of FLIP levels following T cell activation correlates with susceptibility to Fas-mediated apoptosis, CD3-activated T cells were incubated in the presence or the absence of CsA or anti-IL-2-neutralizing Abs and were tested for their susceptibility to Fas-mediated apoptosis. As shown in Fig. 3, CsA and anti-IL-2-neutralizing Abs inhibited the induction of susceptibility to Fas-mediated apoptosis. These results indicate that IL-2 is necessary and sufficient to control FLIP levels and support the role of FLIP as an inhibitor of the Fas signaling pathway.

**A rapamycin-sensitive IL-2 signaling pathway regulates the FLIP protein level**

To understand the mechanism by which IL-2 down-regulates FLIP levels, we asked whether IL-2 signaling is necessary for the modulation of FLIP protein levels. Rapamycin is known to inhibit cell cycle progression and T cell proliferation in response to IL-2 (38–40), thus providing a valuable tool to study whether signal transduction pathways that are triggered by the engagement of IL-2R influence FLIP protein levels. CD3+ T cells were incubated with anti-CD3 or IgG isotype Abs for 72 h in the presence or the absence of rapamycin, followed by the analysis of FLIP levels by immunoblotting. As shown in Fig. 4A, rapamycin prevents the down-regulation of FLIP protein levels induced by anti-CD3 stimulation, but does not affect the levels of FLIP under resting conditions (IgGXL). Since rapamycin prevents the decreased in FLIP levels, we asked whether rapamycin can inhibit susceptibility to Fas-mediated apoptosis. Fig. 4B shows that treatment with rapamycin during the 72 h of T cell activation prevents susceptibility to Fas-mediated apoptosis. These results are in agreement with our previous observations that CsA and anti-IL-2-neutralizing Abs also blocked susceptibility to Fas-mediated apoptosis.

**Regulation of FLIP levels during the cell cycle**

To delineate the IL-2 signaling events that regulate FLIP levels, CD3+ T cells were activated with anti-CD3 Abs in the presence of CsA to inhibit IL-2 production but to prime the cells to respond to IL-2. After 20 h of TCR stimulation and CsA treatment, cells were incubated with IL-2 for different time periods, and FLIP levels

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**FIGURE 2.** Exogenous added FLIP reverts the susceptibility to Fas-mediated apoptosis following anti-CD3 stimulation. CD3+ T cells were activated with IgGXL or CD3XL for 72 h. Activated CD3+ T cells were transduced with different concentrations of HA-TAT-FLIP (A) or TAT-E7 (B) fusion protein for 1 h before addition of 500 ng/ml anti-Fas (CH-11), and cell viability was analyzed 20 h later by trypan blue dye exclusion. The data represent the mean and SD of duplicate points within each experiment. Results shown are from one representative experiment of three performed.

**FIGURE 3.** IL-2 is required to induce decreased levels of FLIP protein and to induce susceptibility to Fas-mediated apoptosis. A, Isotype Ab control (IgGXL) or anti-CD3 Ab (CD3XL)-treated CD3+ T cells were incubated for 72 h in the presence or the absence (f; vehicle control) of 250 nM CsA and immunoblotted with anti-FLIP or anti-actin Abs. B, Same as A, except that CD3+ T cells were treated or not (f) with 30 μg/ml of anti-IL-2 (αIL-2) or anti-IL-6 (αIL-6) Abs. C, CD3+ T cells were activated with anti-CD3 (CD3XL) or IgG isotype Ab (IgGXL) for 72 h in the presence of medium alone (f), CsA (250 nM), or anti-IL-2 neutralizing Abs (αIL-2; 30 μg/ml). Anti-Fas Ab was added (+), or not (−), after 72 h and was incubated for an additional 20 h. Cell viability was analyzed by trypan blue dye exclusion. The data represent the mean and SD of duplicate points within each experiment.
were analyzed. As shown in Fig. 5A, FLIP levels decreased after 48 h of IL-2 stimulation, and further decreased by 72 h. The progressive decrease in FLIP protein levels parallels the increase in cell proliferation as determined by [3H]thymidine incorporation (Fig. 5B). These results provide a link between IL-2-induced cell proliferation and the down-regulation of FLIP protein levels. To determine whether FLIP protein levels fluctuate during the cell cycle, CD3+ T cells were synchronized at different stages of the cell cycle using the different pharmacologic agents: mimosine, which arrests cells in G1 phase, and aphidicolin, which arrests cells in the early S phase (41, 42). After 20 h of anti-CD3 Ab and CsA treatment, cells were incubated with IL-2 for 72 h in the presence or the absence of the cell cycle blockers followed by analysis of FLIP levels by immunoblotting. In parallel, the effectiveness of the cell stimulation and cell cycle inhibitors was confirmed by [3H]thymidine incorporation and flow cytometric analysis. As shown in Fig. 6A, T cells arrested in early S phase with aphidicolin had decreased FLIP levels (lane 6), whereas cells arrested in G1 phase by mimosine had FLIP levels comparable to those in non-stimulated cells (lane 5). These results suggest that the decrease in FLIP levels by IL-2 occurs during the G1 to S phase transition.

To demonstrate that FLIP levels are regulated during the cell cycle and that the decrease in FLIP in the presence of aphidicolin is not secondary to nonspecific effects of cell cycle inhibition, we sorted cells based on their DNA content. Anti-CD3-activated cells were sorted using Hoechst 33342 staining for cell cycle analysis (Fig. 6B), and FLIP levels were analyzed by immunoblot. As shown in Fig. 6C, the levels of FLIP in S phase cells were decreased compared with those in G0/G1 gated cells. Based on these results, we conclude that the levels of FLIP decrease when cells progress to S phase.

Discussion

While T cell activation is known to be required for susceptibility to Fas-mediated apoptosis, the molecular basis for this process remains unknown. In this study we have analyzed the biochemical mechanisms involved in the regulation of susceptibility to Fas-mediated apoptosis following T cell activation. Using TCR activation of primary CD3+ T cells we demonstrate that FLIP protein levels are markedly decreased by 72 h of T cell activation, a time point when the cells are highly susceptible to Fas-mediated apoptosis. We have identified IL-2 as the principal mediator of FLIP protein down-regulation and demonstrated that FLIP protein levels are down-regulated during S phase.

The fact that IL-2 is the critical factor controlling FLIP protein levels explains previous observations in IL-2 and IL-2R knockout mice. Knockout mice develop a pronounced lymphadenopathy as well as associated inflammatory and autoimmune disorders (18, 36). These phenotypes are similar to those observed in mice lacking Fas or FasL (10, 11). In addition, it has been recently shown that activated CD25-/- (IL-2R α-chain) T cells are resistant to Fas-mediated AICD (16), supporting the role of IL-2 as a regulator
of T cell homeostasis. Furthermore, a recent study using IL-2 knockout mice demonstrated that FLIP mRNA levels are decreased following T cell activation (30). Findings from others and our study provide a novel molecular immune mechanism that, if defective, explains the autoimmune lymphoproliferative syndrome characterized by lymphocyte accumulation in the absence of defects in Fas or Fasl (43, 44). Inhibition of IL-2 production by CsA or IL-2 signaling by anti-IL-2-neutralizing Abs or rapamycin is shown here to significantly block susceptibility to Fas-mediated apoptosis by preventing FLIP degradation. CsA, FK506, and rapamycin are being currently used or studied as potent immunosuppressive agents to prevent allograft rejection in transplantation and to decrease autoimmunity in rheumatologic diseases. A better understanding of how these compounds can affect T cell homeostasis by targeting specific second messengers controlling Fas susceptibility of T lymphocytes will help explain some of the side effects of these drugs. Also, the fact that IL-2 directly influences T cell homeostasis should provide more insights into the utilization of this cytokine in boosting the immune system in disease states already characterized by T cell depletion and enhanced T cell activation such as AIDS.

Viral and cellular FLIP have been implicated in the protection of apoptosis against death domain-containing receptors (25, 26). High levels of FLIP are detected in melanoma tumors and in resting T cells, and this correlates with resistance against Fas-mediated apoptosis (26, 31). Previous reports had shown that resting T cells are unable to recruit caspase-8 to the Fas receptor, and an inhibitor of this pathway was suggested to be present in resting T cells (45). Based on our finding, we postulate that after T cell activation, the low protein levels of FLIP are not sufficient to prevent the binding of caspase-8 to the Fas receptor, making the cell competent to receive the Fas-mediated death signal.

Rapamycin inhibits the cell cycle progression triggered by IL-2 stimulation of T cells (40), and thus, the inhibition of FLIP down-regulation by rapamycin further supports the requirement of cell proliferation to decrease FLIP levels. The fact that FLIP levels are down-regulated during S phase provides a molecular mechanism for the previous observation that TCR induced apoptosis occurs preferentially in S phase (46). These findings may also explain why T cell lines, such as Jurkat cells, which are continuously proliferating by virtue of their transformed phenotype have undetectable levels of FLIP and therefore are highly susceptible to Fas-mediated apoptosis.

Our findings implicate cell cycle regulation as essential in deciding cell fate after response to Ag stimulation. It can be hypothesized that components of the cell cycle machinery negatively regulate the Fas apoptotic pathway. Quiescent cells (G0) and cells entering the cell cycle (G1) have a competent apoptotic machinery that is kept inactive by high levels of FLIP. Following cell cycle progression, if the conditions are favorable for proliferation the apoptotic program will be turned off by increasing levels of FLIP, whereas if cell deletion is necessary, as in the case of self Ag recognition, FLIP levels will be down-regulated, allowing for functional apoptotic machinery. A previous report suggested that the late G1, check point is required to determine the fate of the cell (33). Whether FLIP down-regulation occurs in late G1 needs to be further investigated, since the cell cycle blockers used in our study do not distinguish the G1 restriction point.

The mechanism(s) that controls FLIP levels is currently under investigation. A recent study suggests that FLIP down-regulation may be secondary to repressed transcription (30). However, our preliminary data indicate increased FLIP mRNA levels despite lower protein levels (A. Algeciras-Schminich and C. V. Paya, unpublished observation). Therefore, reduction of translation or accelerated protein degradation could account for FLIP down-regulation. Many elements of the cell cycle machinery are modulated at different stages of the cell cycle. The cyclin-dependent kinase inhibitor, p27, is present at high amounts during G0 and G1 phase and decreases as the cell enters the cell cycle. Down-regulation of p27 levels is characterized by constant amounts of p27 mRNA levels and protein synthesis, accompanied by increased ubiquitin-proteasome-mediated protein degradation (47). This scenario could be analogous to the regulation of FLIP protein levels.

In conclusion, our findings link regulation of FLIP levels with cell cycle progression. Elucidation of the mechanism by which FLIP levels are regulated throughout the cell cycle will contribute to the understanding of diseases such as autoimmunity and tumor
malignancies in which resistance to apoptosis by death-inducing receptors may play an important role in pathogenesis.

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