Phosphatidylinositol 3-Kinase Priming Couples c-FLIP to T Cell Activation*

Received for publication, April 14, 2003, and in revised form, October 22, 2003
Published, JBC Papers in Press, October 24, 2003, DOI 10.1074/jbc.M303860200

Li-Wen Fang‡‡, Tsong-Shyhuang Tai§§, Wan-Ni Yušš, Fang Liao††, and Ming-Zong Lai‡‡‡‡**

From the §Graduate Institute of Life Science, National Defense Medical Center, Taipei 11472, the ¶Graduate Institute of Immunology, National Taiwan University, Taipei 10602, and the ¶¶Institute of Molecular Biology and Institute of Biomedical Science, Academia Sinica, Taipei 11529, Taiwan

Cellular FLICE (FADD-like interleukin-1-β-converting enzyme)-inhibitory protein (c-FLIP) inhibits death receptor-induced apoptosis by binding to FADD (Fas-associated death domain protein) and pro-caspase-8. c-FLIP has also been shown to transmit activation signals and to enhance interleukin (IL)-2 production. However, c-FLIP-mediated T cell activation is difficult to detect in most cells. We found that in DO11.10 T cells, c-FLIP expression led to inhibition of IL-2 production, in contrast to the readily detectable c-FLIP-induced activation in Jurkat cells. A direct comparison revealed that distinct signal pathways were regulated by c-FLIP in Jurkat cells and DO11.10 cells. We investigated whether constitutively activated phosphatidylinositol 3-kinase (PI3K) in Jurkat cells stimulated c-FLIP. Inhibition of PI3K in Jurkat cells abrogated a c-FLIP-mediated increase in IL-2 production. In addition, c-FLIP coordinated with active PI3K for ERK activation. Furthermore, introduction of PTEN back into Jurkat cells eliminated the stimulatory effect of c-FLIP on IL-2 production and ERK activation. Our results suggest that priming with PI3K promotes the coupling of c-FLIP to T cell activation.

Apoptosis initiated from Fas and other death receptors begins with the recruitment of FADD, followed by the binding of FADD to pro-caspase-8 (FLICE), which is cleaved and activated at death receptor complexes (1–3). Caspase-8 will then cleave and activate other effector caspases, leading to irreversible cell death. The death receptor apoptotic pathway is specifically regulated by cellular FLICE-inhibitory protein (c-FLIP) (4–10). Two forms of c-FLIP proteins are expressed in cells. The long form of c-FLIP (c-FLIPL) contains two death effector domains (DEDs) and a caspase-like domain lacking the critical cysteine residue for enzyme activity. The short form of c-FLIP (c-FLIPS) contains two death effector domains without the caspase-like domain. c-FLIPL and c-FLIPS bind to FADD and pro-caspase-8 through DED-DED interactions. It is generally believed that through its recruitment to death receptor complexes, c-FLIP inhibits the processing of pro-caspase-8 and blocks apoptosis (9, 10). Many tumors have been found to up-regulate c-FLIP, possibly allowing these cells to escape from death receptor-mediated apoptosis (11–13).

Fas, FADD, and caspase-8 have been shown to participate in the activation of T lymphocytes (14). T cell stimulation reportedly activates caspase-8 and caspase-3 and inhibition of these caspses prevents T cell activation (15–17). Caspase-8 mutation in patients and caspase-8 knockout in mice are associated with defective T cell activation (18–20). Moreover, there is a profound defect in the proliferation of T cells from FADD-deficient chimeric mice and dominant-negative FADD-transgenic mice (21–25). The activation signals transmitted by FADD and caspase-8 remain largely unknown (20, 24). Interestingly, c-FLIP was found to activate ERK through an interaction with Raf-1, increase NF-κB activation by recruiting TNF receptor-associated factor (TRAF) 1/2 and receptor-interacting protein (RIP), and enhance IL-2 production (26). Moreover, transgenic c-FLIP expression results in increased T cell proliferation and IL-2 production when T cells are stimulated with low concentrations of antigens or anti-CD3 (27). It is proposed that activation defects observed in FADD−/− T cells are due to a requirement of c-FLIP for T cell activation (9). In such a scenario (9), the absence of FADD prevents the recruitment of c-FLIP and c-FLIP-transmitted activation signals, resulting in the activation defect in T cells.

The ability of c-FLIP to promote T cell activation is detectable only under restricted conditions. Minimum stimulation of c-FLIP-transgenic T cells results in increased T cell activation, and yet activation with a wide range of concentrations of antigen and CD3 results, for the most part, in inhibition of T cell activation (27). In the present study, we also found that T cell activation was impaired in DO11.10 T cells expressing c-FLIP, in contrast to increases in ERK activation and IL-2 production in Jurkat cells overexpressing c-FLIP. A direct comparison between Jurkat and DO11.10 cells indicates that c-FLIP was targeted to distinct signaling pathways. We examined whether PI3K, constitutively activated in Jurkat cells, may contribute to stimulate c-FLIP. Inhibition of PI3K by wortmannin and LY 294002, or by re-expression of PTEN, abrogated the augmentative effect of c-FLIP on IL-2 expression and ERK activation in Jurkat cells. Expression of active PI3K also primed c-FLIP for ERK activation. Our results suggest that the stimulatory effect of c-FLIP on ERK activation and IL-2 production may be PI3K-dependent.
**PI3K-dependent ERK Activation by c-FLIP**

### EXPERIMENTAL PROCEDURES

#### Retroviral Infection of T Cells—cDNA of human c-FLIP was obtained by PCR using c-FLIP, c-FLIP-L, and c-FLIP-S as templates. The 5′-c-FLIP-specific sequence (5′-GGA AAT TGT TCC ATG) was introduced during PCR. pGCIRES-YFP, a homologue of pGCIRES-GFP (29), was used as a gift from Dr. Garry Nolan (Stanford University). c-FLIP, c-FLIP-S, and c-FLIP-L were cloned into pGCIRES-YFP. Retroviruses were produced by transfecting Phoenix-Eco cells (gifts of Dr. Garry Nolan, Stanford University) with 10 μg of pGC-IREs-YFP, pcG-c-FLIP–IRES-YFP, or pcG-c-FLIP–IRES-YFP plasmids. Phoenix cell supernatants containing retrovirus were collected at 48 and 72 h after transfection. Viral titers were determined using NIH 3T3 cells, and virus stocks with titers greater than 10^6 were used for infection of DO11.10 cells expressing either c-FLIP-S or c-FLIP-L, or in the range of 60%. Twenty-four hours after infection, YFP-expressing DO11.10 cells and Jurkat cells were isolated by sorting on FACStar Plus (BD Biosciences). Transduced cells were constantly monitored on FACS for YFP expression, and cells were discarded when the fraction of YFP-positive cells fell below 90%.

#### Cell Death Measurement—All cultures were performed in RPMI medium with 10% fetal calf serum (both from Invitrogen), 10 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 × 10^{-5} M 2-mercaptoethanol. The extent of apoptosis was determined by propidium iodide staining. At the end of anti-CD3 or anti-Fas stimulation, cells were resuspended in hypotonic fluorochrome solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) (30) and placed at 4°C in the dark overnight. DNA content was analyzed by FACSscan (BD Biosciences). The fraction of cells with sub-G1 DNA content was quantitated using the CELLFIT software program (BD Biosciences).

#### Immuno blotting—Total cell extracts were prepared as described previously (31). Cell extracts (30–50 μg) were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) for 4 h at 20 V. Membranes were washed in rinse buffer (phosphate-buffered saline with 2% Tween 20) at room temperature for 15 min and incubated in blocking buffer (5% nonfat milk in rinse buffer) for 1.5 h. Membranes were then incubated with primary antibodies for 2 h at room temperature and afterward washed three times with rinse buffer. Membranes were then incubated with a 1:1000 diluted horse-antibody followed by development with ECL reagents (Amer sham Biosciences). The primary antibodies used were: anti-c-FLIPc8 (H-202), anti-Mye (9E10), anti-ERK2 (C-20), and anti-JNK1 (C-17) from Santa Cruz Biotechnology; anti-phospho-Thr202/Tyr204 ERK, anti-phospho-Thr180/Tyr182 p38 MAPK, anti-phospho-Thr547/Tyr546 JNK (from Cell Signaling, Beverly, MA).

For densitometry measurements, the developed films were analyzed on a Luminescent Image Analyzer LAS-1000 (Fuji Photo Film Co., Tokyo) using Image Gauge software (Version 3.2, Fuji). Quant mode was used to select the reading area and to subtract background. In short, an area just sufficient to cover each phosphorylated kinase band in a figure was selected. The selected region was then duplicated to enclose every band, and the densitometry reading of each region was recorded. The same region was duplicated on the open area of the film and was used as the background reading for subtraction. After subtraction, the reading of the unstimulated T cell sample was then set as 1 to get the preliminary fold of induction from the activated T cell samples. The quantity of the kinase from each sample was similarly determined. The actual activation fold of the kinase in each sample was obtained by normalizing the preliminary induction fold against the quantity ratio of the kinase.

#### MAPK Assay—T cells were treated with anti-CD3/anti-CD28. Cell lysates were prepared 5, 15, 30 min after activation, and 160 μl of lysate was precipitated with 1 μg of anti-p38 antibody followed by protein A-Sepharose (Amersham Biosciences). The kinase activity of the immune complexes was assessed by the phosphorylation of GST-ATF2(1–109). The reaction mixtures were resolved on SDS-PAGE followed by autoradiography and quantitation by Image Analyzer.

#### Transfection—Full-length human P7T cDNA was purchased from Invitrogen (Carlsbad, CA) and was subcloned into pCDNA4 (Invitrogen). pDeRed2 was obtained from BD-Clontech (Palo Alto, CA) and was subcloned into pCDNA. pCDNA4-P7T was co-transfected with pCDNA3-DeRed2 into Jurkat cells using electroporation in a Bio-Rad Gene Pulser II (Hercules, CA) at 260 nV and 975 microfarads.

---

**RESULTS**

#### c-FLIP Overexpression in DO11.10 and Jurkat Cells—Myc-tagged c-FLIPc and c-FLIPs were subcloned into pGCIRES-YFP, pGCIRES-YFP, pGC-FLIPc–IRES-YFP, and pGC-FLIPS–IRES-YFP were transected into Phoenix-Eco cells. The retroviral progeny was used to infect DO11.10 cells. YFP-positive cells were then isolated by sorting on FACStar Plus. c-FLIP expression was detected by antibodies specific for c-FLIP (A) or by a Myc tag (B). There was little c-FLIP expression only in Jurkat cells expressing YFP (C), which was reduced in c-FLIP-expressing Jurkat cells.

---

**IL-2 Production Was Increased in c-FLIP-Jurkat Cells but Was Reduced in c-FLIP-DO11.10 T Cells—The complete sup-
c-FLIPS were stimulated with immobilized anti-CD3 (OKT3, 10 μg/ml) and anti-CD28 (5 μg/ml). IL-2 production before and 24 h after activation was quantitated using the IL-2-dependent cell line HT-2. A, Jurkat cells expressing YFP, c-FLIP_L, or c-FLIP_S were stimulated with immobilized anti-CD3 (OKT3, 10 μg/ml) and anti-CD28 (5 μg/ml). IL-2 production before and 24 h after activation was quantitated using the IL-2-dependent cell line HT-2. B, YFP-, c-FLIP_L-, or c-FLIP_S-DO11.10 cells were stimulated with different concentrations of ovalbumin-(323–339) (OVA) presented by A20 cells, and the IL-2 produced was quantitated. C, YFP-, c-FLIP_L-, or c-FLIP_S-Jurkat cells were stimulated with plate-bound OKT3 (1 μg/ml), and cell extracts were prepared at the indicated time. Phospho-ERK levels were determined using anti-phosphorylated Thr202/Tyr204 ERK (Cell Signaling) and anti-ERK2 (C-14). The fold induction of ERK was determined by corrected pERK1 and pERK2 densitometry readings divided by normalized ERK2 densitometry readings divided by three measurements. D, YFP-, c-FLIP_L-, or c-FLIP_S-DO11.10 cells were activated with immobilized anti-CD3 (10 μg/ml) for the indicated time, and ERK activation was determined.

Expression of Fas-mediated cell death by c-FLIP (Fig. 1) indicated an effective antagonism of the Fas-FADD-caspase-8 cascade. We next examined the effect of c-FLIP expression on the activation of Jurkat and DO11.10 cells. The production of IL-2, representing successful integration of activation signals (36, 37), was used to evaluate T cell activation. Consistent with previous findings (26), expression of c-FLIP_L or c-FLIP_S in Jurkat cells resulted in a moderate increase of IL-2 production stimulated with plate-bound anti-CD3 and anti-CD28 (Fig. 2A). The enhanced IL-2 expression was observed specifically for activated Jurkat cells. No IL-2 secretion was detected in resting c-FLIP-expressing Jurkat cells. However, a weak signal was detected in c-FLIP-Jurkat cells before TCR engagement (Fig. 2A). For DO11.10 T cells, activation was induced either by the specific antigen ovalbumin-(323–339), presented by I-A^d, or by plate-bound anti-CD3 antibody. Unexpectedly, the IL-2 response was significantly lower for c-FLIP_L- or c-FLIP_S-DO11.10 cells than YFP-DO11.10 cells at all concentrations of ovalbumin peptide tested (Fig. 2B). Up to a 50% reduction in antigen-stimulated IL-2 production was consistently observed in four batches of DO11.10 T cells independently transduced with c-FLIP. There was also a 30% decrease in IL-2 expression in DO11.10 cells expressing c-FLIP_L or c-FLIP_S activated by anti-CD3, both at high concentrations or suboptimal concentrations (data not shown). Therefore, inhibition of the Fas-FADD-caspase-8 cascade by c-FLIP led to an increased T cell activation in Jurkat cells but resulted in defective T cell activation in DO11.10 cells.

c-FLIP Increased ERK Activation in Jurkat but Not in DO11.10 Cells—The differential effect of c-FLIP on IL-2 expression in Jurkat and DO11.10 cells indicates distinct regulation of activation signals. We first examined ERK, known to be activated by c-FLIP (26), in Jurkat and DO11.10 cells. A nearly 2-fold increase in ERK activity was consistently observed in resting c-FLIP-expressing Jurkat cells (Fig. 2C, not shown for c-FLIP_S). The activation of ERK by anti-CD3 was also moderately enhanced in c-FLIP_L- and c-FLIP_S-Jurkat cells. These results agree well with the report that c-FLIP promotes ERK activation in Jurkat cells (26). In contrast, anti-CD3-stimulated ERK phosphorylation was identical in DO11.10 T cells with YFP, c-FLIP_L-, or c-FLIP_S-DO11.10 cells as an average of three measurements. C, peak OKT3-induced calcium influx in YFP-, c-FLIP_L-, or c-FLIP_S-Jurkat cells as an average of three measurements.

c-FLIP Expression Inhibited Calcium Influx and p38 MAPK Activation in DO11.10 but Not in Jurkat Cells—Because ERK activation was normal in c-FLIP-expressing DO11.10 cells, we went on to map the signal defect in these cells. Calcium influx after anti-CD3 engagement was reduced in c-FLIP_L- and c-FLIP_S-DO11.10 cells as shown by using fura-2 as an indicator
(Fig. 3A). A moderate decrease (between 20 and 25%) in peak calcium influx was found for c-FLIPL- and c-FLIPS-DO11.10 cells compared with YFP-DO11.10 T cells. Even though the reduction in calcium mobilization was slight, it was observed in all batches of DO11.10 cells expressing c-FLIPL- and c-FLIPS- (Fig. 3B). Calcium influx, however, was not affected by c-FLIP expression in Jurkat cells. YFP-Jurkat, c-FLIP L-Jurkat, and c-FLIPS-Jurkat cells displayed similar levels of calcium influx upon TCR engagement (Fig. 3C).

p38 MAPK activation was similar for YFP-Jurkat, c-FLIPL-Jurkat, and c-FLIPS-Jurkat cells upon TCR engagement (Fig. 4A). In contrast, a profound inhibition of p38 MAPK phosphorylation was observed in both c-FLIPL- and c-FLIPS-DO11.10 cells (Fig. 4B). Anti-CD3-induced p38 phosphorylation was reduced by nearly 40% in T cells expressing c-FLIPL- and c-FLIPS- during the first hour after stimulation (Fig. 4B). Inhibition of p38 MAPK was also prominent, as measured by p38-directed phosphorylation of GST-ATF2 (Fig. 4C). p38 MAPK and calcium influx are known to be essential for IL-2 expression (38-41). The extent of the decrease in IL-2 production and p38 MAPK activation in DO11.10 cells treated with PI3K inhibitor had no effect on TCR-induced IL-2 production (Fig. 5B). c-FLIP-enhanced ERK activation in the presence of PI3K was abrogated in Jurkat cells, indicating the presence of a PI3K-dependent ERK activation by c-FLIP. We further tested whether re-introduction of PTEN into Jurkat cells eliminated the stimulatory effect of c-FLIP. Expression of PTEN in Jurkat cells mediated by c-FLIP was due to coordination from PTEN and DsRed2, using electroporation, followed by cell sorting-based double expression of DsRed2 and YFP. Elevated IL-2 production was similarly inhibited in both c-FLIPL- and c-FLIPS-expressing DO11.10 cells (Fig. 2), the deficiency in JNK activation is probably independent of the defective IL-2 production observed.
PI3K-dependent ERK Activation by c-FLIP

FIG. 6. Introduction of PTEN into c-FLIPΔ-Jurkat cells abrogated enhancement of IL-2 production and ERK activation. A, reduced Akt phosphorylation in Jurkat cells expressing PTEN. pDNAΔ4-PTEN and pDNAΔ3-DeRed2 were co-transfected in YFP-Jurkat and c-FLIPΔ-Jurkat cells by electroporation. Two days later, the cells expressing both YFP and DeRed2 were sorted on FACStar Plus and cultured with Zeocin for another 2 days. The extent of Akt phosphorylation was determined by immunoblot. B, PTEN-YFP-Jurkat and PTEN-c-FLIPΔ-Jurkat cells were activated with OKT3 (10 μg/ml) plus CD28 (5 μg/ml), and IL-2 production was quantitated. C, PTEN-YFP- and PTEN-c-FLIPΔ-Jurkat cells were stimulated with plate-bound OKT3 (1 μg/ml), and ERK activity was determined as described in the legend for Fig. 2C.

and c-FLIPΔ-Jurkat significantly reduced the extent of Akt phosphorylation (Fig. 6A). In contrast to YFP-Jurkat and c-FLIPΔ-Jurkat cells (Figs. 2A and 5A), enhanced IL-2 production was not observed in c-FLIPΔ-Jurkat cells with PTEN reintroduced (Fig. 6B). Instead, TCR-stimulated IL-2 production was lower in PTEN-c-FLIPΔ-Jurkat than PTEN-YFP-Jurkat cells. Elevated ERK activation in c-FLIPΔ-Jurkat cells was also abrogated by PTEN re-expression (Fig. 6C). ERK activity before and after TCR stimulation in PTEN-c-FLIPΔ-Jurkat cells was nearly identical to that in PTEN-YFP-Jurkat cells. Therefore, c-FLIP-mediated activation events were no longer detectable in Jurkat cells with PTEN re-introduced, supporting the notion that PI3K coupling is required for c-FLIP to transmit activation signals.

DISCUSSION

One of the outstanding questions concerning the roles of FADD and caspase-8 is their potential involvement in T cell activation. The activation signals transmitted by the death receptor, FADD, and caspase-8 are largely unknown. Recently, c-FLIP was shown to interact with Raf-1 and activate ERK, and to increase IL-2 production in Jurkat T cells and c-FLIP-transgenic T cells (26, 27). FADD and caspase-8 may, therefore, participate in T cell activation via the recruitment of c-FLIP and c-FLIP-associated signaling molecules (9). However, the activation signal of c-FLIP is not always detectable. In c-FLIP-transgenic T cells, enhanced activation is evident only when T cells are weakly stimulated, whereas suppression of T cell activation is found with antigen and anti-CD3 at most concentrations used (27). In the present study, we found that the activation of DO11.10 cells decreased regardless of the dose of antigen or anti-CD3 (Fig. 2). Jurkat cells are an exception in that c-FLIP-promoted T cell activation is always detectable (26, Fig. 2). The difficulty in detecting c-FLIP-transmitted activation signals in most T cells suggests the involvement of additional molecules in c-FLIP-mediated activation.

Our results also illustrate the inhibitory nature of c-FLIP. Despite the known ability of c-FLIP to transmit activation signals (9, 26, 27), the observations that expression of c-FLIP inhibits the activation of naive T cells, via higher concentrations of anti-CD3 (27) and activation of DO11.10 cells by TCR engagement (Figs. 2–4), indicates that the inhibitory activity is also part of the indispensable consequences of c-FLIP overexpression. An interesting point is that the reduction of IL-2 production and calcium influx in c-FLIP-DO11.10 cells are analogous to T cells from patients with mutated caspase-8 and from caspase 8-deficient mice (18, 19). Whether the inhibitory phenotype observed in DO11.10 cells is linked to inactivation of caspase-8 by c-FLIP is currently being investigated.

We examined c-FLIP-mediated activation events through a direct comparison of Jurkat and DO11.10 cells. As previously demonstrated, c-FLIP expression enhanced ERK activation in Jurkat cells (Fig. 2A). We further mapped signal defects in DO11.10 cells to p38 MAPK and calcium influx upon c-FLIP overexpression (Figs. 3 and 4). Notably, ERK activation was normal in c-FLIP-DO11.10 cells (Fig. 2B), whereas calcium signals and p38 MAPK were unaffected in c-FLIP-Jurkat cells (Fig. 3C and 4A). There was no overlap between signals activated by c-FLIP and signals suppressed by c-FLIP, suggesting that different mechanisms were involved in the activation and the inhibition mediated by c-FLIP.

Jurkat cells are known for their constitutive PI3K activation due to a lack of PTEN and SHIP (42). We studied the possibility that the distinct role of c-FLIP in the activation of Jurkat and DO11.10 cells (Fig. 2) was due to abnormal signaling in Jurkat cells. We used wortmannin and LY 294002 to inhibit the constitutive PI3K activation (Fig. 5A). Previous reports (42, 45, 46) had established that PI3K inhibitors do not affect CD28-costimulated IL-2 production in Jurkat cells, as also observed in this study in YFP-Jurkat cells (Fig. 5A). In contrast, c-FLIP-enhanced IL-2 production was abolished by PI3K inhibitors, indicating that the augmentative effect of c-FLIP on T cell activation is PI3K-dependent. In addition, expression of c-FLIP with active PI3K led to activation of ERK in 293 cells (Fig. 5B). Notably, PI3K does not exert any direct stimulatory effect on Raf-MEK (MAPK/ERK kinase)-ERK. Instead PI3K-Akt suppresses ERK activity through direct inhibition of Raf activation (43, 44). This finding correlates with our data showing that ERK activity was reduced in cells transfected with P110β only (Fig. 5B). Therefore, the increase of ERK activation when c-FLIP and P110β were co-expressed in this study represents a genuine activating effect of c-FLIP. The ability of c-FLIP to stimulate ERK activation and IL-2 production is apparently PI3K-dependent. This was further supported by the data generated from the introduction of PTEN into Jurkat cells, which inhibited the constitutive activation of PI3K, eliminating the enhanced IL-2 expression and ERK activation mediated by c-FLIP (Fig. 6). ERK activity in PTEN-c-FLIP-Jurkat cells was identical to PTEN-YFP-Jurkat cells, whereas IL-2 production was slightly lower in PTEN-c-FLIP-Jurkat than PTEN-YFP-Jurkat cells. Whether c-FLIP exhibits inhibitory activity in PTEN-Jurkat cells, as seen in DO11.10 cells, is currently being elucidated.

PI3K is involved in multiple pathways of T cell activation (47). For example, PI3K stimulates PKC for cell growth, activates GTPase for actin reorganization, increases vesicle trafficking, and regulates integrin-mediated adhesion (42, 48). It is
reasonable to postulate that coordination of PI3K initiates the coupling of c-FLIP to signaling molecules. Alternatively, c-FLIP expression has also been linked to apoptosis induction (5, 7, 49, 50). PI3K and Akt are prominent anti-apoptotic molecules, and prevention of c-FLIP-initiated cell death is a prerequisite for detection of c-FLIP-mediated activation events. In addition, there is a possibility that constitutive PI3K activation reverses the inhibitory effect of c-FLIP. For example, the activation of Rac, an upstream activator of p38 MAPK, by PI3K in Jurkat cells (42, 48) could alleviate the suppression of p38 MAPK seen in DO11.10 cells (Fig. 4). We are in the progress of elucidating how PI3K participates in the activation of c-FLIP-transmitted signals.

In summary, we have demonstrated in this study that c-FLIP exerts both stimulatory and inhibitory activity on T cells. In addition, the priming of T cells with PI3K facilitates the coupling of c-FLIP to ERK activation and IL-2 production. The presence of PI3K signaling may convert c-FLIP from an inhibitory molecule to a stimulatory molecule. The involvement of PI3K in c-FLIP activation suggests a complicated molecular coupling of c-FLIP to signaling molecules. Alternatively, c-FLIP exerts both stimulatory and inhibitory activity on T cells. In summary, we have demonstrated in this study that c-FLIP-mediated activation events. In

Acknowledgments—We thank Dr. Garry Nolan for Phoenix and Eco-Jurkat cells. Dr. Gina Costa and Dr. Nan-Shih Liao for pGCIR ES-YFP vector, and Dr. Ken Deen for editing the manuscript.

REFERENCES
1. Nagata, S. (1997) Cell 88, 355–365
2. Wallach, D., Varfolomeev, E. E., Malinin, N. L., Golstein, Y. V., Kovalenko, A. V., and Boldin, M. P. (1999) Annu. Rev. Immunol. 17, 331–367
3. Wajant, H. (2002) Science 296, 1635–1636
4. Mirsky, R., Thome, M., Hohne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.-L., Schröter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 190–195
5. Golstein, Y. V., Kovalenko, A. V., Arnold, E., Varfolomeev, E. E., Brodianski, V. M., and Wallach, D. (1997) J. Biol. Chem. 272, 19641–19644
6. Hu, S., Vincenz, C., Ni, J., Gentz, R., and Dixit, V. M. (1997) J. Biol. Chem. 272, 17255–17257
7. Shu, H.-B., Halpin, D. R., and Goeddel, D. V. (1997) Immunity 7, 49, 50). PI3K and Akt are prominent anti-apoptotic molecules, and prevention of c-FLIP-initiated cell death is a prerequisite for detection of c-FLIP-mediated activation events. In addition, there is a possibility that constitutive PI3K activation reverses the inhibitory effect of c-FLIP. For example, the activation of Rac, an upstream activator of p38 MAPK, by PI3K in Jurkat cells (42, 48) could alleviate the suppression of p38 MAPK seen in DO11.10 cells (Fig. 4). We are in the progress of elucidating how PI3K participates in the activation of c-FLIP-transmitted signals.

In summary, we have demonstrated in this study that c-FLIP exerts both stimulatory and inhibitory activity on T cells. In addition, the priming of T cells with PI3K facilitates the coupling of c-FLIP to ERK activation and IL-2 production. The presence of PI3K signaling may convert c-FLIP from an inhibitory molecule to a stimulatory molecule. The involvement of PI3K in c-FLIP activation suggests a complicated molecular coupling of c-FLIP to signaling molecules. Alternatively, c-FLIP exerts both stimulatory and inhibitory activity on T cells. In summary, we have demonstrated in this study that c-FLIP-mediated activation events. In

Acknowledgments—We thank Dr. Garry Nolan for Phoenix and Eco-Jurkat cells. Dr. Gina Costa and Dr. Nan-Shih Liao for pGCIR ES-YFP vector, and Dr. Ken Deen for editing the manuscript.