MOLECULAR AND CELLULAR BIOLOGY, June 1997, p. 3005–3012 Vol. 17, No. 6
0270-7306/97/$04.00+0

T Cells Deficient in Inositol 1,4,5-Trisphosphate Receptor Are Resistant to Apoptosis

THOTTALA JAYARAMAN AND ANDREW R. MARKS*

Molecular Cardiology Program, Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, New York 10032

Received 2 August 1996/Returned for modification 23 September 1996/Accepted 5 March 1997

The type 1 inositol 1,4,5-trisphosphate receptor (IP3R1) calcium release channel is present on the endoplasmic reticulum of most cell types. T lymphocytes which have been made deficient in IP3R1 lack detectable IP3-induced intracellular calcium release and exhibit defective signaling via the T-cell receptor (TCR) (T. Jayaraman, E. Ondriasova, K. Ondrias, D. Harnick, and A. R. Marks, Proc. Natl. Acad. Sci. USA 92:6007–6011, 1995). We now show that IP3R1-deficient T cells are resistant to apoptosis induced by dexamethasone, TCR stimulation, ionizing radiation, and Fas. Resistance to TCR-mediated apoptosis in IP3R1-deficient cells is reversed by pharmacologically raising cytoplasmic calcium levels. TCR-mediated apoptosis can be induced in calcium-free media, indicating that extracellular calcium influx is not required. These findings suggest that intracellular calcium release via the IP3R1 is a critical mediator of apoptosis.

As a general paradigm, excitatory events at the plasma membrane trigger the release of intracellular stores of calcium. Cellular processes activated by the resulting increase in intracellular (cytoplasmic) calcium concentration ($[Ca^{2+}]_i$) include diverse phenomena such as muscle contraction, cellular proliferation, synaptic transmission, fertilization, and antigen-specific T-cell activation. In T cells, after stimulation of the T-cell receptor (TCR), a sustained elevation in $[Ca^{2+}]_i$ is one of the signals that lead to transcriptional activation of the interleukin-2 (IL-2) gene (15). IL-2 promotes cellular proliferation required for antigen-specific T-cell activation (53). Upon TCR stimulation an initial rapid elevation in $[Ca^{2+}]_i$ is due to the release of intracellular calcium stores via inositol 1,4,5-trisphosphate receptor (IP3R) calcium release channels on the endoplasmic reticulum. The resulting depletion of endoplasmic reticulum calcium stores activates a calcium channel in the plasma membrane (11, 60), causing a prolonged influx of calcium, a process called capacitative calcium entry (CCE) (38, 39). Inhibiting the expression of the type 1 IP3R (IP3R1) by stable transfection of antisense IP3R1 cDNA prevents TCR-mediated release of intracellular calcium and CCE and blocks IL-2 production (17).

Apoptosis, or programmed cell death, is a fundamental process that is required for the normal development and function of the immune system. Apoptosis can be induced in thymocytes by diverse stimuli, including glucocorticoids (35, 56), γ-irradiation (43), antibody to CD3 (αCD3) (33, 42, 45, 46, 48), antibody to Fas antigen (24, 36), CD2 antigen (2), and removal of essential growth factors (7, 37, 50).

It has been proposed that calcium activates endonucleases that cause DNA fragmentation and death in apoptotic cells. Increases in $[Ca^{2+}]_i$ have been shown to trigger apoptosis (19), and inducers of apoptosis that lead to increases in $[Ca^{2+}]_i$ include glucocorticoids (20, 32), γ-irradiation (47), and αCD3 (46). Pharmacological elevation of $[Ca^{2+}]_i$ with calcium ionophores (56) or the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin can initiate apoptosis (18). The antideath protein Bcl-2 reduces calcium release from the endoplasmic reticulum via an unknown mechanism (26), and chelation of intracellular calcium inhibits apoptosis (32, 47). Thus, it would appear that elevation of $[Ca^{2+}]_i$ is a generalized trigger for apoptosis. However, in some instances $[Ca^{2+}]_i$ elevation blocks cell death, but this occurs in cells lacking calcium-dependent endonuclease activity in their nuclei, suggesting that other mechanisms for DNA fragmentation are present (41).

In addition to calcium-activated nucleases, other key molecules associated with the regulation of apoptosis include cytokines, the cysteine protease IL-1β-converting enzyme (ICE), and the transcription factor p53. The role of cytokines in the regulation of TCR-dependent T-cell apoptosis remains controversial. IL-2 has been shown to induce apoptosis in mature T cells (27), whereas it prevents apoptosis in human medullary thymocytes and activated peripheral T cells (13). Antibodies to Fas (CD95), a cell surface protein with homology to tumor necrosis factor receptor, can induce apoptosis (49, 58). T cells deficient in ICE are resistant to Fas-mediated apoptosis, suggesting that ICE is one of the components in the Fas pathway (29). ICE-deficient mice develop normally, and their thymocytes undergo apoptosis induced by glucocorticoids or ionizing radiation but are resistant to apoptosis induced by Fas antibody (25, 28). The tumor suppressor p53 is required for DNA-damaging agents to induce apoptosis. Thymocytes from p53-deficient mice are resistant to apoptosis induced by radiation (30). It appears that, rather than activating transcription, p53 acts as a repressor of genes necessary for cell survival or as a component of the enzymatic machinery involved in apoptotic DNA fragmentation (3).

In order to explore the role of IP3R1 in T-cell signaling, we made Jurkat T cells that were deficient in this intracellular calcium release channel. We initially observed that IP3R1-deficient T cells have defective TCR-mediated signaling, despite normal levels of TCR (17). Moreover, after pharmacologic depletion of intracellular calcium stores with thapsigargin, IP3R1-deficient T cells exhibited normal CCE, indicating that the calcium influx pathway was intact. Stimulating IP3R1-deficient T cells with ionomycin and phorbol myristate acetate resulted in normal production of IL-2, indicating that except for the defect in intracellular calcium release, the signaling machinery required for T-cell activation was intact (17).

* Corresponding author. Mailing address: Molecular Cardiology Program, P&S 9-401, College of Physicians and Surgeons of Columbia University, 630 W. 168th St., New York, NY 10032. Phone: (212) 305-0270. Fax: (212) 305-0156. E-mail: arm42@columbia.edu.

Seemingly, no two cellular functions are more disparate than proliferation and programmed cell death. However, in the current study we demonstrate that IP3R1-deficient T cells that are defective in TCR-mediated signaling are also resistant to apoptosis induced by TCR stimulation, Fas, glucocorticoids, and ionizing radiation. TCR-mediated upregulation of CD95 is abrogated in the IP3R1-deficient cells. Pharmacologically elevating $[Ca^{2+}]_i$ with thapsigargin restores the cells' sensitivity to apoptotic induction via TCR stimulation, and chelation of extracellular calcium with EGTA does not block apoptosis in wild-type cells. Together these findings demonstrate that IP3R1 plays a role in generating the calcium signal during induced apoptosis. These observations suggest that the IP3-gated intracellular calcium release channel on the endoplasmic reticulum is central to divergent signaling pathways that lead either to proliferation or to cell death, and that whether cells live or die must be determined by additional costimulatory events.

MATERIALS AND METHODS

Cell culture and characterization. Jurkat cells were grown in RPMI medium containing 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 μg of streptomycin/ml. IP3R1 expression was inhibited in the Jurkat cells by stable transfection with an antisense 2.9-kb fragment of the 5′-most portion of the IP3R1 cDNA with hygromycin selection as described previously (17). For all experiments, the same construct in the sense orientation and sense and antisense vectors alone were used as transfection controls. αCD3 (HIT3a, an immunoglobulin G2a antibody) (Pharmingen) was used to stimulate the TCR as described previously (17). Exponentially growing cells were γ-irradiated at a dose of 20 Gy with ^{137}Cs as the source (Memorial Sloan-Kettering Cancer Center, New York, N.Y.). Cells were then plated for an additional 40 h, and the percentage of dead cells was scored by trypan blue dye positivity. The percentage of cells that were dead after γ-irradiation was compared with that of cells that had not been irradiated. Cell cycle progression was examined with propidium iodide (50 μg/ml)-stained cells analyzed by flow cytometry. Cells were made quiescent by removal of serum for 24 h, after which serum (10% fetal calf serum) was added. Cells were stained with propidium iodide at various time points and analyzed by flow cytometry with a Coulter analyzer.

IP3R protein analyses. IP3R immunoblotting with a polyclonal antibody specific for IP3R1 (14) or for IP3R2 (54) or with a monoclonal antibody specific for IP3R3 (Transduction Laboratories) was performed as described previously (14). Proteins were size fractionated by sodium dodecyl sulfate–6% polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antibodies, followed by signal detection with enhanced chemiluminescence and Western blot reagents (Amersham).

Calcium measurements. Jurkat cells in RPMI 1640 solution were placed on coverslips coated with Cell-Tak (Biocoat) and incubated for 20 min at 37°C. After incubation, 5 μM Fluo-3 AM (Molecular Probes, Eugene, Oreg.) was added and the mixture was incubated for 40 min at 37°C, diluted 10-fold, and incubated for an additional 30 min at 37°C. The cells were washed three times with Krebs-Ringer solution containing 1 mM calcium or 40 μM calcium (the latter was considered calcium-free). Coverslips were mounted in a microscope tissue chamber. Measurements of intracellular calcium in the Jurkat cells were performed with a Nikon inverted microscope. Data analysis was performed with Kinephoto software (Kinetek, Yonkers, N.Y.). The cells were stimulated with αCD3. At the conclusion of each trial, maximal fluorescence (F_{max}) was obtained by the addition of 6 μM digitonin and 5 mM $CaCl_2$. Minimal fluorescence (F_{min}) was obtained by exchanging the calcium solution for 6 μM digitonin with 20 mM EGTA. $[Ca^{2+}]_i$ was calculated from these values as $K_d(F - F_{min})/(F_{max} - F)$, where K_d is 400 nM (21). Fluorescence measurements were performed at room temperature.

Calcium levels in populations of cells were determined by analyzing Indo-1 AM (Molecular Probes)-loaded cells by flow cytometry. Cells were incubated with Indo-1 AM (3 μM) for 40 min at 37°C as described previously (5). Calcium levels were determined by calculating the ratio of Indo-1 fluorescence emission at 395 nm and at 500 nm with a Coulter flow cytometer.

DNA and nuclear fragmentation assays. We cultured 5×10^3 cells per well in 24-well plates with or without plate-bound αCD3. At various time points, 20 μl of cells was removed from each well, mixed with an equal volume of trypan blue, and counted with a hemocytometer. Cells taking up trypan blue were scored as dead and were expressed as a percentage of the total number of cells counted. DNA fragmentation was assessed with propidium iodide-stained cells as described previously (34). We plated 5×10^3 cells per well in 24-well plates with or without platebound αCD3; at various time points, cells were collected, resuspended in a hypotonic buffer (0.1% sodium citrate and 0.1% Triton X-100) containing 50 μg of propidium iodide/ml, and analyzed with a Coulter flow cytometer. The percentage of cells undergoing DNA fragmentation was determined based on the number of cells that had a DNA content in the subdiploid range that is characteristic of apoptotic cells.

For DNA fragmentation assays (4), cells were lysed in a solution containing 5 mM Tris-Cl (pH 8.0), 5% glycerol, 0.05% bromophenol blue, and 5 mg of RNase A/ml. The sample was loaded onto a discontinuous agarose gel with the part above the sample wells containing 1% agarose, 2% sodium dodecyl sulfate, and 64 μg of proteinase K/ml and the part below the wells containing 2% agarose. Gels were run at 60 mV for 14 h at room temperature. DNA was visualized by ethidium bromide staining as described previously (31).

DNA synthesis was determined by measuring [^{3}H]thymidine incorporation as described previously (16). Triplicate cultures containing 5×10^3 cells per well were cultured in a 96-well plate precoated with various concentrations of αCD3. Cells were harvested at various time points, and [^{3}H]thymidine incorporation was measured with a scintillation counter.

Analysis of CD95 expression. Cells (5×10^5 per well) were stimulated with plate-bound αCD3 for a set period at 37°C and analyzed for CD95 expression with human anti-CD95 antibody (αCD95; Pharmingen). A minimum of 3,000 cells were analyzed for each sample.

RESULTS

Apoptotic resistance in IP3R1-deficient Jurkat cells. To examine the role of IP3-gated Ca^{2+} channels in signaling apoptosis, we used a T-cell (Jurkat) line that is deficient in the expression of IP3R1 and that fails to mobilize calcium upon TCR stimulation (17). The Jurkat T cells from which the IP3R1-deficient cells were derived express three forms of IP3R which can be detected by immunoblotting with type-specific antibodies. The most abundant form appears to be IP3R1, with lower levels of IP3R2 and IP3R3 (Fig. 1A); however, possible differences in the avidity of the different antibodies used in these experiments limit the significance of the relative signals seen by immunoblotting. IP3R1-deficient T cells had no detectable IP3R1 and had decreased but detectable levels of IP3R2 and IP3R3 protein detected by immunoblot analyses (Fig. 1A). Compared to the controls (which were sense vector transfected), IP3R1-deficient T cells were defective in intracellular calcium release after activation with αCD3 (Fig. 1B).

TCR stimulation with αCD3 induces apoptosis in Jurkat cells (33, 46). In sense vector-transfected (control) Jurkat T cells, activation with αCD3 inhibited cell growth (Fig. 2A) and DNA synthesis (Fig. 2B) in a concentration-dependent manner and induced apoptosis marked by DNA fragmentation in ~40% of the cells (Fig. 2C). In contrast, αCD3 had no significant effect on cell growth and did not induce apoptosis in IP3R1-deficient cells (Fig. 2A to C).

In addition to TCR stimulation with αCD3, well-characterized inducers of apoptosis in T cells include Fas ligand (58), glucocorticoids (32), irradiation (56), and serum deprivation (7). Jurkat cells cultured with αCD95 exhibited apoptosis marked by an increase in the percentage of cells exhibiting DNA fragmentation, but IP3R1-deficient cells did not (Fig. 3A).

Glucocorticoids induce DNA damage and apoptosis possibly via a calcium-dependent mechanism (4). Dexamethasone induced apoptosis in the control but not in IP3R1-deficient cells (Fig. 3B). Irradiation induces DNA damage and apoptosis via a p53-dependent pathway (30). The role of calcium in p53-mediated apoptosis is not well understood. Irradiation induced apoptosis in the control Jurkat T cells but not in IP3R1-deficient cells (Fig. 3C). For Fig. 3A to C flow cytometry was used to determine the percentage of cells exhibiting DNA fragmentation. In addition, DNA fragmentation was visualized with agarose gels (Fig. 3D). Control cells, but not IP3R1-deficient cells, exhibited DNA laddering consistent with apoptosis following stimulation with αCD3, αCD95, γ-irradiation, and dexamethasone (Fig. 3D). Taken together, these results suggest that four pathways leading to apoptosis—TCR stimula-

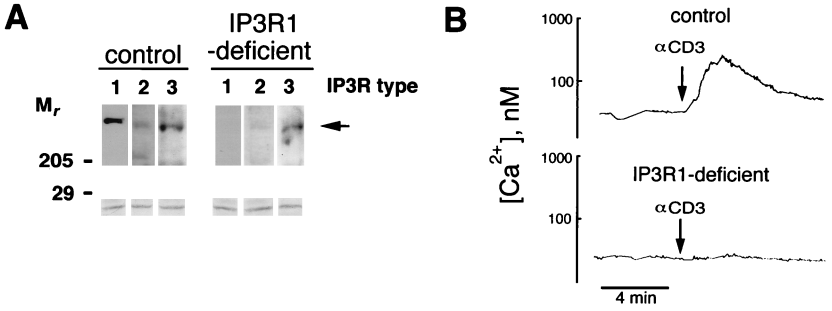

FIG. 1. IP3R1-deficient Jurkat cells have defective intracellular calcium release in response to TCR stimulation. (A) Immunoblot analyses of proteins from lysates of parental and IP3R1-deficient Jurkat T cells with antibodies specific for IP3R1, IP3R2, or IP3R3. IP3R1 is the most abundant form of IP3R expressed in the parental Jurkat T cells. It is not detected by immunoblot analysis in Jurkat T cells stably transfected with antisense IP3R1 cDNA. Lower levels of IP3R2 and IP3R3 are detected in both parental and IP3R1-deficient Jurkat T cells. The arrow indicates the position of the IP3R proteins which migrate at ~300 kDa. The same nitrocellulose filters were incubated with αCD3 to indicate the relative levels of a common antigen detected on each immunoblot. Molecular weight (in thousands) is indicated on the left. (B) Addition of soluble αCD3 to activate the TCR elicited a rapid and sustained rise in $[Ca^{2+}]_i$ in sense vector-transfected (control) Jurkat cells but not in IP3R1-deficient cells. Fluorescence measurements were performed on single cells loaded with the calcium-sensitive dye Fluo-3 AM. αCD3 was added at the times indicated by the arrows. Each experiment was repeated more than 10 times, and representative results are shown.

tion, CD95/Fas, glucocorticoids, and irradiation—involve intracellular calcium release via the IP3R1.

Growth factor withdrawal has been reported as an inducer of apoptosis in fibroblasts, IL-2-dependent T cells, and Jurkat T cells (7, 10, 59). Growth factor withdrawal induced apoptosis in both control and IP3R1-deficient cells (Fig. 4). Thus, in contrast to TCR stimulation, Fas, glucocorticoids, and irradiation, growth factor withdrawal induces apoptosis despite the defect in IP3-mediated intracellular calcium release in IP3R1-deficient Jurkat cells, suggesting that a calcium-independent signaling pathway is important for this form of apoptosis.

Cytosolic calcium levels were elevated in sense vector-transfected Jurkat cells, but not in IP3R1-deficient cells, in response to Fas stimulation, γ-irradiation, and dexamethasone, in addition to TCR stimulation (Fig. 5). Interestingly, in contrast to TCR stimulation, which induces an elevation in $[Ca^{2+}]_i$ within minutes, the other three apoptotic stimuli cause elevations in $[Ca^{2+}]_i$ 24 to 48 h after exposure of the cells (Fig. 5). To address the question of whether this late $[Ca^{2+}]_i$ rise in cells treated with Fas, γ-irradiation, and glucocorticoids can be blocked by EGTA (implying that this rise might be due to calcium entry through a damaged or leaky membrane), cells were stimulated at time zero, EGTA (2 mM, sufficient to chelate extracellular calcium to the level of ~10 μM) was added at 24 h, and cells were loaded with Indo-1 AM at 48 h for determination of $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ following Fas stimulation, γ-irradiation, and dexamethasone occurred between 24 and 48 h after stimulation and was not blocked by chelation of extracellular calcium.

Regulation of the Fas pathway in IP3R1-deficient Jurkat cells. The Fas pathway is an important regulator of apoptosis, and upregulation of CD95 on the cell surface correlates with increased kinetics of activation-induced apoptosis in T cells (51). As noted above, stimulation of Fas with αCD95 induced apoptosis in Jurkat cells but not in IP3R1-deficient cells (Fig. 3A). The resistance to Fas-mediated apoptosis led us to examine the levels of Fas/APO-1 on the IP3R1-deficient cells compared to those on (control) cells. Using flow cytometry of cells stained with αCD95, we detected similar levels of Fas/APO-1 in unactivated (control) cells and in IP3R1-deficient cells. However, upregulation of CD95 levels occurred only in the (control) cells after TCR stimulation and not in the IP3R1-deficient cells (Fig. 6).

Induction of apoptosis in calcium-free media. IP3R1-deficient T cells are defective in intracellular calcium release, and as a result of the inability to deplete the intracellular calcium store after TCR stimulation, there is no calcium influx (17). To determine whether it is intracellular calcium release or calcium influx that signals apoptosis, Jurkat T cells were stimulated with αCD3 in calcium-free medium, and apoptosis was determined. TCR-mediated apoptosis occurred whether or not extracellular calcium was present, as long as intracellular calcium release occurred (Fig. 7A). IP3R1-deficient T cells cross-linked with αCD3 and treated with thapsigargin (to pharmacologically deplete the intracellular calcium stores and raise $[Ca^{2+}]_i$) in the absence of extracellular calcium exhibited degrees of apoptosis similar to those shown by (control) cells after TCR stimulation, indicating that pharmacologically releasing intracellular calcium stores restores the cells' susceptibility to induced apoptosis (Fig. 7A). Exposure of cells to low concentrations of thapsigargin (10 and 20 nM) did not induce apoptosis in either control cells or IP3R1-deficient cells (Fig. 7B). Low concentrations of thapsigargin (10 and 20 nM) did not restore the cells' sensitivity to the four inducers of apoptosis that were examined, αCD3, Fas, γ-irradiation, and glucocorticoids (Fig. 7C and 8). These results suggest that a rise in $[Ca^{2+}]_i$ rather than depletion of calcium stores is the signal required for apoptosis.

DISCUSSION

We report that T cells deficient in intracellular calcium release via the IP3R1 have defective TCR-mediated signaling and are resistant to induced apoptosis. These findings are consistent with earlier reports showing that calcium plays a role in antigen-specific TCR signaling and in apoptosis. The identification of IP3R1 as a regulator of induced apoptosis indicates that intracellular calcium release via this channel is involved in both T-cell activation and apoptosis. How calcium itself interacts with these other apoptotic signals remains uncertain, although activation of calcium-dependent enzymes (e.g., proteases and/or nucleases) may be a common feature of multiple pathways leading to apoptosis.

Activation of parental Jurkat cells via TCR stimulation with αCD3 resulted in a rapid rise of calcium due to both an initial intracellular calcium release from internal stores and a subsequent sustained calcium influx through a plasma membrane (Fig. 1B). When the same experiment was conducted with essentially Ca^{2+}-free extracellular solution, calcium release but not calcium influx occurred, indicating that the initial rise in

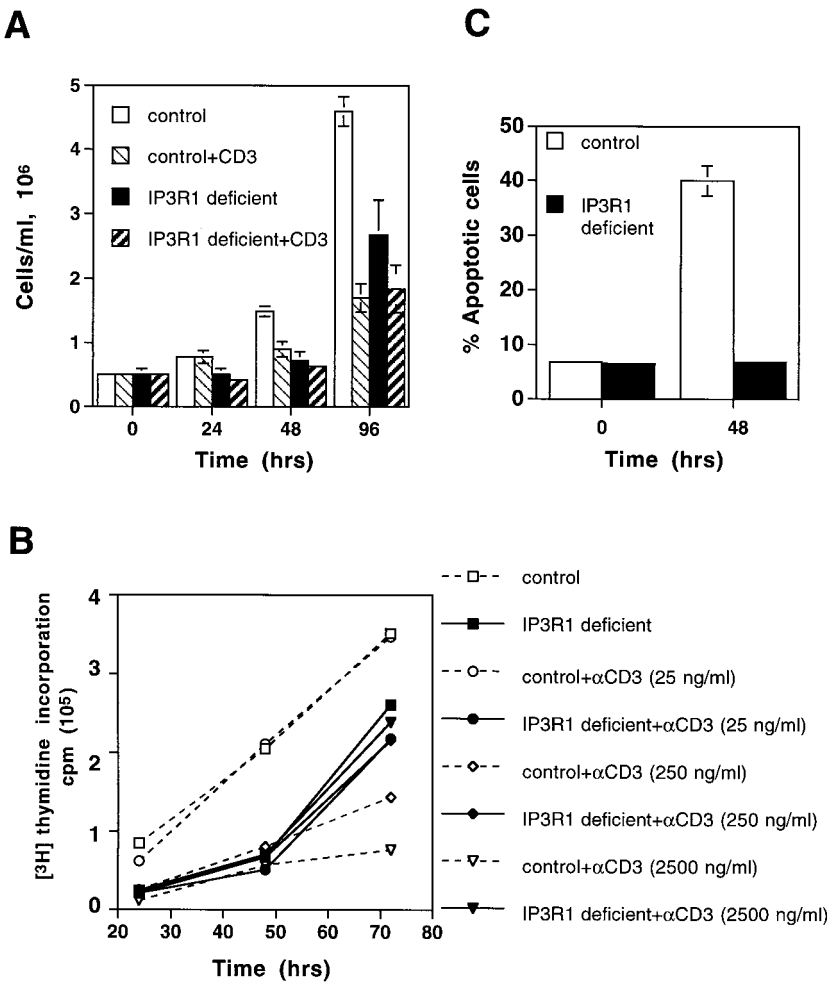

FIG. 2. TCR stimulation induces apoptosis in control Jurkat cells but not in IP3R1-deficient cells. (A) Sense vector-transfected (control) Jurkat T cells exhibit reduced cell growth after TCR stimulation with αCD3 compared to that of unstimulated cells growing in culture. In contrast, no significant reduction in cell numbers occurs when IP3R1-deficient T cells are stimulated with αCD3. Cell numbers were determined for Jurkat cells growing in medium plus 10% FBS with or without plate-bound αCD3. (B) DNA synthesis was inhibited in (control) cells after TCR stimulation with αCD3. In contrast, there was no inhibition of DNA synthesis after TCR stimulation in IP3R1-deficient T cells over a range of concentrations of αCD3. DNA synthesis was determined by measuring [^{3}H]thymidine incorporation in triplicate samples for each data point. (C) The percentage of cells exhibiting DNA fragmentation, a marker for apoptosis, was increased in (control) cells after TCR stimulation with αCD3 but not in IP3R1-deficient T cells. DNA fragmentation was determined in propidium iodide-stained cells by flow cytometry. Error bars represent standard errors of the means.

$[Ca^{2+}]_i$ is due to the release of internal calcium stores in the parental Jurkat cells (17).

The antisense cDNA used to inhibit IP3R1 expression was 2.9 kb long and had 60 to 75% homology with IP3R2 and IP3R3 at the nucleotide level (14, 57). Thus, expression of all three types of IP3R could have been inhibited. To determine whether IP3R2 or IP3R3 is expressed in IP3R1-deficient T cells, immunoblotting was conducted with antibodies specific for either IP3R2 or IP3R3. Immunoblot analyses did not detect IP3R1 protein, but low levels of IP3R2 and IP3R3 were present in IP3R1-deficient and parental Jurkat T cells (Fig. 1A). These data suggest that in IP3R1-deficient Jurkat T cells neither IP3R2 nor IP3R3 is sufficient to provide the calcium signal required for T lymphocytes to undergo TCR-mediated activation or induced apoptosis. Interestingly, it appears that IP3R1 is most abundant in Jurkat cells, with lower levels of IP3R2 and IP3R3 (Fig. 1A), in contrast to previous reports that IP3R3 is highly expressed in Jurkat cells and is more abundant than IP3R1 mRNA (57). It is possible that the discrepancy in the relative levels of the IP3R subtypes could be due to differences in the avidities of the antibodies rather than to the relative amounts of the antigens.

It has been proposed that there may be a calcium-sensitive step involved in Fas signaling (52) and that αCD3-induced apoptosis is mediated, at least in part, through Fas/CD95 expression (53). Unstimulated cells from both wild-type and IP3R-deficient T cells showed comparable low levels of Fas expression (Fig. 6). Activation of sense vector-transfected (control) Jurkat T cells with αCD3 resulted in a rapid upregulation of Fas levels, whereas no such upregulation of Fas was seen in IP3R1-deficient T cells. Cells with increased levels of Fas are more susceptible to apoptosis, and Fas appears to play a role in mediating immune privilege (12). Upregulation of Fas has been reported to increase the kinetics of activation-induced apoptosis in T cells (51). Increased levels of Fas on T cells may contribute to the pathology of autoimmune diseases, such as the lymphopenia seen with systemic lupus erythematosus (1). The present study suggests that upregulation of Fas after TCR stimulation depends on intact TCR signaling and may be calcium dependent as well.

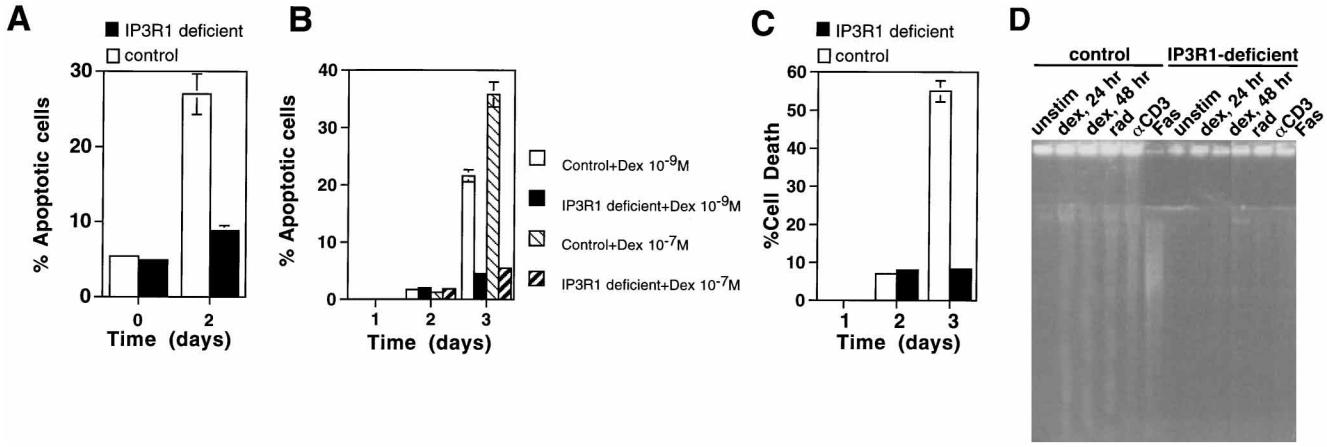

FIG. 3. Resistance to inducers of apoptosis in IP3R1-deficient cells. (A) Activation of the Fas pathway. Cells were activated by cross-linking plate-bound αCD95 as described previously (22). We added 0.5×10^3 cells per well from sense vector-transfected (control) Jurkat T cells or IP3R1-deficient T cells to αCD95-coated wells. The cells were collected after 48 h in culture, washed, and stained with propidium iodide, and apoptotic nuclei were detected as described in Materials and Methods. (B) Dexamethasone (Dex) was added to cells at the indicated concentrations. After 48 h cells were collected, washed, stained with propidium iodide, and analyzed as described in Materials and Methods. (C) Cells were γ-irradiated with 20 Gy and cultured in RPMI medium containing 10% FBS for an additional 40 h at 37°C. The number of dead cells was determined by using the trypan blue exclusion test and expressed as a percentage of the total number counted. (D) DNA fragmentation was examined by agarose gel electrophoresis for each of the following conditions: unstimulated (unstim), dexamethasone (10^{-7} M) for 24 h (dex, 24 h), dexamethasone (10^{-7} M) for 48 h (dex, 48 h), irradiated as for panel C (rad), 48 h after stimulation with αCD3 (αCD3), and 48 h after stimulation with αCD95 as for panel A (Fas). Error bars show standard errors of the means. No error bars are shown when the range of the standard error is small.

The finding that $[Ca^{2+}]_i$ is elevated 24 to 48 h after certain apoptotic stimuli (including Fas, dexamethasone, and γ-irradiation) in parental and control cells but not in IP3R1-deficient cells (Fig. 5) provides further support for the hypothesis that an increase in $[Ca^{2+}]_i$ is an important signal for apoptosis induced by these agents. TCR stimulation induced an immediate rise in $[Ca^{2+}]_i$ (Fig. 1B), whereas other inducers caused elevations hours after the initial stimulation. This suggests that an elevation of $[Ca^{2+}]_i$ at any point within the first 24 to 48 h after an initial signal is sufficient to allow apoptosis to proceed. Data in the present study indicate that the late $[Ca^{2+}]_i$ rise in cells treated with Fas, γ-irradiation, and glucocorticoids is due to intracellular calcium release, not calcium flux through the plasma membrane. Moreover, depletion of the endoplasmic reticulum calcium pool with low concentrations of thapsigargin does not restore the cells' sensitivity to inducers of apoptosis in IP3R1-deficient cells (Fig. 7C and 8), suggesting that a rise in $[Ca^{2+}]_i$ rather than depletion of calcium stores is the signal required for apoptosis. Addition of EGTA to cell culture medium such that the extracellular calcium concentration is reduced to 10 μM prevents calcium entry through the voltage-gated calcium channels and is considered to be equivalent to calcium-free medium. It should be noted, however, that even in essentially calcium-free medium a nonspecific leak in the plasma membrane (with unknown biophysical properties) could theoretically permit calcium to enter the cell because there would still be a 100-fold concentration gradient for calcium (e.g., 10 μM extracellular and 100 nM intracellular).

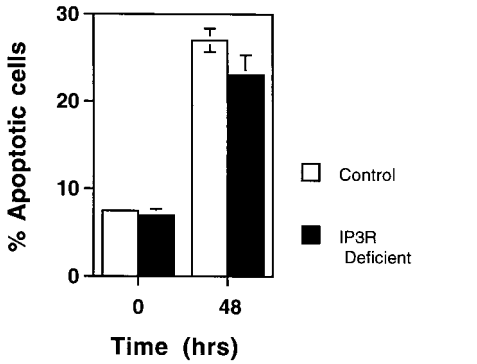

FIG. 4. Serum withdrawal induces apoptosis in both sense vector-transfected (control) Jurkat cells and IP3R1-deficient T cells. Cells growing exponentially in medium containing 10% FBS were washed three times and placed in RPMI medium with no serum. At the indicated time points, cells were collected, stained with propidium iodide, and analyzed as described in Materials and Methods. Error bars represent standard errors of the means.

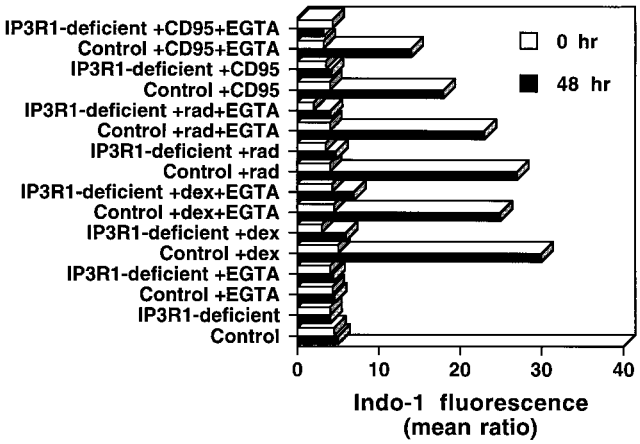

FIG. 5. Effects of inducers of apoptosis on cytosolic calcium levels in control and IP3R1-deficient Jurkat T cells. Apoptotic inducers (dexamethasone [dex], γ-irradiation [rad], and αCD95) all induce a rise in $[Ca^{2+}]_i$ at 48 h that is likely due to intracellular calcium release. Cytosolic calcium levels were determined in Indo-1-loaded sense vector-transfected (control) and IP3R1-deficient Jurkat T cells at the indicated times following stimulation with dexamethasone (10^{-7} M), γ-irradiation at 20 Gy, or αCD95. Addition of EGTA (2 mM) did not block the late rise in $[Ca^{2+}]_i$. In each case cells were stimulated as described in Materials and Methods and in the legends to Fig. 2 and 3. One hour prior to analysis by flow cytometry, cells were loaded with Indo-1 AM (3 μM). The mean Indo-1 ratio of 395-nm/500-nm fluorescence emission is plotted at baseline (unstimulated cells) and at 48 h.

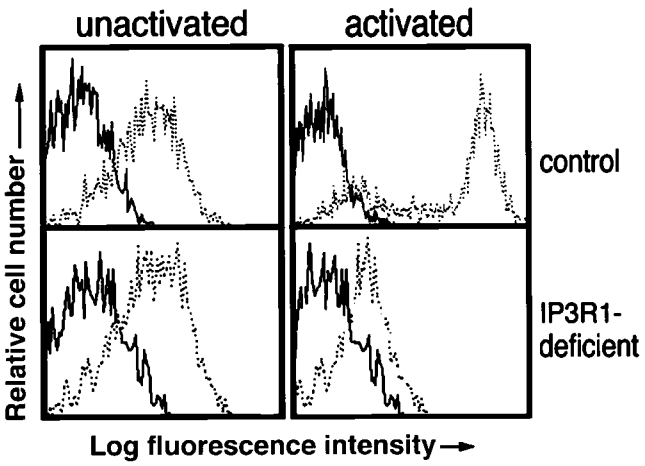

FIG. 6. Defective upregulation of Fas in TCR-stimulated IP3R1-deficient cells. Fas levels were similar in unactivated sense vector-transfected (control) Jurkat cells and IP3R1-deficient cells (left panels). Fas levels increased 48 h after TCR stimulation in control cells but not in IP3R1-deficient T cells (right panels). Fas expression was determined by flow cytometry with cells immunostained with αCD95 conjugated to fluorescein isothiocyanate. Cells were activated by cross-linking to αCD3. The thick line represents cells stained with preimmune serum; the dotted line represents cells stained with αCD95.

Apoptosis during growth factor withdrawal has been described for systems in which the retinoblastoma protein (Rb) pathway is activated without the concurrent activation of other mitogenic pathways. For example, blocking Rb action by overexpression of adenovirus E1A protein, a stoichiometric inhibitor of Rb (8, 9), or circumventing it by E2F overexpression induces apoptosis when cells are placed in serum-free medium (40, 44). This process appears to involve a p53-dependent checkpoint that triggers apoptosis in response to an imbalance of mitogenic signals (6, 55). Indeed, it has been proposed that inactivation of Rb by hyperphosphorylation may cause apoptosis when cells are deprived of growth factors (6, 40, 44, 55). The hyperphosphorylated form of Rb persisted in both control and IP3R1-deficient Jurkat cells after growth factor withdrawal (data not shown), indicating that Rb was inactivated and possibly explaining why these cells were driven to apoptosis, rather than growth arrest, by serum deprivation.

Although Rb hyperphosphorylation was also observed in cells after TCR activation and after Fas stimulation (data not shown), it is unlikely that it was the cause of apoptosis in these situations, since inactivation of Rb has not been shown to cause apoptosis without concomitant growth factor withdrawal. The fact that the IP3R1-deficient Jurkat cells can undergo apoptosis when growth factors are withdrawn indicates that the final common pathways that result in programmed cell death are intact in the antisense vector-transfected cells. In other words, the IP3R1-deficient Jurkat cells are capable of undergoing apoptosis under some conditions (presumably triggered by non-calcium-dependent stimuli).

The argument could be made that the expression of antisense IP3R1 mRNA in Jurkat cells disrupted a number of signaling pathways, resulting in a generalized defect in these cells, one of the consequences of which is resistance to apoptosis. Several points argue against this possibility, including the fact that the cells produce normal amounts of IL-2 and CD69 when they are stimulated with phorbol myristate acetate and ionomycin (17). Moreover, data showing that mitogen withdrawal induces apoptosis in IP3R1-deficient cells (Fig. 4) and that pharmacologically raising $[Ca^{2+}]_i$ restores the sensitivity of the cells to inducers of apoptosis (e.g., αCD3, as shown in Fig. 7A) underscores the point that apoptotic signaling is intact, with the exception of IP3R1. Thus, the defect in the IP3R1-deficient cells that results in resistance to apoptosis appears to be specific for the IP3R1.

A delay in cell cycle progression results in a reduced growth rate in the IP3R1-deficient cells (data not shown). However,

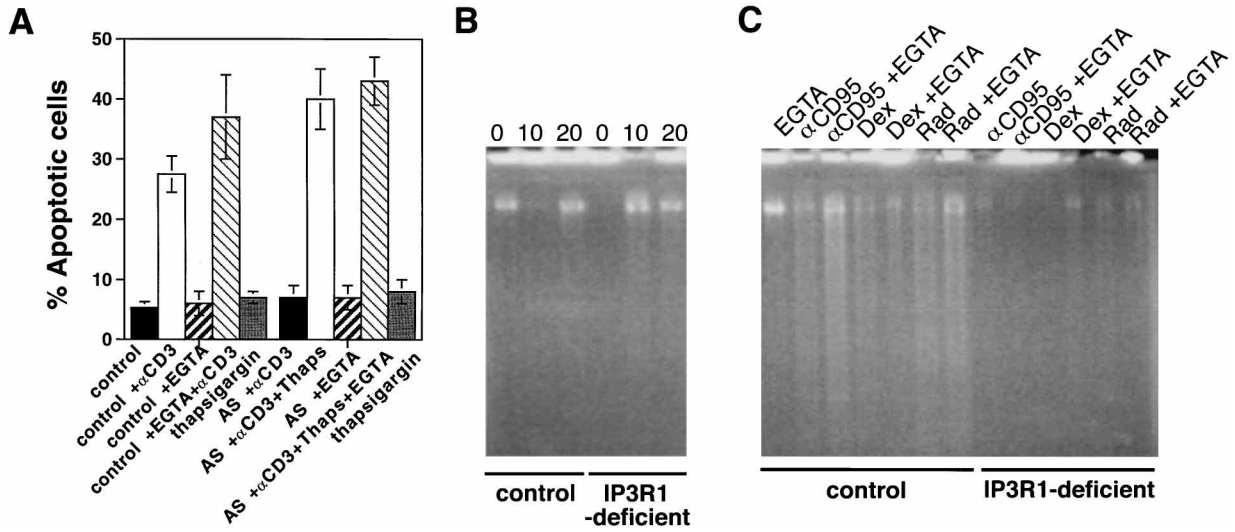

FIG. 7. Role of intracellular calcium release in induced apoptosis. (A) Apoptosis was induced 48 h after TCR stimulation without extracellular calcium. Cells were stimulated by cross-linking to αCD3 in media containing physiological concentrations of extracellular calcium (control + αCD3) and in media in which the extracellular calcium was chelated with 1.0 mM EGTA (control +EGTA+αCD3). Similar results were observed when 2.5 mM EGTA was used to chelate extracellular calcium. Sensitivity to TCR-mediated apoptosis was restored in IP3R1-deficient T cells (AS) by pharmacologically increasing cytosolic calcium levels with the calcium ATPase inhibitor thapsigargin (100 nM) (AS +αCD3+Thaps). Similar results were observed when extracellular calcium was chelated with EGTA (1.0 mM) (AS +αCD3+Thaps+EGTA). Thapsigargin alone (100 nM) did not induce apoptosis in either control cells or IP3R1-deficient cells. The percentage of cells exhibiting DNA fragmentation was determined by flow cytometry of propidium iodide-stained cells. (B) Low concentrations of thapsigargin (10 and 20 nM) applied for 48 h did not induce apoptosis as determined by agarose gel electrophoresis in either control or IP3R1-deficient cells. (C) Chelation of extracellular calcium with EGTA (2 mM) did not inhibit apoptosis induced by Fas (αCD95), γ-irradiation (Rad), or glucocorticoids (Dex).

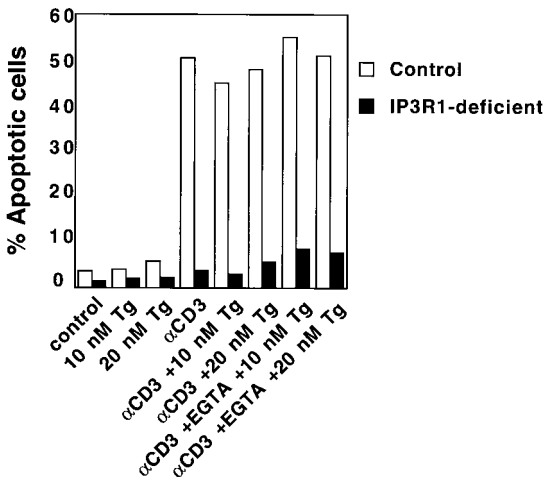

FIG. 8. Depletion of intracellular calcium stores does not restore sensitivity of cells to apoptosis in IP3R1-deficient Jurkat cells. Low concentrations (10 or 20 nM) of thapsigargin (Tg) did not induce apoptosis in either sense vector transfected (control) or IP3R1-deficient Jurkat cells. Moreover, exposure to 10 or 20 nM thapsigargin for up to 48 h did not restore the cells' sensitivity to αCD3-mediated apoptosis. Addition of EGTA (2 mM) to prevent CCE secondary to depletion of calcium stores did not inhibit αCD3-mediated apoptosis. The percentage of cells exhibiting DNA fragmentation was determined by flow cytometry of propidium iodide-stained cells.

the decrease in apoptosis in IP3R1-deficient cells is out of proportion to the delay in cell cycle progression. Compared to control cells, IP3R1-deficient cells exhibited a delay in progression to G_2, but the percentage of cells in S phase was only reduced by 15% (9.5 versus 11.1% for IP3R1-deficient versus control cells). This relatively small reduction in S-phase cells cannot explain the ~80% reduction in the number of IP3R1-deficient cells that undergo apoptosis compared to the number of control cells that do so.

The data showing that raising $[Ca^{2+}]_i$ with thapsigargin in IP3R1-deficient cells, even in calcium-free media (Fig. 7A), restores the ability of TCR stimulation with αCD3 to induce apoptosis further implicates intracellular calcium release as opposed to calcium influx as the critical source of calcium during induction of apoptosis. Moreover, since the thapsigargin-induced increase in $[Ca^{2+}]_i$ in calcium-free medium in IP3R1-deficient cells is sufficient to allow αCD3 to trigger apoptosis, it appears that CCE is not required for induced apoptosis. In support of the lack of a requirement for calcium influx (i.e., CCE) during induced apoptosis is the finding that αCD3 can trigger apoptosis in calcium-free media in the parental (data not shown) and control (Fig. 7A) Jurkat T cells.

The finding that extracellular EGTA does not block αCD3-induced apoptosis is in disagreement with earlier studies showing that removal of extracellular Ca^{2+} blocks apoptosis induced by αCD3 or by glucocorticoids (32, 33). One possible explanation for this discrepancy is the difference in the concentrations of EGTA used to remove extracellular Ca^{2+} in each study. McConkey et al. (32, 33) used 8 mM EGTA as opposed to the 1 or 2.5 mM EGTA used in the present study. In the present study the concentration of EGTA was chosen by calculating how much EGTA would be required to buffer the calcium concentration in the cell culture media to ~10 μM, or >10-fold higher than cytosolic $[Ca^{2+}]$. At 8 mM, EGTA might remove intracellular cations and possibly even deplete the endoplasmic reticulum, resulting in inhibition of both calcium influx and intracellular calcium release. In contrast, we have shown that under the conditions considered to be calcium free IP3-induced calcium release is preserved, whereas calcium influx does not occur (17).

While this article was under review, Khan et al. reported that blocking expression of IP3R3, but not IP3R1, in B and T lymphocytes with antisense oligonucleotides inhibited triggered apoptosis (23). They suggest that calcium influx via IP3R3 in the plasma membrane is required for triggered apoptosis. Our results are in agreement with those of Khan et al. in terms of the fundamental observation that calcium flux via an IP3R is required for some forms of triggered apoptosis. We differ in that our data indicate that calcium influx is not essential for triggered apoptosis, as evidenced by the fact that apoptosis was induced in calcium-free media (Fig. 7A and C and 8). Moreover, in our studies IP3R1-deficient cells were resistant to apoptosis despite the presence of IP3R3 (Fig. 1). Khan et al. found that IP3R3 levels were highest and IP3R1 levels were lowest in cells after 80% had undergone apoptosis. These results of Khan et al. suggest that cells with the highest levels of IP3R3 and lowest levels of IP3R1 are relatively resistant to apoptosis and thus survive longer, which is in agreement with our findings that cells deficient in IP3R1 are in fact resistant to apoptosis.

The role of calcium in apoptosis has been controversial. The present study substantially strengthens the argument that calcium is important for apoptosis induced by TCR stimulation, glucocorticoids, Fas, and ionizing radiation. Moreover, our data show that IP3-induced intracellular calcium release, or pharmacologically increased $[Ca^{2+}]_i$ in IP3R1-deficient cells, is sufficient to support calcium-dependent forms of apoptosis. Since IP3-gated intracellular calcium release channels are widely expressed in most types of cells, we propose that they serve as a common pathway for releasing intracellular calcium stores after cells have been stimulated by a wide array of signals, including growth factors and inducers of apoptosis. Accordingly, while calcium release is essential, whether cells live or die is more likely determined by other signals associated with activation of specific pathways that lead either to cellular proliferation or to apoptosis.

ACKNOWLEDGMENTS

We thank R. Wojcikiewicz for providing antibody to IP3R2 and J. Putney for helpful comments.

This work was supported by grants to A.R.M. from the National Institutes of Health (RO1 AI39794 and HL56180) and the Muscular Dystrophy Association and to A.R.M. and T.J. from the American Heart Association. A.R.M. is a Bristol-Myers Squibb Established Investigator of the American Heart Association.

REFERENCES

1. **Amasaki, Y., S. Kobayashi, T. Takeda, N. Ogura, S. Jodo, T. Nakabayashi, A. Tsutsumi, A. Fujisaku, and T. Koike.** 1995. Up-regulated expression of Fas antigen (CD95) by peripheral naive and memory T cell subsets in patients with systemic lupus erythematosus (SLE): a possible mechanism for lymphopenia. Clin. Exp. Immunol. **99:**245–250.
2. **Bierer, B. E., S. L. Schreiber, and S. J. Burakoff.** 1991. The effect of the immunosuppressant FK506 on alternate pathways of T cell activation. Eur. J. Immunol. **21:**439–445.
3. **Caelles, C., A. Helmberg, and M. Karin.** 1994. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature **370:** 220–223.
4. **Cohen, J. J., and R. C. Duke.** 1984. Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J. Immunol. **132:**38–42.
5. **Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.).** 1990. Current protocols in immunology, vol. 1. Wiley Interscience, New York, N.Y.
6. **Debbas, M., and E. White.** 1993. Wild-type p53 mediates apoptosis by E1QA, which is inhibited by E1B. Genes Dev. **7:**546–554.
7. **Duke, R. C., and J. J. Cohen.** 1986. IL-2 addition: withdrawal of growth factor activates a suicide program in dependent T cells. Lymphokine Res. **5:**289–299.

8. **Dyson, N., K. Buchkovich, P. Whyte, and E. Harlow.** 1989. The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and JC virus. Cell **58:**249–255.
9. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–937.
10. **Evan, G. I., A. H. Wyllie, C. S. Gilbert, T. D. Littlewood, H. Land, M. Brooks, C. M. Waters, L. Z. Penn, and D. C. Hancock.** 1992. Induction of apoptosis in fibroblasts by c-myc protein. Cell **69:**119–128.
11. **Fanger, C. M., M. Hoth, G. R. Crabtree, and R. S. Lewis.** 1995. Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for physiological roles of CRAC channels. J. Biol. Chem. **131:**655–667.
12. **Griffith, T. S., T. Brunner, S. M. Fletcher, D. R. Green, and T. A. Ferguson.** 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. Science **270:**1189–1192.
13. **Groux, H., D. Monte, B. Plouvier, A. Capron, and J.-C. Ameisen.** 1993. CD3 mediated apoptosis of human medullary thymocytes and activated peripheral T cells: respective roles of interleukin-1, interleukin-2, interferon γ, and accessary cells. Eur. J. Immunol. **23:**1623–1629.
14. **Harnick, D. H., T. Jayaraman, L. Go, Y. Ma, P. Mulieri, and A. R. Marks.** 1995. The human type 1 inositol 1,4,5-trisphosphate receptor from T lymphocytes: structure, localization and phosphorylation. J. Biol. Chem. **270:** 2833–2840.
15. **Imboden, J. B., and A. Weiss.** 1987. The T-cell antigen receptor regulates sustained increases in cytoplasmic free Ca^{2+} through extracellular Ca^{2+} influx and ongoing intracellular Ca^{2+} mobilization. Biochem. J. **247:**695–700.
16. **Jayaraman, T., and A. R. Marks.** 1993. Rapamycin-FKBP12 blocks proliferation, induces differentiation, and inhibits cdc2 kinase activity in a myogenic cell line. J. Biol. Chem. **268:**25385–25388.
17. **Jayaraman, T., E. Ondriasova, K. Ondrias, D. Harnick, and A. R. Marks.** 1995. The inositol 1,4,5-trisphosphate receptor is essential for T cell receptor signaling. Proc. Natl. Acad. Sci. USA **92:**6007–6011.
18. **Jiang, S., S. C. Chow, P. Nicotera, and S. Orrenius.** 1994. Intracellular Ca^{2+} signals activate apoptosis in thymocytes: studies using the Ca^{2+}-ATPase inhibitor thapsigargin. Exp. Cell Res. **212:**84–92.
19. **Kaiser, N., and I. S. Edelman.** 1977. Calcium dependence of glucocorticoid-induced lymphocytolysis. Proc. Natl. Acad. Sci. USA **74:**638–642.
20. **Kaiser, N., and I. S. Edelman.** 1978. Further studies on the role of calcium in glucocorticoid-induced lymphocytolysis. Endocrinology **103:**936–942.
21. **Kao, J. P. Y., A. T. Harootunian, and R. Y. Tsien.** 1989. Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. J. Biol. Chem. **264:**8179–8184.
22. **Katsikis, P. D., E. S. Wunderlich, C. A. Smith, L. A. Herzenberg, and L. A. Herzenberg.** 1995. Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals. J. Exp. Med. **181:**2029–2036.
23. **Khan, A. A., M. J. Soloski, A. H. Sharp, G. Schilling, D. M. Sabatini, S.-H. Li, C. A. Ross, and S. H. Snyder.** 1996. Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor. Science **273:**503–507.
24. **Klas, C., K. M. Debatin, R. R. Jonker, and P. H. Krammer.** 1993. Activation interferes with the APO-1 pathway in mature T cells. Int. Immunol. **5:**625–630.
25. **Kuida, K., J. A. Lippke, G. Ku, M. W. Harding, D. J. Livingston, M. S. Su, and R. A. Flavell.** 1995. Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. Science **267:**2000–2003.
26. **Lam, M., G. Dubyak, L. Chen, G. Nunez, R. L. Miesfeld, and C. W. Distelhorst.** 1994. Evidence that BCL-2 represses apoptosis by regulating endoplasmic reticulum-associated Ca^{2+} fluxes. Proc. Natl. Acad. Sci. USA **91:** 6569–6573.
27. **Lenardo, M. J.** 1991. Interleukin 2 programs mouse T lymphocytes for apoptosis. Nature **353:**858–861.
28. **Li, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, and J. Salfeld.** 1995. Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell **80:**401–411.
29. **Los, M., M. Van de Craen, L. C. Penning, H. Schenk, M. Westendorp, P. A. Baeuerle, W. Droge, P. H. Krammer, W. Fiers, and K. Schulze-Osthoff.** 1995. Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. Nature **375:**81–83.
30. **Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks.** 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature **362:**847–849.
31. **Luo, Y., S. Marx, A. Koff, J. Massague, and A. R. Marks.** 1996. Rapamycin resistance tied to defective regulation of $p27^{kip1}$. Mol. Cell. Biol. **16:**6744–6751.
32. **McConkey, D. J.** 1989. Glucocorticoids activate a suicide process in thymocytes through elevation of cytosolic Ca^{2+} concentration. Arch. Biochem. Biophys. **269:**365–370.
33. **McConkey, D. J., P. Hartzell, J. F. Amador-Perez, S. Orrenius, and M. Jondal.** 1989. Calcium-dependent killing of immature thymocytes by stimulation via the CD3/T cell receptor complex. J. Immunol. **143:**1801–1806.
34. **Nicolleti, I., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi.** 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods **139:** 271–279.
35. **Nieto, M. A., and A. Lopez-Rivas.** 1989. IL2 protects T lymphocytes from glucocorticoid induced DNA fragmentation and cell death. J. Immunol. **143:**4166–4170.
36. **Owen-Schaub, L. B., S. Yonehara, W. L. Crump, and E. Grimm.** 1992. DNA fragmentation and cell death is selectively triggered in activated human T lymphocytes by fas antigen engagement. Cell. Immunol. **140:**197–205.
37. **Perandones, C. E., V. A. Illera, D. Peckham, L. L. Stunz, and R. F. Ashman.** 1993. Regulation of apoptosis in vitro in mature murine spleen T cells. J. Immunol. **151:**3521–3529.
38. **Putney, J. W.** 1986. A model for receptor-regulated calcium entry. Cell Calcium **7:**1–12.
39. **Putney, J. W., and G. S. J. Bird.** 1993. The signal for capacitative calcium entry. Cell **75:**199–201.
40. **Qin, X., D. Livingston, W. Kaelin, and P. Adams.** 1994. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc. Natl. Acad. Sci. USA **91:**10918–10922.
41. **Rodriguez-Tarduchy, G., P. Malde, A. Lopez-Rivas, and M. Collins.** 1992. Inhibition of apoptosis by calcium ionophores in IL-3-dependent bone marrow cells is dependent on production of IL-4. J. Immunol. **148:**1416–1422.
42. **Russal, J. H., C. L. White, D. Y. Loh, and P. Meleedy-Rey.** 1991. Receptor stimulated death pathway is opened by antigen in mature T cells. Proc. Natl. Acad. Sci. USA **88:**2151–2155.
43. **Sellins, K. S., and J. J. Cohen.** 1987. Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J. Immunol. **139:**3199–3206.
44. **Shan, B., and W. H. Lee.** 1994. Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. Mol. Cell. Biol. **14:**8166–8173.
45. **Shi, Y., R. P. Bissonnette, N. Parfrey, M. Szaley, R. T. Kubo, and D. R. Green.** 1991. In vivo administration of monoclonal antibodies to the CD3 T cell receptor complex induces cell death in immature thymocytes. J. Immunol. **146:**3340–3346.
46. **Smith, C. A., G. T. Williams, R. Kingston, E. J. Jenkininson, and J. J. Owen.** 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature **337:**181–184.
47. **Story, M. D., L. C. Stephens, S. P. Tomasovic, and R. E. Meyn.** 1992. A role for calcium in regulating apoptosis in rat thymocytes irradiated *in vitro*. Int. J. Radiat. Biol. **61:**243–251.
48. **Takahashi, S., H. T. Maecker, and R. Levy.** 1989. DNA fragmentation and cell death mediated by T cell antigen receptor/CD3 complex on a leukemia T cell line. Eur. J. Immunol. **19:**1911–1919.
49. **Trauth, B. C., C. Klas, A. M. J. Peters, S. Matzku, P. Moller, W. Falk, K.-M. Debatin, and P. Krammer.** 1989. Monoclonal antibody-mediated tumor regression by inducers of apoptosis. Science **245:**301–305.
50. **Tsuda, H., R. W. Huang, and K. Takatsuki.** 1993. Interleukin 2 prevents programmed cell death in adult T cell leukemia cells. Jpn. J. Cancer Res. **84:**431–437.
51. **Tucek-Szabo, C. L., S. Andjelic, E. Lacy, K. B. Elkon, and J. Nikolic-Zugic.** 1996. Surface T cell Fas receptor/CD95 regulation, in vivo activation, and apoptosis. Activation-induced death can occur without Fas receptor. J. Immunol. **156:**192–200.
52. **Vito, P., E. Lacana, and L. D'Adamio.** 1996. Interfering with apoptosis: Ca^{2+}-binding protein ALG-2 and Alzheimer's disease gene *ALG-3*. Science **271:**521–525.
53. **Weiss, A., and D. R. Littman.** 1994. Signal transduction by lymphocyte antigen receptors. Cell **76:**263–274.
54. **Wojcikiewicz, R. J. H.** 1995. Type I, II, and III inositol 1,4,5-trisphosphate receptors are equally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. J. Biol. Chem. **270:** 11678–11683.
55. **Wu, X., and A. J. Levine.** 1994. p53 and E2F-1 cooperate to mediate apoptosis. Proc. Natl. Acad. Sci. USA **91:**3602–3606.
56. **Wyllie, A. H.** 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature **284:**555–556.
57. **Yamamoto-Hino, M., T. Sugiyama, K. Hikichi, M. Mattel, K. Hasegawa, S. Sekine, K. Sakurada, A. Miyakawa, T. Furuichi, M. Hasegawa, and K. Mikoshiba.** 1994. Cloning and characterization of human type 2 and type 3 inositol 1,4,5-trisphosphate receptors. Recept. Channels **2:**9–22.
58. **Yonehara, S., A. Ishii, and M. Yonehara.** 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-down regulated with the receptor of tumor necrosis factor. J. Exp. Med. **169:**1747–1756.
59. **Zauli, G., D. Gibellini, A. Caputo, A. Bassini, M. Negrini, M. Monne, M. Mazzoni, and S. Capitani.** 1995. The human immunodeficiency virus type-1 Tat protein upregulates Bcl-2 gene expression in Jurkat T-cell lines and primary peripheral blood mononuclear cells. Blood **86:**3823–3834.
60. **Zhu, X., M. Jiang, M. Peyton, G. Boulay, R. Hurst, E. Stefani, and L. Birnbaumer.** 1996. *trp*, a novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. Cell **85:**661–671.