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Transcriptional Program of Apoptosis Induction following Interleukin 2 Deprivation: Identification of RC3, a Calcium/Calmodulin Binding Protein, as a Novel Proapoptotic Factor

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Apoptosis of mature T lymphocytes preserves immune system homeostasis by counteracting transient increases in T-cell number. This process is regulated, at least in part, by the cytokine interleukin 2 (IL-2): T cells deprived of IL-2 undergo apoptosis. The mechanism of apoptosis induction by IL-2 deprivation remains to be determined but is known to require RNA synthesis, implying the existence of transcriptionally activated genes whose products induce cell death. To identify such genes, we have performed expression profiling in IL-2-dependent T cells following cytokine deprivation. Our results reveal an intricate transcriptional program entailing the induction of known proapoptotic factors and the simultaneous repression of known antiapoptotic factors. Surprisingly, one gene whose transcription substantially increased was *RC3* (also called *neurogranin*), which encodes a calmodulin binding protein thought to be a neural-specific factor involved in learning and memory. We show that ectopic expression of *RC3* in IL-2-dependent T cells increases the intracellular Ca^{2+} concentration and induces apoptosis even in the presence of cytokine. Buffering the Ca^{2+} increase with the cytoplasmic Ca^{2+} chelator BAPTA-AM [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*1,*N*-tetraacetic acid] blocks RC3-induced apoptosis, indicating that the rise in intracellular Ca^{2+} is required for apoptotic death. RC3 mutants unable to bind calmodulin fail to increase intracellular Ca^{2+} levels and to induce apoptosis. Based upon these results, we propose that IL-2 deprivation raises the level of RC3 and other apoptotic factors, which induce apoptosis by increasing the intracellular Ca^{2+} concentration.

A complex network of cytokines controls the development, maturation, homeostasis, and responses of the immune system. An immune response typically involves a large increase in activated T cells, which must be eliminated following termination of the response to preserve homeostasis. This is achieved by apoptosis (also called programmed cell death), which can result from either cytokine deprivation or T-cell receptor activation (19). The cytokine interleukin 2 (IL-2) is produced and secreted by activated T cells and controls lymphocyte survival and cell cycle progression (17), and paradoxically it can also facilitate a particular apoptotic program in T lymphocytes termed activation-induced cell death (25). When deprived of IL-2, cycling T cells cease to proliferate and rapidly undergo apoptosis (19).

A key component of the apoptotic machinery is a proteolytic system involving an evolutionarily conserved family of cysteine aspartate proteases called caspases. Although this machinery is common to many forms of apoptosis, the precise mechanisms by which it becomes activated can vary considerably. Two pathways leading to caspase activation have been characterized: the extrinsic pathway, which involves so-called death receptors, and the intrinsic pathway, which involves the release of proapoptotic proteins from the mitochondria. In addition, some apoptotic pathways are transcription dependent, whereas others entail a purely posttranscriptional signal transduction cascade.

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There have been several proposals for the mechanism by which IL-2 deprivation leads to apoptosis, including regulation of Bcl-2 family members, modulation of second messengers, and alteration of the activity of protein kinases or phosphatases (1, 5, 11). However, none of these regulatory events explain the requirement of de novo RNA or protein synthesis for induction of apoptosis following IL-2 deprivation (10). Here we use expression profiling to identify death-promoting genes that are transcriptionally activated in lymphocytes following IL-2 deprivation.

MATERIALS AND METHODS

Plasmids and cell culture. *RC3* cDNA was hemagglutinin tagged at the 3′ end and cloned into pcDNA3.1+ and pcDNA3.1− vectors (Invitrogen). IL-2-dependent murine HT-2 (T helper) cells, CTLL-2 (cytotoxic T) cells, myeloid 32D cells, HEK-293 cells, and human T-cell leukemia Jurkat cells were all obtained from the American Type Culture Collection; IL-3-dependent murine FL5.12 cells (pro-B lymphocytes) were provided by Craig Thompson (University of Pennsylvania). HT-2 and CTLL-2 cells were maintained in RPMI 1640 medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (GIBCO-BRL), 1 mM sodium pyruvate (GIBCO-BRL), 100 U of penicillin (Sigma) per ml, 100 μg of streptomycin (Sigma) per ml, 0.05 mM 2-mercaptoethanol (GIBCO-BRL), and 200 U of recombinant IL-2 (PharMingen) per ml or 10% rat T-STIM factor with concanavalin A (Becton Dickinson). FL5.12 and 32D cells were maintained in RPMI medium supplemented with 10% FBS, 1 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.05 mM 2-mercaptoethanol, and 3 ng of recombinant IL-3 (PharMingen) per ml. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 1 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml. Jurkat cells were maintained in RPMI medium supplemented with 10% FBS, 1 mM L-glutamine, 100 U of penicillin per ml, and 100 mg of streptomycin per ml.

Human peripheral blood lymphocytes from healthy donors were initially cultured in RPMI medium supplemented with 10% FBS, 1 mM L-glutamine, and 5

μg of phytohemagglutinin (Sigma) per ml for 2 days. Cells were then collected and placed in RPMI containing 10% FBS and 10% human IL-2 (Advanced Biotechnologies) for 4 days with a medium change every 48 h. Staurosporine (Sigma) and thapsigargin (Calbiochem) were added to the media to final concentrations of 10 and 2 μM, respectively. BAPTA-AM [1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*1,*N*-tetraacetic acid] (Sigma) was added to the media to a final concentration of 25 μM.

Microarray analysis. HT-2 cells were cultured in the presence of IL-2 and subjected to IL-2 deprivation as described previously (10). Poly(A)$^+$ mRNA was isolated at 8 h following IL-2 withdrawal by using an Oligotex direct mRNA isolation kit (Qiagen). The integrity of mRNA was monitored by a Northern blot assay with a glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) probe. Two micrograms of mRNA was used to generate a cDNA library by using an oligo(dT) T7 primer and Superscript cDNA synthesis system (GIBCO-BRL) according to the manufacturer's instructions. The cDNA library was in vitro transcribed with biotinylated nucleotides by using the T7 Megascript kit (Ambion) according to the manufacturer's instructions. The resulting cRNA was fragmented by heating at 94°C for 30 min. GeneChip Murine 19K and 11K Affymetrix oligonucleotide arrays representing ~30,000 known genes or expressed sequence tags were hybridized with fragmented cRNA overnight at 37°C. Following washing, the chips were scanned and analyzed with Affymetrix microarray suite software. The complete data set is available upon request.

Transient transfections and apoptotic assays. HT-2, CTLL-2, and FL5.12 cells were transfected with plasmids by using Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. Cells were collected 24 h after transfection and stained with annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (Oncogene). For cotransfection experiments, HT-2 cells were cotransfected with 1 μg of enhanced green fluorescent protein (GFP) plasmid (Clontech) and 4 μg of pcDNA3.1+/RC3 (sense orientation) or pcDNA3.1−/RC3 (antisense orientation). Cotransfected cells were stained with annexin V-phycoerythrin (PE) (PharMingen) and analyzed by fluorescence-activated cell sorting (FACS) analysis. At least two independent experiments were performed, and the results are presented as the averages ± standard deviations.

For HEK-293 fibroblasts, cells were seeded into a Lab-Tek II chamber slide (Nunc) at 6×10^5 cells per slide and cotransfected with 0.5 μg of β-galactosidase reporter plasmid (Clontech) and 4 μg of expression plasmid (as indicated), using Superfect transfection reagent. Following transfection, cells were washed three times with phosphate-buffered saline, fixed with 0.5% glutaraldehyde, and stained with 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (Promega) as described previously (20). The percentage of transfected (blue) cells with apoptotic morphology was determined. At least two independent experiments were performed, and the results are presented as the averages ± standard deviations.

For DNA fragmentation analysis, DNA from 2×10^6 cells was isolated by phenol extraction 24 h following transfection and analyzed on a 1% agarose gel as previously described (32).

Fluorospectrometric analysis of intracellular calcium concentration. To measure intracellular Ca^{2+} concentrations, 5×10^6 HT-2 cells were stained with 2 mM fura-2 AM (Molecular Probes), a fluorescent Ca^{2+} indicator, in 1 ml of saline containing 1 mM $CaCl_2$. Cells were stained for 30 min at 37°C and for 10 min at room temperature. The cells were washed two times with saline containing 1 mM $CaCl_2$ and resuspended at 10^6 cells/ml in the same solution. Spectrometric analysis was performed at 380 and 340 nm as described previously (36). The Ca^{2+} ionophore ionomycin was added to a final concentration of 10 mM at the end of each experiment to determine whether the limits of saturation of fura-2 AM had been reached. At least two independent experiments were performed, and the results are presented as the averages ± standard deviations.

Single-cell Ca^{2+} imaging. Intracellular Ca^{2+} was measured in primary human T lymphocytes and in HT-2 cells transfected with the *RC3* expression plasmid. HT-2 cells were cotransfected with 5 μg of either pcDNA3.1+/RC3 or pcDNA3.1 and a plasmid expressing the truncated human CD4 molecule as a marker to select transfected cells. Transfected cells were then isolated from untransfected cells by using the MACSelect-transfected cell isolation kit (Miltenyi Biotech) according to the manufacturer's instructions. Transfected cells were then stained with 3 μM fura-2 AM in RPMI for 20 min at room temperature. Cells were washed three times with RPMI and plated onto poly-D-lysine-coated glass-bottom dishes (Mattek Corporation) for 10 min at room temperature. Cells were washed once with Ringer lactate solution. Images were collected at 340- and 380-nm wavelengths. Single-cell measurements of intracellular Ca^{2+} ion concentration were calculated from the 340 nm/380 nm ratios (generated from background-corrected 340 nm/380 nm images) by using the equation of Grynkiewicz et al. (18) and a K_d of 250 nM for fura-2 AM. At least two independent experiments were performed, and the results are presented as the averages ± standard deviations.

RESULTS

Identification of genes transcriptionally activated in T cells following IL-2 deprivation. The mouse HT-2 T helper cell line requires IL-2 for growth, and in its absence HT-2 cells undergo apoptosis (Fig. 1A) (34). For expression profiling, HT-2 poly(A)$^+$ mRNA was isolated 8 h after IL-2 withdrawal and used to interrogate Affymetrix DNA chips that contained ~30,000 genes or expressed sequence tags. Comparison of the transcription profiles of cells grown in the presence or absence of IL-2 revealed that ~97% of the genes were unaffected by cytokine deprivation (Fig. 1B). Of the genes whose transcription was altered by cytokine deprivation, surprisingly, approximately three-quarters were stimulated and one-quarter were repressed (Fig. 1C).

Several of the genes that underwent significant transcriptional changes following IL-2 deprivation are listed in Table 1. Three of the genes that underwent transcriptional stimulation were *CAPN5*, encoding a calpain cysteine protease; *DRP-1*, which encodes death-associated protein kinase (DAPK)-related protein 1 (21); and *RC3*/*neurogranin*, encoding a calmodulin (CaM) binding protein (4). The apoptotic roles of calpains and *DRP-1* are well documented (21, 24). *DRP-1* encodes a CaM-regulated serine/threonine kinase that is highly similar to DAPK. Both kinases contain a CaM binding domain, and, interestingly, ectopic expression of either *DRP-1* or *DAPK* induces apoptosis in a wide variety of cell types (6, 21).

Conversely, transcription of a number of genes decreased following IL-2 deprivation, including the *Pim1* oncogene (33) and *ATFx*, which encodes a basic-region leucine zipper (bZIP) transcription factor (28) (Table 1). Significantly, expression of *Pim1* and *ATFx* is also repressed following cytokine deprivation in IL-3-dependent FL5.12 cells (8), and both genes encode proteins with antiapoptotic activities (30, 35).

The finding that *RC3* was transcriptionally activated in lymphocytes was surprising, because the RC3 protein was previously characterized as a neurally specific factor involved in synaptic plasticity and spatial learning (29, 37). Therefore, *RC3* represented a putative novel gene involved in lymphocyte apoptosis, and we sought to test this hypothesis and study the mechanism of RC3 action. Northern blot analysis confirmed the results of the DNA microarray experiments: IL-2 withdrawal resulted in transcriptional activation of *RC3* and repression of *ATFx* (Fig. 2A). A time course analysis revealed that *RC3* transcriptional induction preceded the onset of apoptosis (Fig. 2B). Immunoblot analysis showed that like the mRNA levels, RC3 protein levels were also up-regulated following IL-2 withdrawal (Fig. 2C).

Specificity of *RC3* transcriptional activation. To investigate the specificity of *RC3* transcriptional activation, we analyzed several other cytokine-dependent cell lines: IL-2-dependent CTLL-2 cells, IL-2-dependent primary human T lymphocytes, IL-3-dependent murine pro-B lymphocytic FL5.12 cells, and IL-3-dependent murine myeloid 32D cells. Similar to the results obtained with the IL-2-dependent HT-2 cell line, transcription of *RC3* was also activated in CTLL-2 cells (Fig. 3A) and in IL-2-dependent primary human T lymphocytes (Fig.

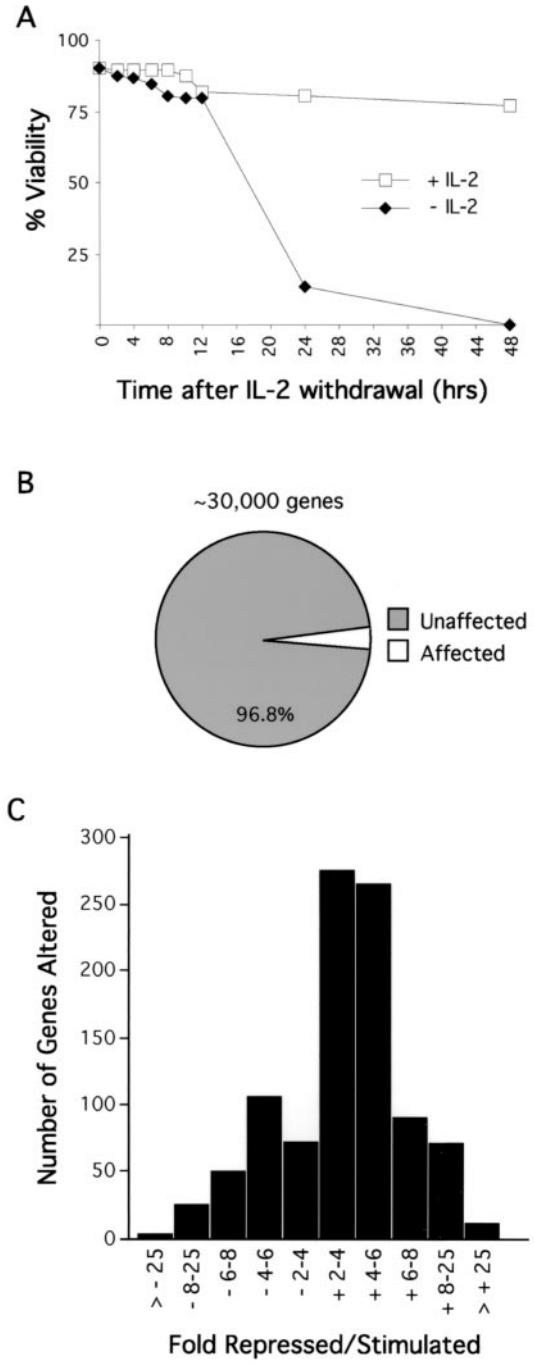

FIG. 1. A transcriptional program of apoptosis induction following IL-2 deprivation. (A) Time course of apoptosis following IL-2 deprivation. The percentage of viable HT-2 cells was determined by annexin V-FITC staining. (B) Summary of microarray analysis. Affected is defined as a twofold or greater change following IL-2 deprivation. (C) Graphical representation of the fold changes of genes stimulated or repressed.

3B) following cytokine deprivation. However, the results with the IL-3-dependent cell lines differed. Although *RC3* mRNA could be detected in FL5.12 cells grown in the presence of IL-3, the mRNA levels were not significantly increased following cytokine deprivation (Fig. 3C). In IL-3-dependent 32D cells, *RC3* mRNA was not detected in either the presence or absence of cytokine (Fig. 3D). Transcriptional activation of *RC3* also did not occur in cytokine-deprived IL-7-dependent D1-F4 cells or serum-deprived human fibroblasts (data not shown). Finally, we analyzed *RC3* expression in a cytokine-independent T-cell line (human T-cell leukemia Jurkat cells). Apoptosis was induced in Jurkat cells with staurosporine, and

TABLE 1. Selected genes whose transcription was altered following IL-2 deprivation of HT-2 cells[a]

Gene	Fold change in transcription
CAPN5	+26.7
DRP-1	+25.0
RC3	+15.6
ATFx	−5.5
Pim1/2 kinase gene	−78.0

[a] Known proapoptotic genes that underwent more than a 15-fold induction and known antiapoptotic genes that underwent more than a 5-fold repression are shown. *RC3* is the subject of this study.

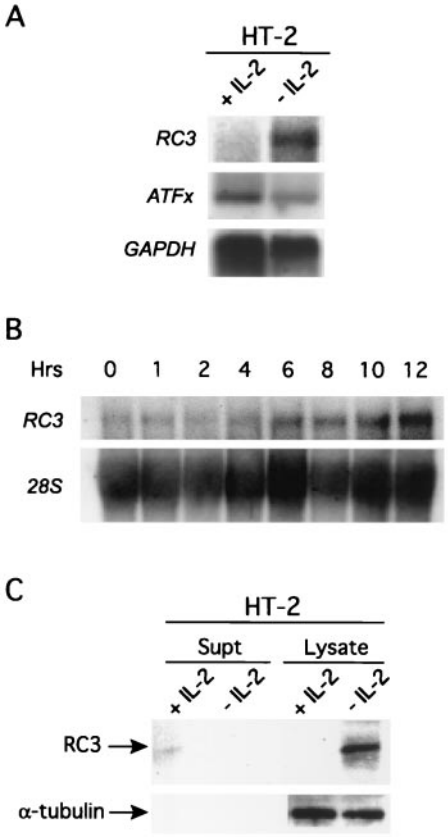

FIG. 2. Induction of *RC3* transcription following IL-2 deprivation. (A) Transcriptional activation of *RC3* upon IL-2 deprivation. Expression of *RC3*, *ATFx*, and *GAPDH* (as a loading control) in HT-2 cells was assessed by Northern blot analysis with 1 μg of poly(A)$^+$ mRNA or 10 μg of total RNA 8 h after IL-2 deprivation. (B) Time course of *RC3* induction. *RC3* mRNA levels in HT-2 cells were analyzed at the indicated times following IL-2 deprivation. The Northern blot was stripped and reprobed with a 28S rRNA probe as a loading control. (C) Immunoblot analysis of whole-cell extracts (Lysate) or culture supernatants (Supt) from HT-2 cells grown in the presence or absence of IL-2. The same blot was stripped and reprobed with an α-tubulin antibody (Sigma) as a loading control. The exposure time was 10 s.

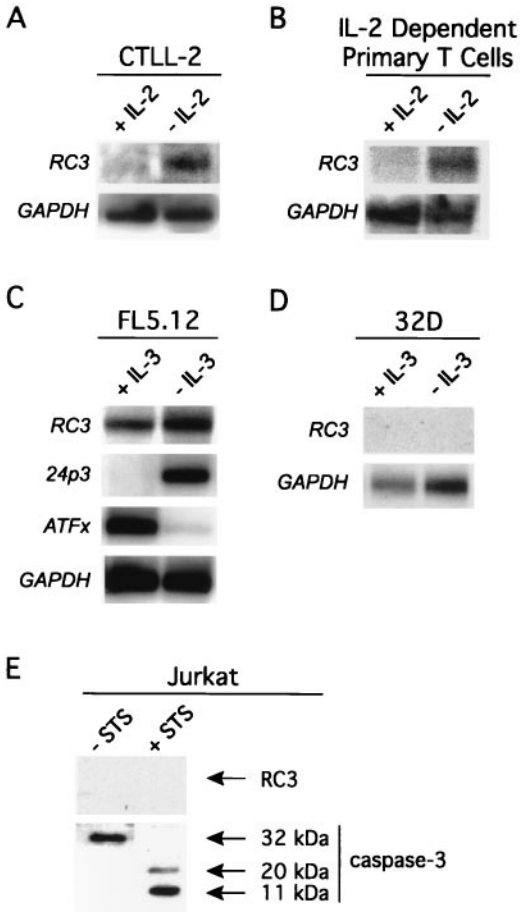

FIG. 3. Cell specificity of *RC3* transcription activation. (A) *RC3* is transcriptionally activated in CTLL-2 cells upon IL-2 deprivation. Expression of *RC3* and *GAPDH* (as a loading control) was assessed by Northern blot analysis with 1 μg of poly(A)$^+$ mRNA or 10 μg of total RNA 8 h after IL-2 removal. (B) Northern blot analysis of poly(A)$^+$ mRNA prepared from primary human T lymphocytes 12 to 14 h after IL-2 removal. (C) Northern blot analysis of poly(A)$^+$ mRNA prepared from FL5.12 cells 8 h after IL-3 removal. The same blot was sequentially stripped and reprobed with *24p3* (as an induction control), *ATFx* (as a repression control), and *GAPDH* probes. (D) Northern analysis of poly(A)$^+$ mRNA from 32D cells. The blot was probed sequentially with *RC3* and *GAPDH* probes. (E) Immunoblot analysis of whole-cell extracts from Jurkat cells after staurosporine (STS) treatment, which induces apoptosis. The same blot was stripped and reprobed with a caspase-3 antibody (Santa Cruz Biotechnology) as a control; STS treatment results in proteolytic activation of caspase 3.

RC3 protein levels were analyzed by immunoblot analysis. Although staurosporine treatment resulted in proteolytic activation of the apoptotic marker caspase 3, expression of RC3 was not detected (Fig. 3E). Collectively, these results indicate that *RC3* transcriptional activation is not a general consequence of apoptosis induction due to growth factor deprivation.

Ectopic *RC3* expression induces apoptosis. To investigate whether overexpression of *RC3* was sufficient to induce apoptosis, we transfected IL-2-dependent HT-2 and CTLL-2 cells and IL-3-dependent FL5.12 cells with a plasmid expressing *RC3*. After 24 h, cells were stained with annexin V-FITC-propidium iodide to monitor apoptosis. Figure 4A shows that expression of *RC3* induced apoptosis in HT-2 and CTLL-2 cells but not in FL5.12 cells. As expected, transfection of cells with the expression vector pcDNA3.1 had no effect on either cell line. Immunoblot analysis revealed that the differential response of HT-2 and FL5.12 cells was not due to variations in RC3 expression (Fig. 4B).

The ability of RC3 to induce apoptosis in HT-2 cells was also investigated by using two other independent assays. In one assay, the *RC3* expression plasmid was cotransfected with a plasmid expressing GFP, which provided a marker for transfected cells. Quantitation of cells that were both GFP positive and annexin V-PE positive by FACS analysis confirmed that ectopic *RC3* expression induced apoptosis (Fig. 4C). As expected, cotransfection with a plasmid lacking the *RC3* coding sequence (pcDNA3.1 vector alone) or with *RC3* cloned in the antisense orientation failed to induce apoptosis (Fig. 4C). In the second assay, DNA extracted from HT-2 and CTLL-2 cells transfected with an *RC3* expression plasmid was analyzed by using a DNA fragmentation assay. Figure 4D shows that in both cell types *RC3* expression resulted in a characteristic DNA ladder, confirming that cell death was apoptotic.

A functional RC3 CaM binding domain is required for induction of apoptosis. Alterations in intracellular ion levels are a feature of several apoptotic pathways, and the involvement of Ca^{2+} has been well documented. For example, Ca^{2+} ionophores, which directly mobilize Ca^{2+} from internal stores, induce apoptosis in diverse cell types (27). Conversely, agents that decrease intracellular Ca^{2+} concentration can inhibit apoptosis; for example, one of the many activities of the antiapoptotic Bcl-2 protein is to prevent the loss of Ca^{2+} from intracellular stores, thereby blocking apoptosis (2).

Many of the physiological effects of Ca^{2+} are mediated by CaM, a small, ubiquitous protein that regulates a wide variety of cellular processes by activating Ca^{2+}/CaM-dependent enzymes that, in turn, modulate signaling cascades. Like intracellular Ca^{2+}, CaM may also regulate apoptosis; for example, increased CaM expression has been correlated with apoptosis (9), and, conversely, CaM antagonists can interfere with apoptosis (26).

RC3 contains a well-characterized 20-amino-acid sequence known as the IQ motif (3), which functions as a binding site for CaM (12). Specific single amino acid substitutions in the IQ domain eliminate binding of RC3 to CaM in vivo (31). In the brain, CaM appears to be the major protein that interacts with RC3, and the RC3-CaM interaction is believed to be essential for RC3 function (12, 31). To investigate whether the ability of RC3 to induce apoptosis correlated with its ability to bind CaM, we analyzed the apoptotic activity of RC3 derivatives bearing mutations in the CaM binding domain by using two different assays. In the first assay, HT-2 cells cultured in the presence of IL-2 were transfected with plasmids expressing either wild-type RC3 or an RC3 mutant, and the percentage of apoptotic cells was determined by annexin V-FITC staining. Figure 5A shows that mutation of Ile-33 to Gln (I33Q), which completely inhibits RC3 binding to CaM (31), also eliminated its proapoptotic activity. Similarly, mutation of either Ser-36 to Asp (S36D) or Arg-38 to Gln (R38Q), which weakens the RC3-CaM interaction (31), also reduced the proapoptotic activity of RC3. In contrast, the Ser-36-to-Ala mutation (S36A),

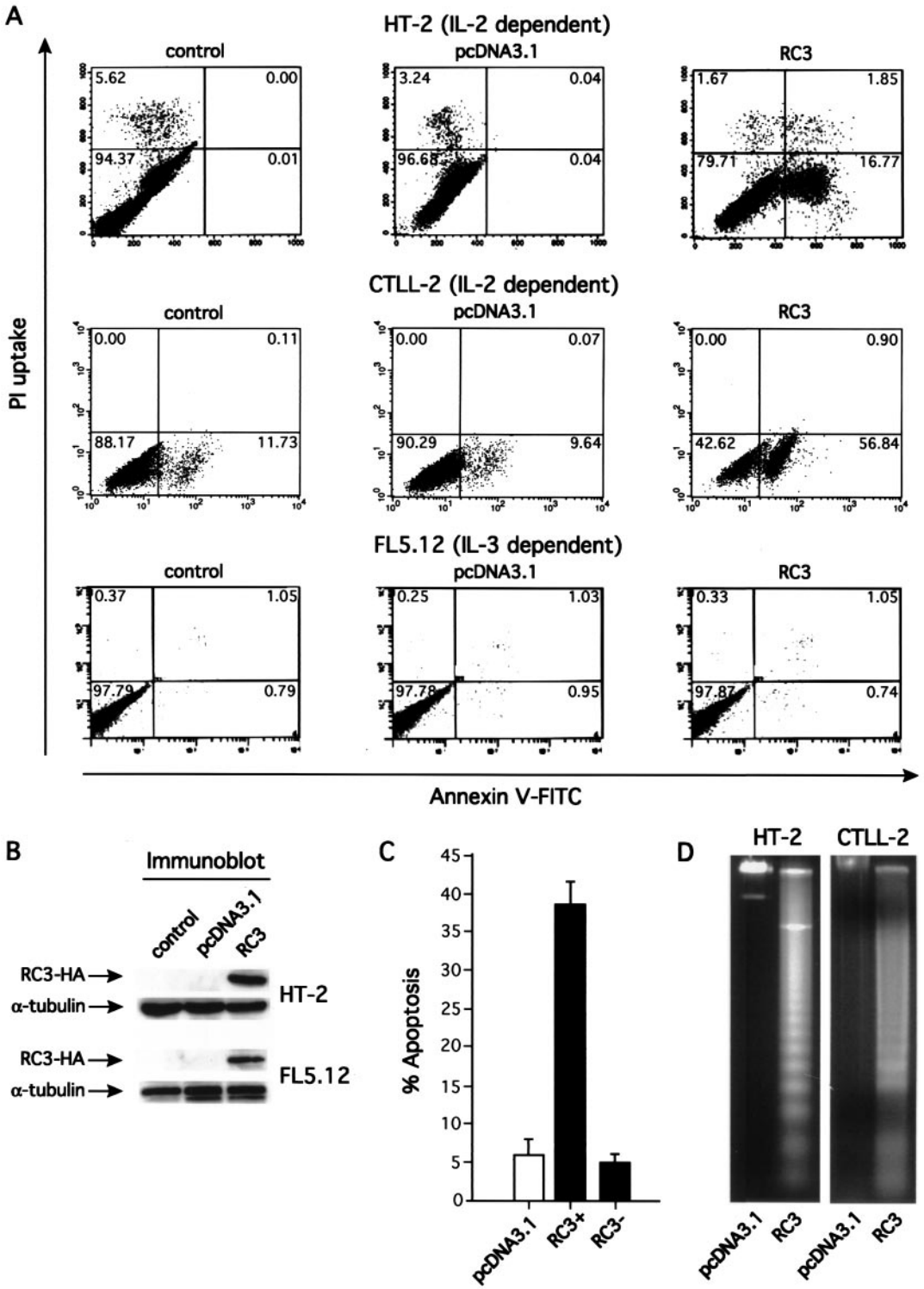

FIG. 4. Expression of RC3 induces apoptosis in HT-2 and CTLL-2 cells. (A) HT-2 and CTLL-2 cells were transiently transfected with 5 μg of either pcDNA3.1 (empty vector) or pcDNA3.1/RC3 or left untransfected (control). One day after transfection, cells were collected and stained with annexin V-FITC and analyzed by FACS. PI, propidium iodide. (B) Extracts were prepared from a portion of the transfected cells and analyzed by immunoblotting with an antihemagglutinin (anti-HA) monoclonal antibody or an α-tubulin antibody. (C) HT-2 cells were cotransfected with 1 μg of GFP plasmid and 4 μg of either pcDNA3.1 (empty vector), pcDNA3.1+/RC3 (sense orientation), or pcDNA3.1-/RC3 (antisense orientation). Cotransfected cells were stained with annexin V-PE and analyzed by FACS. The percentage of GFP-positive cells that were also positive for annexin V-PE was quantitated and plotted. Error bars indicate standard deviations. (D) HT-2 and CTLL-2 cells transfected with empty vector plasmid (pcDNA3.1) or an *RC3* expression plasmid were subjected to DNA fragmentation analysis.

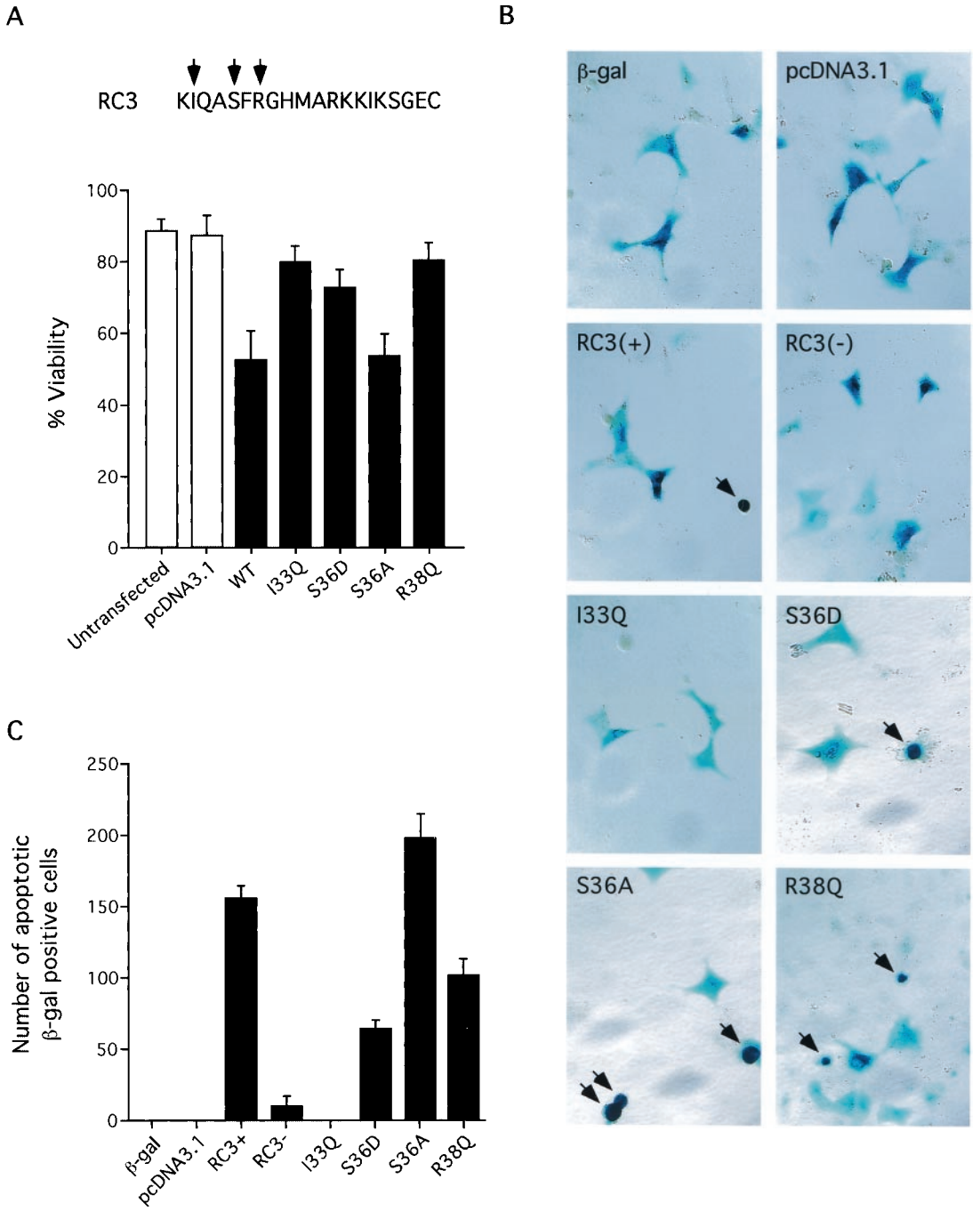

FIG. 5. A functional RC3 CaM binding domain is required for induction of apoptosis. (A) HT-2 cells grown in the presence of IL-2 were transfected with 4 μg of empty vector plasmid (pcDNA3.1), wild-type RC3 (WT), or RC3 mutants. Cells were collected 24 h after transfection, stained with annexin V-FITC, and subjected to FACS analysis to monitor cell viability. The protein sequence of the 20-amino-acid CaM binding domain (IQ motif) of RC3 is shown; mutated residues are indicated by arrows. Error bars indicate standard deviations. (B) HEK-293 cells were transiently cotransfected with a β-galactosidase (β-gal) reporter plasmid (0.5 μg) and the experimental plasmid (4 μg), as indicated. The cells were washed 24 h after transfection, fixed, and stained with 0.2% X-Gal. (C) Quantitative analysis of the experiment for panel B. Four hundred β-galactosidase-positive cells were analyzed, and the number of blue cells displaying an apoptotic morphology was assessed.

which does not affect the RC3-CaM interaction (31), induced apoptosis at levels similar to those for wild-type RC3.

In the experiment shown in Fig. 5A, apoptosis was analyzed in a population in which there was a high background of untransfected cells (~60%). To confirm these results, we performed a second assay that measured apoptosis only in transfected cells. For these experiments, which involve staining individual cells, we used the adherent cell line HEK-293. HEK-293 cells were cotransfected with a β-galactosidase reporter plasmid and a plasmid encoding either the wild-type or mutant

RC3 protein. The cells were then stained with the chromogenic substrate X-Gal, which marks transfected cells, and apoptotic cells were identified by their characteristic condensed morphology (Fig. 5B) and quantitatively assessed (Fig. 5C). The results of this second apoptotic assay were similar to those of Fig. 5A. Collectively, these results reveal a strong correlation between the abilities of RC3 to bind CaM and to induce apoptosis.

Both IL-2 deprivation and *RC3* expression increase intracellular calcium concentration. An increased intracellular Ca^{2+} concentration is known to induce apoptosis. We therefore reasoned that increased RC3 levels that occur during IL-2 deprivation might trigger apoptosis by sequestering CaM, thereby releasing Ca^{2+} and increasing the intracellular Ca^{2+} concentration. As an initial test of this hypothesis, we measured intracellular Ca^{2+} levels in HT-2 cells following IL-2 deprivation. As a positive control, HT-2 cells were treated with thapsigargin, which releases Ca^{2+} from intracellular pools and induces apoptosis. Figure 6A shows that thapsigargin treatment increased the intracellular Ca^{2+} concentration 10-fold, whereas IL-2 deprivation increased the intracellular Ca^{2+} concentration 5-fold. We conclude that IL-2 deprivation increases intracellular Ca^{2+} concentration.

We next asked whether *RC3* expression would, like IL-2 deprivation, increase the intracellular Ca^{2+} concentration. HT-2 cells were transfected with a plasmid expressing RC3 or an RC3 mutant. Figure 6B shows that the intracellular Ca^{2+} concentration in cells transfected with an *RC3* expression plasmid was 2.5-fold higher than that in untransfected cells or cells transfected with the pcDNA3.1 expression plasmid. After correcting for transfection efficiency (~40%), we concluded that *RC3* expression increased the intracellular Ca^{2+} concentration approximately sixfold, which was very similar to the result observed following IL-2 deprivation. Analysis of two RC3 mutants (R38Q and S36A) again revealed a correlation between the abilities of RC3 to interact with CaM and to increase intracellular Ca^{2+}. We conclude that RC3 increases the intracellular Ca^{2+} concentration.

To directly quantify the Ca^{2+} increase induced by *RC3* expression, we performed single-cell imaging experiments. HT-2 cells transfected with either an *RC3* expression plasmid or pcDNA3.1 were isolated and subjected to single-cell intracellular Ca^{2+} imaging analysis. The pseudocolor image in Fig. 6C shows that *RC3* expression resulted in an increased intracellular Ca^{2+} concentration. Quantitation revealed that *RC3* expression increased the intracellular Ca^{2+} concentration approximately eightfold, comparable to the approximately ninefold increase observed upon IL-2 withdrawal in the same assay, confirming the results of Fig. 6A and B. Finally, a similar analysis for IL-2-dependent primary human T lymphocytes again indicated that *RC3* expression increased the intracellular Ca^{2+} concentration (Fig. 6D).

An increase in intracellular calcium is required for induction of apoptosis. To determine if the Ca^{2+} increase that occurs following IL-3 withdrawal or *RC3* expression is directly responsible for triggering apoptosis, we asked whether depletion of intracellular Ca^{2+} could protect cells from apoptotic death. Incubation in the presence of BAPTA-AM, an intracellular Ca^{2+} chelator, inhibited apoptosis induced by cytokine deprivation in IL-2-dependent HT-2 cells (Fig. 7A) and in IL-2-dependent primary human T lymphocytes (Fig. 7B). Similarly, BAPTA-AM treatment abolished the apoptotic effects of *RC3* expression (Fig. 7C). These results indicate that intracellular free Ca^{2+} accumulation plays a critical role in apoptosis induced by either IL-2 deprivation or *RC3* expression.

DISCUSSION

We have found that induction of apoptosis due to cytokine deprivation in IL-2-dependent lymphocytes involves an orchestrated transcriptional program in which genes encoding proapoptotic factors are transcriptionally activated and genes encoding antiapoptotic factors are transcriptionally repressed. In addition, we have identified a novel proapoptotic factor, RC3, a member of the calpacitin family, whose members bind and release CaM in response to Ca^{2+} flux (13). The role of RC3 in modulating intracellular Ca^{2+} and CaM levels in neurons is well documented (13). Our studies reveal that RC3 also has a function in lymphocytes. We find that *RC3* is transcriptionally activated in IL-2-dependent T cells following cytokine deprivation and that ectopic expression of *RC3* in these cells induces apoptosis. These results suggest a model in which RC3 plays a role in eliminating activated T cells following an immune response. Consistent with a potential role in the immune response, previous studies have shown that in addition to the brain, *RC3* is expressed in the spleen and thymus (16, 37). *RC3* knockout mice have been derived (29), and it will be important to carefully evaluate their immunological status.

Although the mechanism by which IL-2 promotes transcription has been well characterized (reviewed in reference 15), the pathway by which IL-2 deprivation increases transcription of *RC3* and other genes remains to be elucidated. One potential clue has come from studies with IL-2-dependent mouse lymphoid cells, which show that expression of the AP-1 transcription factors c-Fos and c-Jun is rapidly induced following growth factor deprivation (7). The *RC3* promoter contains putative AP-1 binding sites (22), suggesting a possible mechanism for transcriptional activation of *RC3* following IL-2 deprivation.

The proapoptotic activity of RC3 requires a functional CaM binding domain, indicating that the ability of RC3 to induce apoptosis is related to its role in modulating intracellular CaM and/or Ca^{2+} levels. Recent computer-based modeling of calpacitin-CaM interactions predicts that increased RC3 levels would lead to dissociation of the Ca^{2+}-CaM complex, thereby increasing intracellular Ca^{2+} levels (14). Consistent with this prediction, we found that overexpression of *RC3* resulted in an increased intracellular Ca^{2+} concentration (Fig. 6).

Based on the findings presented in this report and previous studies on calpacitin regulation and function, we propose the following model for the induction of apoptosis by RC3 in lymphoid cells. IL-2 deprivation induces *RC3* expression, and the elevated levels of RC3 sequester CaM and stabilize it in its Ca^{2+}-free form, which, as described above, contributes to an increase in intracellular Ca^{2+} and triggers apoptosis. In support of this model, antisense-mediated blocking of the Ca^{2+} channel renders T cells resistant to apoptosis, indicating that intracellular Ca^{2+} levels play a role in T-cell apoptosis (23).

IL-2 deprivation may also decrease protein kinase C (PKC) activity (17). PKC phosphorylates RC3 and blocks the RC3-

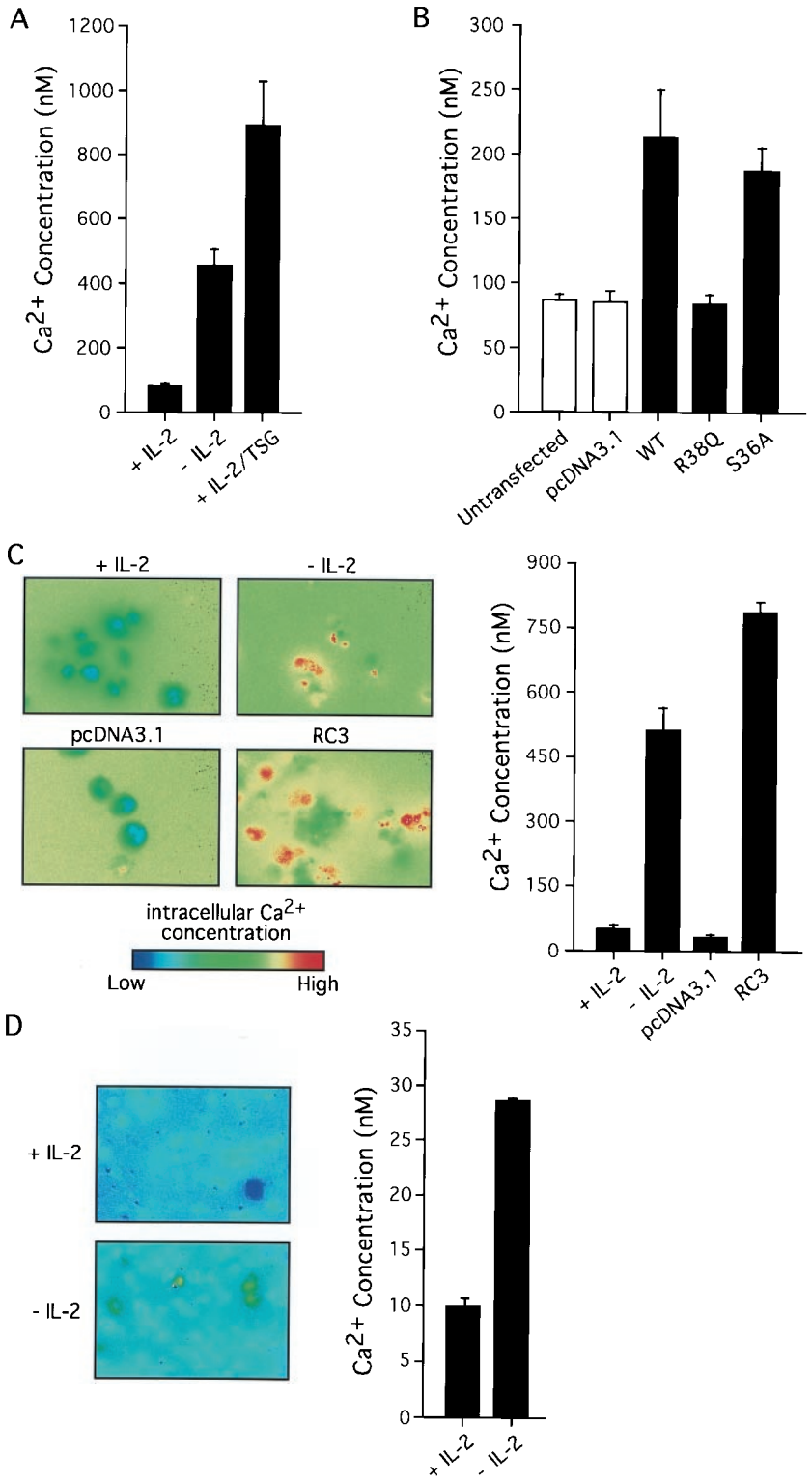

FIG. 6. Both IL-2 deprivation and *RC3* expression increase intracellular Ca^{2+} concentration. (A) HT-2 cells grown in the presence or absence of IL-2 for 12 h were stained with the fluorescent Ca^{2+} indicator fura-2 AM, and the intracellular Ca^{2+} concentration was measured (see Materials and Methods for details). As a control, HT-2 cells were treated with 2 μM thapsigargin (TSG), a Ca^{2+} store depletor, for 1 min before staining with fura-2 AM. Error bars indicate standard deviations. (B) HT-2 cells were transfected with a plasmid expressing RC3 or an RC3 mutant and analyzed as described for panel A. WT, wild type. (C) HT-2 cells were transfected with either pcDNA3.1 or an *RC3* expression plasmid; transfected cells were isolated from untransfected cells (see Materials and Methods for details) and, in parallel with controls with or without IL-2, stained with fura-2 AM. The left panels show the pseudocolor images of intracellular Ca^{2+} within fura-2-loaded cells; high Ca^{2+} levels are shown in red, and low Ca^{2+} levels are shown in blue or violet. Cells cultured in the absence of IL-2 or expressing RC3 display an apoptotic morphology. The average intracellular Ca^{2+} concentration of the cells shown in each field of view was calculated from background corrected images (see Materials and Methods) and is shown in the panel on the right. (D) Primary human T lymphocytes were grown in the presence or absence of IL-2 for 12 to 14 h and stained with fura-2 AM. The left panels show the pseudocolor images, and the right panel shows the average intracellular Ca^{2+} concentration as described for panel C.

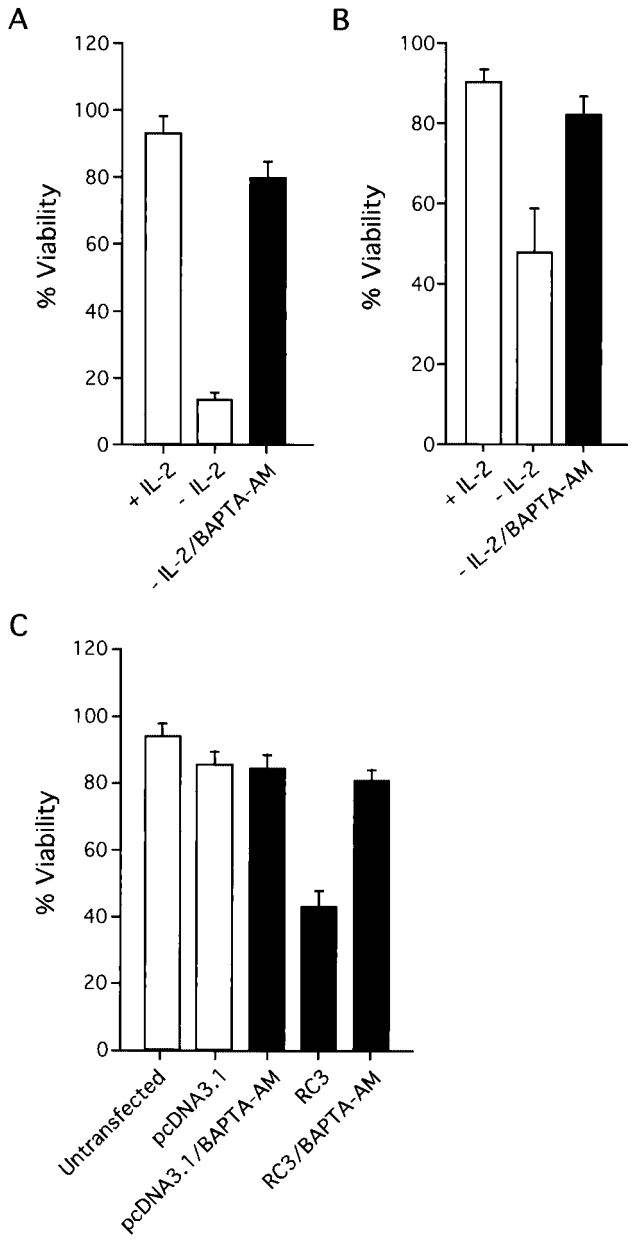

FIG. 7. An increase in intracellular Ca^{2+} is required for induction of apoptosis. (A) HT-2 cells grown in the presence or absence of IL-2 were incubated with BAPTA-AM, and the number of cells undergoing apoptosis was quantitated by annexin V-FITC staining and FACS analysis. Error bars indicate standard deviations. (B) Primary human T lymphocytes grown in the presence or absence of IL-2 were incubated with BAPTA-AM, and the number of cells undergoing apoptosis was quantitated as described for panel A. (C) HT-2 cells transfected with either pcDNA3.1 or an *RC3* expression plasmid were treated with BAPTA-AM, and the number of cells undergoing apoptosis was analyzed as described for panel A.

CaM interaction (12). Thus, a reduction in PKC phosphorylation should stabilize the Ca^{2+}-free form of CaM, which would also increase the intracellular Ca^{2+} concentration. Finally, RC3 could also increase intracellular Ca^{2+} and induce apoptosis by regulating the association of Ca^{2+}/CaM with various proteins, thereby modulating other CaM-dependent pathways. One such protein may be the CaM-dependent plasma membrane Ca^{2+}-ATPase; a decreased efflux of intracellular Ca^{2+} could contribute to the Ca^{2+} increase.

The ability of a CaM binding protein to induce apoptosis when overexpressed has also been demonstrated with two proapoptotic serine/threonine kinases: DAPK and DRP-1. Intriguingly, like that of *RC3*, expression of *DRP-1* is also increased following IL-2 deprivation (Table 1). DRP-1 has been shown to induce apoptosis when expressed in a variety of cell lines (21), and both RC3 and DRP-1 have similar IQ motif CaM binding domains. It therefore appears that IL-2 deprivation increases the level of multiple CaM-interacting proteins that activate parallel apoptotic pathways, thus ensuring efficient onset of apoptosis.

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