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Analysis of Ras-Dependent Signals That Prevent Caspase-3 Activation and Apoptosis Induced by Cytokine Deprivation in Hematopoietic Cells[1]

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In hematopoietic cells, Ras has been implicated in signaling pathways that prevent apoptosis triggered by deprivation of cytokines, such as interleukin-3 (IL-3). However, the mechanism whereby Ras suppresses cell death remains incompletely understood. We have investigated the role of Ras in IL-3 signal transduction by using the cytokine-dependent BaF3 cell line. Herein, we show that the activation of the pro-apoptotic protease caspase-3 upon IL-3 removal is suppressed by expression of activated Ras, which eventually prevents cell death. For caspase-3 suppression, the Raf/extracellular signal-regulated kinase (ERK)- or phosphatidylinositol 3-kinase (PI3-K)/Akt-mediated signaling pathway downstream of Ras was required. However, inhibition of both pathways did not block activated Ras-dependent suppression of cell death-associated phenotypes, such as nuclear DNA fragmentation. Thus, a pathway that is independent of both Raf/ERK and PI3-K/Akt pathways may function downstream of Ras, preventing activated caspase-3-initiated apoptotic processes. Conditional activation of c-Raf-1 also suppressed caspase-3 activation and subsequent cell death without affecting Akt activity, providing further evidence for a PI3-K/Akt-independent mechanism.

Cells of the hematopoietic lineage require multiple cytokines for their survival, growth, and differentiation (1). Immortalized cell lines that proliferate in a cytokine-dependent manner have been used for the investigation of cytokine signal transduction. By the use of interleukin-3 (IL-3)- or granulocyte/macrophage colony-stimulating factor (GM-CSF)-dependent cells, it has been demonstrated that the low molecular weight GTP-binding protein Ras acts downstream of the common β chain of IL-3 and GM-CSF receptors (2, 3).

The role of Ras in IL-3/GM-CSF signal transduction was investigated through the use of receptor and Ras mutants (4–6). Inducible expression of dominant-negative Ras that blocks the downstream Raf/extracellular signal-regulated kinase (ERK) pathway did not affect cell number increase, leading to the notion that Ras is not required for cell proliferation (6). On the other hand, a mutant GM-CSF receptor that is unable to stimulate the Ras pathway failed to block cell death, while inducing transient mitogenic response (5). Moreover, ectopic expression of activated Ras effectively prevented apoptosis triggered by cytokine deprivation (5, 6). Overall, Ras is thought to be implicated in anti-apoptosis signaling particularly in cytokine-dependent cells. Downstream of Ras, a diverse array of signaling pathways, including Raf/ERK, phosphatidylinositol 3-kinase (PI3-K), and RalGDS pathways, have been identified (7). Through the use of several effector domain mutants of Ras, which can activate a distinct set of downstream effectors, the involvement of Raf/ERK and PI3-K in preventing apoptosis was demonstrated (8) although precise mechanisms downstream of these molecules remain elusive.

The caspase family of cysteine proteases plays a pivotal role in apoptosis (9, 10). In particular, caspase-3 is characterized as an executioner of cell death because it cleaves multiple key substrates that are directly involved in apoptotic signaling. Caspases are activated through proteolytic digestion at specific sites by auto- or trans-activation mechanisms. The activation of caspase-3 is triggered in response to death signals mediated in mitochondria-dependent

Abbreviations used: DTT, dithiothreitol; ERK, extracellular signal-regulated kinase; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL-3, interleukin-3; MEK, mitogen-activated protein kinase/ERK kinase; PI3-K, phosphatidylinositol 3-kinase; PMSF, phenylmethylsulfonyl fluoride.

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and -independent manners (9, 10). A mitochondria-independent pathway consisting of adaptor proteins, such as FADD, and the initiator caspase-8 functions downstream of certain receptors, such as Fas and the tumor necrosis factor receptor. On the other hand, Apaf-1, a mammalian homologue of the *Caenorhabditis elegans* CED-4 protein, is activated through complex formation with cytochrome c, which is released from mitochondria, in the presence of adenosine nucleotides. Apaf-1, in turn, activates another initiator caspase-9. Deprivation of cytokines including IL-2 and IL-3 also causes caspase-3 activation in hematopoietic cells, which is required for the progression of cell death (11–13). Yet, the signaling cascade linked to caspase-3 activation following cytokine removal is totally unknown.

Herein, we show that Ras prevents hematopoietic cell death by suppressing caspase-3 activation. Either the Raf/ERK or PI3-K/Akt pathway downstream of Ras is necessary for caspase-3 suppression, whereas these pathways are not essential for prevention of cell death, suggesting a Ras-dependent anti-apoptotic mechanism downstream of caspase-3.

MATERIALS AND METHODS

Materials. Purified mouse IL-3 is a generous gift of Robert Kastelein and Satish Menon (DNAX Research Institute of Molecular and Cellular Biology, CA). The recombinant DFF45 protein (14) is a generous gift of Hiroshi Itoh (Tokyo Institute of Technology, Yokohama, Japan). Antibodies against ERK1/2 (sc-154G), Akt1 (06-558), phosphorylated MAPK (V8031), and phosphorylated Akt1 (Thr 308) (#9275) were purchased from Santa Cruz Biotechnology, Upstate Biotechnology, Promega, and New England Biolabs, respectively. LY294002, U0126, β-estradiol, Ac-DEVD-MCA, and trypan blue stain solution (355-52) were obtained from Calbiochem-Novabiochem, Promega, Sigma, Peptide Institute (Osaka, Japan), and Nacalai Tesque (Kyoto, Japan), respectively.

Cell culture. BaF3-V2 cells (6) were cultured in RPMI 1640 supplemented with fetal bovine serum (10% (v/v)), mouse IL-3 (approximately 1 nM), G418 (1 mg/ml), and hygromycin (1 mg/ml). BaF3-ΔRaf(DD)13 and BaF3-ΔRaf(DD)14 cell lines were isolated using blasticidin (15 μg/ml) from BaF3 cells transfected with the mammalian expression vector pWZLblast3-EGFPΔRaf-1:ER[DD] (kindly provided by Martin McMahon, University of California, San Francisco, CA) (15, 16). These cell lines were cultured in RPMI 1640 supplemented with fetal bovine serum (10% (v/v)), mouse IL-3 (approximately 1 nM). EGFPΔRaf-1:ER[DD] in BaF3-ΔRaf(DD)13 and BaF3-ΔRaf(DD)14 cells was activated by adding β-estradiol (1 μM) to the culture medium.

Assay for caspase-3-like protease activity. Cells (1–2 $\times$ 10^5 per point) were lysed in lysis buffer (10 mM HEPES-NaOH (pH 7.0), 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM dithiothreitol (DTT), 1% (v/v) Nonidet P-40), and centrifuged at 15,000*g* for 10 min at 4°C. Supernatants were incubated within reaction buffer (10 mM HEPES-NaOH (pH 7.0), 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT) containing 25 μM Ac-DEVD-MCA for 30 min at 37°C. After incubation was stopped by adding an equal volume of stopping buffer (0.2 M glycine-HCl (pH 2.8)), fluorescence intensity of released 7-amino-4-methylcoumarin was measured using a fluorescence spectrophotometer (Hitachi F2000) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

DNA fragmentation in vivo. Fragmentation of the chromosomal DNA during apoptotic cell death was assayed as follows. Cells (5×10^5 per point) were dissolved in lysis buffer, and cell lysates were obtained by centrifugation at 15,000 $\times$ *g* for 10 min at 4°C. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation, which were then dissolved in water containing loading buffer (Takara, Kyoto, Japan). Samples were subjected to agarose gel electrophoresis (2% (w/v) agarose). Subsequently, gel was treated with ribonuclease A (100 μg/ml) for 60 min at 37°C. DNA was stained with ethidium bromide and visualized under ultraviolet light.

Preparation of nuclei. Mouse liver nuclei were prepared essentially as described previously (17). Minced mouse liver was homogenized in solution A (15 mM PIPES-NaOH (pH 7.4), 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 250 mM sucrose) using a Dounce homogenizer. After filtration through four layers of gauze, an equal volume of solution B (15 mM PIPES-NaOH (pH 7.4), 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM PMSF, 2.3 M sucrose) was added and mixed thoroughly. Homogenates were layered over solution B and centrifuged at 22,000 rpm for 90 min at 4°C with the Beckman SW41Ti rotor. Nuclei were resuspended in solution A at a concentration of 1×10^5 nuclei/μl and stored at −80°C.

Cell-free DNA fragmentation. Cell lysates were prepared essentially as described previously (18–20) with minor modifications. BaF3-V2, BaF3-ΔRaf(DD)13, or BaF3-ΔRaf(DD)14 cells cultured under various conditions were washed with extraction buffer (50 mM PIPES-NaOH (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 μM cytochalasin B, 1 mM DTT, 40 mM β-glycerophosphate, 1 mM PMSF, 1.8 μg/ml aprotinin, 1 μg/ml leupeptin) and collected in a glass Dounce homogenizer. Cells were disrupted through a cycle of freezing and thawing, followed by 5 to 6 strokes of a pestle, and centrifuged at 15,000 $\times$ *g* for 20 min at 4°C. Cell lysates were diluted with extraction buffer to a final concentration of 20 mg protein/ml, and then incubated with or without nuclei ($2 \sim 5 \times 10^5$ per point) for 30 min at 37°C. Nuclei were dissolved in lysis buffer, and centrifuged at 15,000*g* for 10 min at 4°C. Nucleic acids were purified by phenol/chloroform extraction and ethanol precipitation, which were then dissolved in water containing loading buffer. Samples were subjected to agarose gel electrophoresis (2% (w/v) agarose). Subsequently, gel was treated with ribonuclease A (100 μg/ml) for 60 min at 37°C. DNA was stained with ethidium bromide and visualized under ultraviolet light.

Immunoprecipitation and immunoblotting. Cells (5×10^5 per point) were dissolved in IP buffer (50 mM HEPES-NaOH (pH 7.3), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM NaPPi, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin), and the supernatant of centrifugation at 15,000*g* for 10 min at 4°C was used as a cell lysate. Protein G-Sepharose 4 Fast Flow (Amersham Pharmacia) and an anti-Akt1 antibody (06-558, 1.5 μg) were mixed gently with lysates for 2 h at 4°C, and precipitates were washed twice with IP buffer. Precipitated proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was stained with an antibody against phosphorylated Akt1 (Thr 308) (#9275, 1.5 μg/ml) and enhanced chemiluminescence detection reagents (DuPont/NEN). Following this, the membrane was treated with stripping buffer (62.5 mM Tris-HCl (pH 6.8), 0.02% (w/v) SDS, 0.7% (v/v) 2-mercaptoethanol) for 30 min at 55°C and reprobed with an antibody against Akt1 (06-558, 1.5 μg/ml). Cell lysates were applied to SDS-PAGE and proteins were transferred onto a nitrocellulose membrane. The membrane was stained with an antibody against phosphorylated MAPK (V8031, 1:5,000 dilution) and reprobed with an antibody against ERK1/2 (sc-154G, 1 μg/ml).

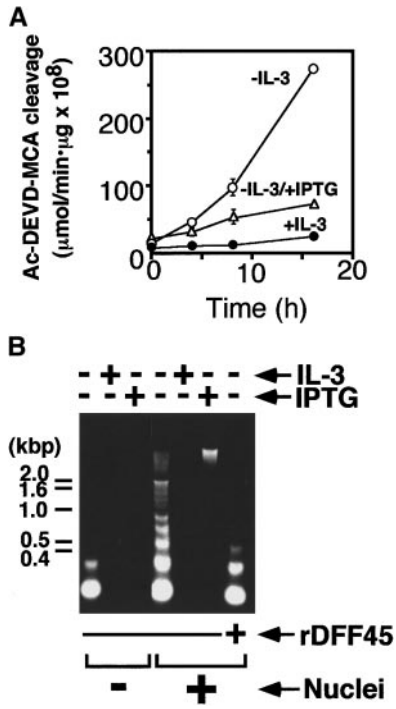

FIG. 1. Suppression of caspase-3 activation upon IL-3 withdrawal by activated Ras. (A) Time course of the activation of caspase-3-like protease activity after IL-3 withdrawal. BaF3-V2 cells were washed and cultured in the presence of IL-3 (~1 nM) or IPTG (5 mM) as indicated during specified periods. For Ras(G12V) induction, cells were treated with IPTG (5 mM) for 16 h prior to IL-3 deprivation. Caspase-3-like protease activity was determined by the use of Ac-DEVD-MCA as a substrate. Results are shown as mean ± SE (n = 3). (B) Cell-free DNA fragmentation. BaF3-V2 cells were cultured for 20 h in the presence of IL-3 (~1 nM) or IPTG (5 mM) as indicated. For Ras(G12V) induction, cells were treated with IPTG (5 mM) for 16 h prior to IL-3 deprivation. DNA fragmentation-inducing activity was determined by cell-free DNA fragmentation assays. Where indicated, recombinant DFF45 (40 μg/ml) was added. Representative results of at least three independent experiments are shown.

Trypan blue exclusion assay. BaF3-V2 cells cultured under various conditions were mixed with trypan blue stain solution. The percentage of dead cells was determined by counting dead versus total cell number on a haemocytometer, using trypan blue exclusion as a criterion of cell viability.

RESULTS AND DISCUSSION

Activation of caspase-3 upon IL-3 deprivation and its suppression by activated Ras is shown in Fig. 1. BaF3-V2 cells conditionally express activated Ha-Ras (G12V) upon IPTG treatment (6). Caspase-3 activity was measured by two assays: cleavage of the fluorescent-labeled substrate Ac-DEVD-MCA (Fig. 1A) and cell-free induction of nuclear DNA fragmentation (18–20) (Fig. 1B). In the latter, extracts of BaF3-V2 cells cultured under various conditions were incubated with nuclei isolated from mouse liver. Fragmented DNA bands observed in a nucleus-dependent and recombinant DFF45/ICAD (14, 21, 22)-sensitive manner are yielded in this cell-free assay. DNA bands in the first lane of Fig. 1B are derived from BaF3-V2 cell extracts. Furthermore, cell-free DNA fragmentation in this assay represents caspase-3 activity in extracts, but not nuclease activity that has been activated prior to extract preparation as evidenced by the potent inhibitory effect of the caspase-3-specific inhibitor Ac-DEVD-CHO added to the extract (data not shown). In both assays, IPTG-induced activated Ras effectively prevented caspase-3 activation following IL-3 removal. Ras-dependent prevention of apoptosis triggered by a variety of death signals has been well documented in many types of cells. Particularly, the downstream effector PI3-K plays a pivotal role serving as an activator of Akt, which in turn phosphorylates and inactivates several pro-apoptotic molecules such as Bad and caspase-9 (23, 24). Another effector Raf is also responsible for anti-apoptotic function of Ras in some cases although the mechanism is less understood (see below).

Thus, we examined the role of Raf/ERK and PI3-K/Akt pathways downstream of Ras in the prevention of caspase-3 activation in response to IL-3 removal. As shown in Fig. 2, U0126 and LY294002, specific inhibitors for mitogen-activated protein kinase/ERK kinase (MEK) and PI3-K, respectively, completely blocked Ras-induced phosphorylation/activation of ERK1/2 and Akt1. Effects of these inhibitors on caspase-3 activity are illustrated in Fig. 3. IL-3-dependent suppression of caspase-3 was totally insensitive to U0126 and LY294002. In marked contrast, these inhibitors, when added in combination, potently blocked the ability of Ras to suppress caspase-3. Therefore, the Raf/ERK or PI3-K/Akt pathway is required for caspase-3 suppression downstream of Ras, whereas IL-3 can suppress caspase-3 through multiple signaling pathways includ-

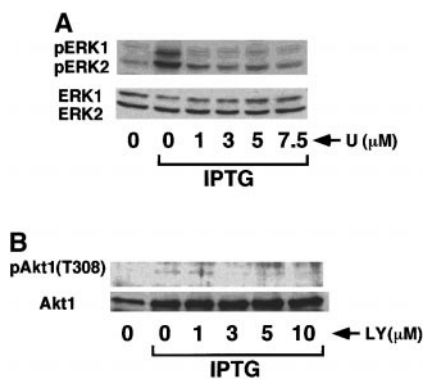

FIG. 2. Inhibition of activated Ras-dependent ERK1/2 (A) and Akt1 (B) phosphorylation by specific inhibitors. BaF3-V2 cells were cultured for 20 h in medium containing IPTG (5 mM) but not IL-3 in the presence of various concentrations of U0126 (U) or LY294002 (LY). For Ras(G12V) induction, cells were treated with IPTG (5 mM) for 16 h prior to IL-3 deprivation. Phosphorylated ERK1/2 and Akt1 were detected by immunoblotting as described under Materials and Methods. Representative results of at least three independent experiments are shown.

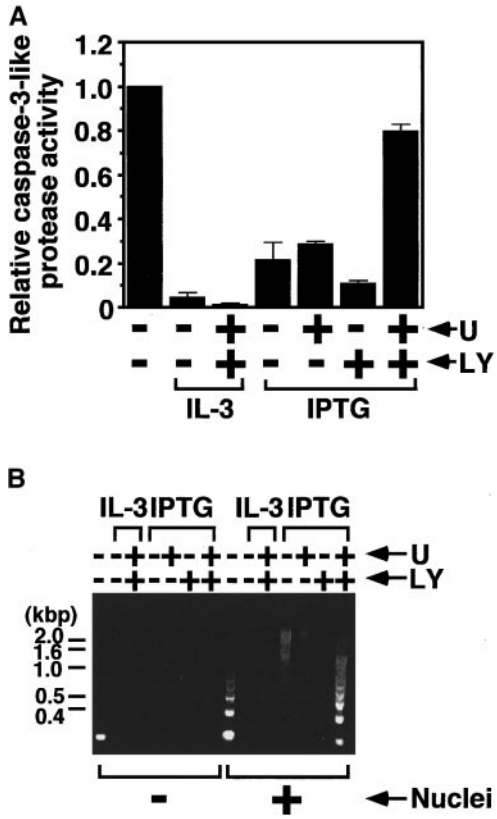

FIG. 3. Inhibition of activated Ras-dependent suppression of caspase-3 by specific inhibitors. (A) Caspase-3-like protease activity. BaF3-V2 cells were cultured for 20 h in medium containing IL-3 (~1 nM) or IPTG (5 mM) in the presence of U0126 (U, 5 μM) or LY294002 (LY, 10 μM) as indicated. For Ras(G12V) induction, cells were treated with IPTG (5 mM) for 16 h prior to IL-3 deprivation. Caspase-3-like protease activity was determined by the use of Ac-DEVD-MCA as a substrate. Results are shown as mean ± SE (n = 3). (B) Cell-free DNA fragmentation. Cell lysates prepared as in A were subjected to cell-free DNA fragmentation assays. Representative results of at least three independent experiments are shown.

ing U0126 and LY294002-insensitive and probably Ras-independent pathways. In fact, cAMP-dependent protein kinase, for instance, phosphorylates a specific site of Bad, leading to its inactivation in response to IL-3 (25). Furthermore, the STAT5 pathway is responsible for IL-3-dependent regulation of Bcl-x_L (26). Both Bad inactivation and Bcl-x_L induction result in blockade of mitochondria-mediated pro-apoptotic signaling including the activation of caspase-3.

Cell survival as determined by dye exclusion assays and chromosomal DNA fragmentation after treatment with U0126 and LY294002 are shown in Fig. 4. While IL-3-dependent cell survival remained unaffected upon treatment with these inhibitors, Ras-dependent survival was partially sensitive to each of the inhibitors. Treatment with these inhibitors in combination also caused cell death only to a limited extent when activated Ras was expressed in the absence of IL-3. However, as shown in Fig. 4B, Ras-dependent as well as IL-3-dependent prevention of DNA fragmentation was resistant to the inhibitory action of U0126 and LY294002. Hence, Ras is still able to prevent DNA fragmentation and cell death, at least in part, under conditions where caspase-3 is no longer repressed as defined by *in vitro* assays.

Prevention by Ras of cell death-associated phenotypes such as chromosomal DNA fragmentation after caspase-3 induction is interesting in that virtually all

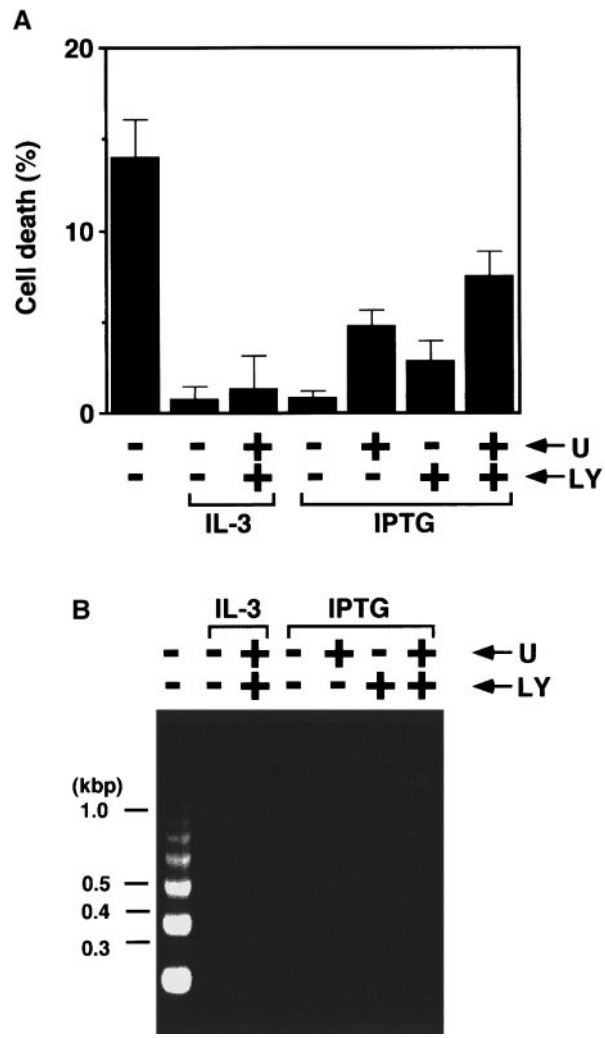

FIG. 4. Effect of specific inhibitors on activated Ras-dependent suppression of cell death. (A) Trypan blue exclusion assays. BaF3-V2 cells were cultured for 30 h in medium containing IL-3 (~1 nM) or IPTG (5 mM) in the presence of U0126 (U, 5 μM) or LY294002 (LY, 10 μM) as indicated. For Ras(G12V) induction, cells were treated with IPTG (5 mM) for 16 h prior to IL-3 deprivation. Percentage of dead cells was determined by trypan blue exclusion assays. Results are shown as mean ± SE (n = 3). (B) DNA fragmentation *in vivo*. BaF3-V2 cells were cultured for 20 h in medium containing IL-3 (~1 nM) or IPTG (5 mM) in the presence of U0126 (U, 5 μM) or LY294002 (LY, 10 μM) as indicated. For Ras(G12V) induction, cells were treated with IPTG (5 mM) for 16 h prior to IL-3 deprivation. DNA fragmentation *in vivo* was assessed as described under Materials and Methods. Representative results of at least three independent experiments are shown.

documented anti-apoptotic signals mediated by Ras culminate in the inactivation of caspase-3. Signaling pathways downstream of Ras responsible for the above effect are independent of Raf/ERK and PI3-K/Akt pathways although the Ras effector(s) that regulates these pathways remains obscure. Prevention of cell death may be ascribable to the action of a protein that becomes able to bind and inhibit activated caspase-3 itself in response to Ras signals in undisrupted cells, but not in a cell free system. Such a protein may belong to the inhibitor-of-apoptosis family of proteins, yet the Ras-dependent regulatory mechanism remains largely unsolved. On the other hand, Ras signals may modulate individual pathways, for instance, the CAD/ICAD pathway that leads to DNA fragmentation (21, 22) and the Acinus pathway for chromatin condensation (27). In this respect, it should be noted that Ras may inhibit only some (including DNA fragmentation), but not all activated caspase-3-triggered phenotypes associated with apoptosis. Indeed, cells expressing activated Ras ultimately undergo cell death within 2 days when treated with both inhibitors, suggesting that MEK and PI3-K-independent signals triggered by Ras may be insufficient for complete inhibition of cell death.

To further investigate the role of the Raf/ERK pathway in apoptosis after IL-3 withdrawal, we isolated BaF3-derived clones that express conditionally activated c-Raf-1 (15, 16). Two representative clones, designated BaF3-ΔRaf(DD)13 and BaF3-ΔRaf(DD)14, respectively, were used for further analyses. ΔRaf(DD)-ER, an activated c-Raf-1 mutant fused to the ligand binding domain of the estrogen receptor, is activated upon β-estradiol treatment, leading to the activation of the downstream ERK pathway (Fig. 5A). Under these conditions, no phosphorylation/activation of Akt1 was detected (Fig. 5B). Chromosomal DNA fragmentation *in vivo* upon IL-3 removal was effectively blocked by activated c-Raf-1 (Fig. 6), which is similar to previously reported observations (8, 28). On the basis of these biological activity, we next examined whether activated c-Raf-1 is sufficient to suppress caspase-3 activation. As shown in Fig. 7, activated c-Raf-1 completely blocked caspase-3 activation upon IL-3 removal. Therefore, not only the well-documented PI3-K/Akt pathway, but also the Raf/ERK pathway is sufficient to prevent caspase-3 activation although the mechanism remains elusive. In support of this, it has been shown that expression of dominant-negative PI3-K in BaF3 cells abrogated IL-3-induced proliferation and Bad phosphorylation without significantly affecting levels of apoptosis (29), suggesting that other signaling pathways such as the Raf/ERK pathway are also important for preventing cell death.

It has been shown that the Raf/ERK pathway is involved in prevention of cell death in diverse cell types, including neuron-like (30) and fibrosarcoma (31) cells. In particular, the Raf/ERK pathway determines sensitivity to Fas-induced apoptosis by modulating caspase-8 activity (32). Furthermore, cytokine-induced phosphorylation of Bad, leading to cell survival, is mediated, at least in part, by the Raf/ERK pathway (33). In Rat-1 fibroblast cells, on the other hand, B-Raf, through the action of MEK, prevents cell death after growth factor deprivation by inhibiting caspase-3 activation following cytochrome c release (34). Taken together with our results described here, the Raf/ERK pathway contributes to blockade of apoptosis at various points of signaling in a wide range of cell types. Further investigation to clarify the mechanism

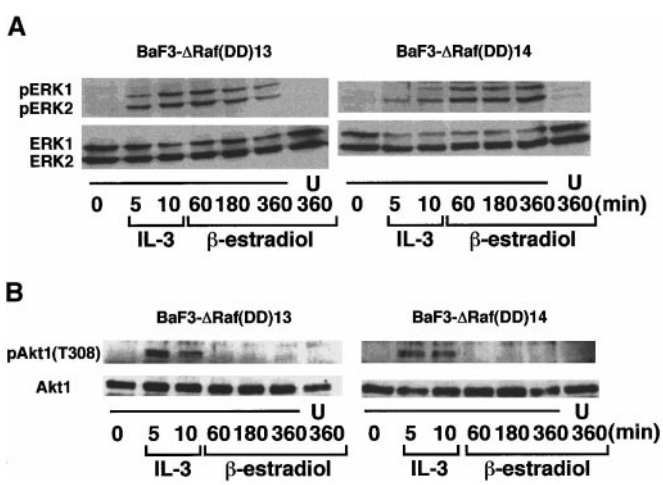

FIG. 5. Activated c-Raf-1-induced phosphorylation of ERK1/2 (A), but not Akt1 (B). BaF3-ΔRaf(DD)13 or BaF3-ΔRaf(DD)14 cells were cultured in IL-3-free medium for 20 h, and stimulated with IL-3 (~1 nM) or β-estradiol (1 μM) for indicated periods. "U" indicates U0126 treatment (10 μM) during β-estradiol stimulation. Phosphorylated ERK1/2 and Akt1 were detected by immunoblotting as described under Materials and Methods. Representative results of at least three independent experiments are shown.

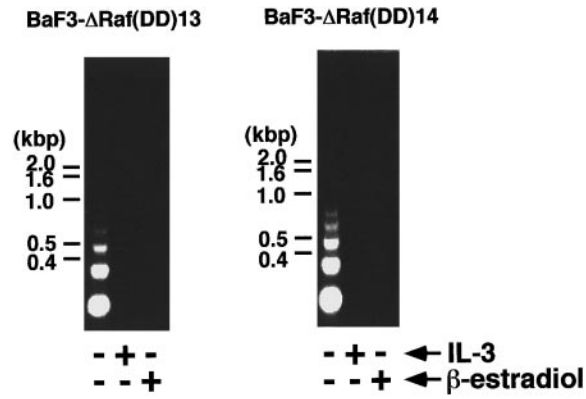

FIG. 6. Suppression of DNA fragmentation *in vivo* by activated c-Raf-1. BaF3-ΔRaf(DD)13 or BaF3-ΔRaf(DD)14 cells were cultured for 20 h in the presence of IL-3 (~1 nM) or β-estradiol (1 μM) as indicated. DNA fragmentation *in vivo* was assessed as described under Materials and Methods. Representative results of at least three independent experiments are shown.

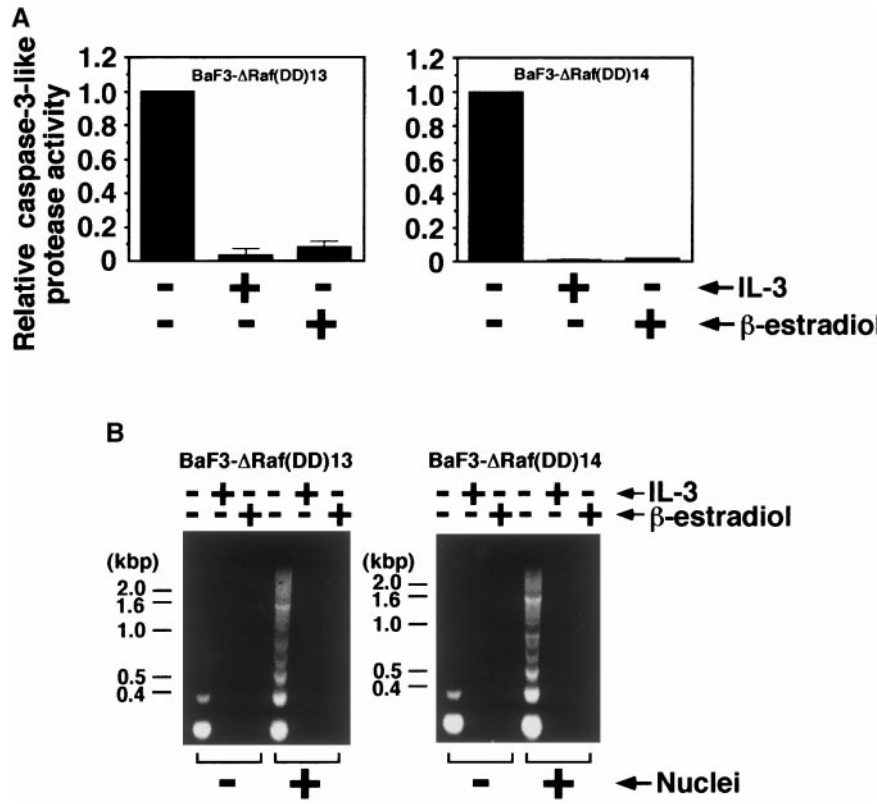

FIG. 7. Suppression of caspase-3 activation upon IL-3 withdrawal by activated c-Raf-1. (A) Caspase-3-like protease activity. BaF3-ΔRaf(DD)13 or BaF3-ΔRaf(DD)14 cells were cultured for 20 h in the presence of IL-3 (~1 nM) or β-estradiol (1 μM) as indicated. Caspase-3-like protease activity was determined by the use of Ac-DEVD-MCA as a substrate. Results are shown as mean ± SE (n = 3). (B) Cell-free DNA fragmentation. Cell lysates prepared as in A were subjected to cell-free DNA fragmentation assays. Representative results of at least three independent experiments are shown.

whereby apoptosis is suppressed by Ras in hematopoietic cells will be important.

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