Differential Regulation of Discrete Apoptotic Pathways by Ras*

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The products of the ras genes are known to regulate cell proliferation and differentiation; recently, they have been found to play a role in apoptosis. The expression of oncogenic p21ras in a number of cell types, including Jurkat (a human T lymphoblastoid cell line) and murine fibroblasts, makes the cells susceptible to apoptosis following suppression of protein kinase C (PKC) activity (PKC/Ras-mediated apoptosis). Engagement of Fas antigen, a potent effector of apoptosis, activates cellular p21ras, which may be required for completion of the cell death program. To further investigate the role of p21ras in the regulation of apoptosis, the cellular mechanisms employed in these two apoptotic processes in which Ras activity is involved (PKC/Ras-related and Fas-triggered apoptosis), was explored. Increasing p21ras activity by expressing v-ras or by treatment with an antisense oligonucleotide to the GTPase-activating protein was found to accelerate the Ras-mediated apoptotic process in Jurkat and mouse LF cells. PKC/Ras-related apoptosis was associated with, and required, cell cycle progression, accompanied by the expression of the G1/S cyclins. In contrast, Fas engagement, although inducing a vigorous and PKC-independent activation of endogenous p21ras, did not alter cell cycle progression, nor did it require such progression for apoptosis. Both the protein synthesis inhibitor cycloheximide and cy- clin E antisense oligonucleotides partially abolished PKC/Ras-mediated apoptosis but had only a moderate effect on Fas-induced apoptosis. In contrast, the CED-3/ interleukin-1β-converting enzyme (ICE) protease inhibitor Z-VADfmk efficiently suppressed Fas-induced apo- ptosis and only marginally inhibited PKC/Ras-mediated apoptosis. Induction of both pathways resulted in activation of the Jun NH2-terminal kinase/JUN signaling system. These results suggest that different cell death programs, such as PKC/Ras-mediated and Fas-mediated apoptosis, may be interconnected via p21ras and perhaps Jun NH2-terminal kinase/JUN. In response to various death stimuli, p21ras may act as a common intermediate regulator in the transduction of apoptotic signals.

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1 The abbreviations used are: PKC, protein kinase C; PMA, phorbol myristate acetate; Z-VADfmk, N-benzylxycarbonyl-Val-Ala-asp-flu- oromethylketone; ICE, interleukin-1β-converting enzyme; JNK, Jun NH2-terminal kinase; Ab, antibody; MOPS, 4-morpholino propanesulfonic acid.

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activate cellular p21ras, possibly via a sphingomyelin/ceramide pathway (35), and this p21ras activity appears to be absolutely required for efficient execution of the death program. Thus, p21ras, a critical mediator of cell growth, also appears to play an important role in regulation of programmed cell death.

Fas/Apo-1 (CD95) belongs to the tumor necrosis factor receptor superfamily (33, 36). Fas is expressed on the surface of a variety of transformed cell lines and chronically stimulated T cells and can mediate apoptosis in vitro and in vivo after ligation with Fas-specific antibodies or Fas-ligand (37). Mutations in Fas or its ligand are responsible for lymphadenopathy, lymphoaccumulation, and accelerated autoimmune defects in lpr and gld homozygous mice, respectively (38–44). Although the molecular mechanisms of Fas-induced apoptosis are not fully understood, it is clear that multiple pathways might be involved in Fas signaling, including those mediated by the CED-3 homolog ICE-like protease family, and the sphingomyelin/ceramide activation of p21ras signaling. In the first pathway, MORT1/FADD and FLICE can bind to an essential intracellular domain of Fas, the death domain, and upon binding, together with other caspases, they form a death-inducing signaling complex (45–48). It is not clear how, or whether, CED-3/ICE signals interact with the sphingomyelin/Ras pathway. Fas ligation appears to activate the Jun NH2-terminal kinase (JNK)/stress-activated protein kinase and p38, through a pathway at least partially independent of MORT1/FADD (49). JNK and its substrate c-Jun are downstream effectors of p21ras, and although they were initially characterized as mitogenic effectors, JNK/Jun can promote apoptosis (35, 50–52). Conversely, there is evidence that activation or mitogenic signals, as well as apoptotic signals, are delivered by Fas (53). It has been reported that developing thymocytes and some T lymphocytes undergo activation-induced cell death, which requires Fas/FasL activity and cell cycle progression. For example, in a T cell hybridoma, activation-induced T-cell death is cell cycle-dependent and is accompanied by elevations of p34cdc2 and cyclin B-associated histone H1 kinase (54). The involvement of p21ras as a common mediator of both mitogenic and apoptotic signals might resolve these seemingly contradictory or opposing activities.

Despite the reports demonstrating a requirement for p21ras activity in the operation of some apoptotic programs, relatively little is known about the molecular and biochemical mechanisms of Ras in the regulation of apoptosis. Given the evidence that Ras can regulate two important and seemingly very different biological processes, cell proliferation and apoptosis, we wished to determine whether Ras might function differentially in two potentially distinct types of programmed cell death: the Fas-induced and the PKC/Ras-mediated apoptotic processes. We investigated the behavior of Jurkat cells. Jurkat cell lines stably transfected with v-Ha-ras (PH1), mouse thymoma cells (LF1210), and LF1210 cells transfected with either a fos gene (LF(+)) or an antisense fos gene (LF(−)) under the conditions of suppression of PKC activity or engagement of Fas antigen. An additional method for inducing the activation of endogenous cellular Ras that was not dependent upon stable transfections was also utilized to further define the role of Ras in these studies. Inhibition of Ras-GTPase-activating protein (GAP) proteins is almost certainly one mechanism used by T cell receptor to physiologically regulate endogenous Ras activity (55–57). GAP-specific antisense oligonucleotides were employed to suppress the level of GAP expression, so as to increase the endogenous cellular Ras activity transiently. In the presence of GAP antisense oligonucleotides, LF(+) cells underwent apoptosis after suppression of PKC and also became more sensitive to Fas ligation. During PKC/Ras-mediated apoptosis, PH1 cells remained in the cell cycle without G1 arrest, with continued expression of G1/S cyclins. The addition of cycloheximide or cyclin E antisense oligonucleotides, but not the protease inhibitor peptide Z-VADfmk, suppressed the PKC/Ras-mediated apoptotic process. In contrast, although increasing p21ras activity greatly accelerated Fas-mediated apoptosis, cyclin E antisense oligonucleotides, and protein synthesis inhibitors had only minor effects on Fas-induced apoptosis. These data therefore suggest a mechanism by which different apoptotic pathways, such as PKC/Ras-mediated and Fas-mediated apoptosis, may interconnect. p21ras, as an intermediate regulator, may recruit and modulate signals at various steps in otherwise discrete apoptotic processes.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Transfections—The human lymphoblastoid cell line Jurkat (American Tissue Culture Collection, Rockville, MD) was obtained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated newborn calf serum (Hazelton Research Products, Inc., Lenexa, KS), 2 mM L-glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml. Jurkat cells were stably transfected by electroporation as described previously (58). The V-Ha-ras expressing vector used was PH1, a circularly permuted clone of the Harvey sarcoma virus (the generous gift of E. Scolnick, Merck Pharmaceuticals). This gene was co-transfected with a selectable marker conferring resistance to G418, and transfectants (called PH1 lines) were selected for resistance to Geneticin. The independent lines and clones resulting, as well as control Jurkat lines transfected with the selectable marker alone, have been described previously (58). After transfection, cells were carried in the same growth medium plus 0.7 mg/ml of Geneticin. LF1210 mouse thymoma cells were transfected with fos gene (LF(+)) or antisense fos gene (LF(−)) (generous gifts from Dr. S.-T. Ju, Boston University) and cultured in medium containing 0.7 mg/ml of Geneticin.

Cell Surface Staining—Cells (1 × 106), under normal growth conditions or after 24 h of high-dose phorbol myristyl acetate (PMA) treatment to down-regulate PKC activity, were incubated with a mouse monoclonal Ab to human Fas (PanVera Corp., Madison, WI) or a hamster Ab to mouse Fas (Pharmingen, San Diego, CA). After the cells were washed twice with 1× phosphate-buffered saline, they were stained with secondary Abs conjugated with fluorescein. The profiles of the surface staining was analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

Cell Viability Assay—Jurkat, PH1, LF1210, LF(+) and LF(−) cells (1 × 106 cells/ml) were incubated in 500 μl of PMA for 24 h, washed twice with 1× phosphate-buffered saline, and seeded into 100-mm Petri dishes (0.5 × 106 cells/ml). For the Fas ligation experiment, the cells were incubated with 1.5 μg/ml of anti-human Fas antibody (PanVera Corp., Madison, WI) for Jurkat and PH1 cells or 5 μg/ml mouse anti-mouse Fas antibody (Pharmingen, San Diego, CA) for LF cells. Cells were collected at the times indicated and enumerated using trypan blue dye exclusion to assess viability. For the inhibitor assays, the concentrations of cycloheximide and Z-VADfmk were 10 and 1 μg/ml, respectively. The cell cultures were treated with the inhibitors for the last 6 h following 24 h of high-dose PMA treatment or for 30 min prior to Fas ligation. For the antisense oligonucleotide experiments, 20 μM of GAP antisense or sense oligonucleotides or 5 μM of cyclin E antisense or sense oligonucleotides were added to the cell cultures simultaneously with the addition of 500 μl of PMA or 48 h in advance for Fas ligation experiments. The sequences of the GAP sense and antisense oligonucleotides were 5′-AACATGATGGCGGCCGAG-3′ and 5′-CTCGGCCGCCATCATGTT-3′, respectively. The sequences of the cyclin E sense and antisense oligonucleotides were 5′-GCCATGCACCAGGAAGAAGCTGCA-3′ and 5′-TCCGATTCCTCTCCCTTGAGATGCGATC-3′, respectively.

Cell Cycle Analysis—After treatment with either 500 nM of PMA for 24 h or anti-Fas antibody as described above, or culture under normal conditions, cells (1 × 106) were washed twice with 1× phosphate-buffered saline, fixed with 70% ethanol, and treated with 10 ng/ml RNase. Subsequently, the cells were stained with propidium iodide. A Cytofluorometric analysis was performed with a FACSScan (Becton Dickinson, CA). The data analysis and display were performed using the Cell-Fit program, which provides data from the flow cytometer and real-time statistical analysis of the data, computed at 1-s intervals, and also discriminates doublets or adjacent particles. Those cells with less than G1 DNA content were excluded on the basis of wide and low angle scatter.
Measurement of p21<sup>w</sup>—Activation—Cells (20 × 10<sup>6</sup> cells/per cell line) were washed with phosphate-free RPMI medium containing 10% diazoyed fetal bovine serum and cultured in the same medium with 0.5 mM ortho-[<sup>32</sup>P]P for 4 h. Subsequently, cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.5), 1% Triton X-114, 20 mM MgCl<sub>2</sub>, 150 mM NaCl, and 100 µg of aprotonin and leupeptin per ml. The amounts of cell lysates were immunoprecipitated with anti-Ras antibody (Y13–259) (Oncogene Science, Uniondale, NY), followed by addition of goat anti-rat immunoglobulin-conjugated antibody A-Sepharose beads. Precipitates were washed with lysis buffer, and bound nucleotides were eluted with elution buffer containing 20 mM EDTA and 25 µM each of ATP and GTP at 65 °C. The eluted products were separated on PEI-cellulose plates. Quantitation was performed by densitometric scanning of autoradiograms using a laser densitometer.

JNK Activity and Protein Expression—The solid-phase JNK assays were carried out by using a GST-c-Jun (1–79) fusion protein coupled to glutathione beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as a substrate. The cells (10 × 10<sup>6</sup>) were treated with either 500 nM of PMA for 24 h or anti-Fas Ab for 60 min and subsequently lysed in lysis buffer (25 mM HEPES, pH 7.5, 0.3 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 20 µM β-glycerophosphate, 0.1 mM Na<sub>2</sub>VO<sub>3</sub>, 2 µg of leupeptin/ml, 1 mM phenylmethylsulfonyl fluoride). One hundred µg of the lysates were mixed with glutathione-agarose beads to which 20 µg of GST-c-Jun were bound. The beads were incubated with glutathione buffer (pH 7.5, 1% Triton X-100, 20 mM MgCl<sub>2</sub>, 150 mM NaCl, 100 µg of aprotonin and leupeptin per ml). The phosphorylated proteins were resolved on a 10% SDS-polyacrylamide gel electrophoresis gel and visualized by autoradiography. For the immunoblot assay of JNK1 protein, the cells were treated as described above. Equal amounts of the cell lysates were immunoprecipitated and subsequently immunoblotted with anti-JNK1 Ab (Santa Cruz Biotechnology, Inc.). The immunoblots were developed with the appropriate antibody alkaline phosphatase reagent (Oncogene Science).

PKC Enzymatic Assay—Jurkat and PH1 cells (1 × 10<sup>6</sup> cells/ml) were cultured in flat wells of a 6-well plate with 10 ml of medium per well, containing different reagents as indicated. For single stimulation experiments, the cells were exposed to either PMA or anti-Fas antibody alone for 15 min. For PKC activity inhibitor experiments, cells were exposed either to 500 nM of PMA for 30 h or to 0.5 µM of 2-aminoethoxydextran (2-AM, 200 µM) for 30 h. After lysis in 25 mM Tris-HCl (pH 7.5), 1% Triton X-100, 20 mM MgCl<sub>2</sub>, and 150 mM NaCl, the lysates were normalized for protein concentration, and 100 µg of protein were analyzed for PKC activity using a PKC assay kit containing a specific substrate peptide for PKC and the inhibitor mixture, which blocks PKA activity by using a PKC assay kit containing a specific antibody alone for 15 min. For PKC activity inhibitory experiments, cells were treated as described above. Equal amounts of the cell lysates were immunoprecipitated with anti-cyclin E antibody.

For DNA fragmentation assays confirmed the differences in the induction of Fas-mediated apoptosis between these two cell lines (data not shown). Jurkat cells transfected with the empty vector alone demonstrated similar cell growth kinetics in response to Fas engagement to the parental Jurkat cells (data not shown). These findings suggested that the expression of activated p21<sup>w</sup> may accelerate the Fas-mediated apoptotic process. For murine LF cells, the numbers of LF<sup>+<sup>/</sup>-</sup> and LF1210 cells started to decrease by 10 h following addition of anti-Fas Ab. The delay of the execution in response to Fas ligation may be due to intrinsic, physiological differences in programmed cell death between human and murine systems. LF<sup>+</sup> cells were more susceptible to Fas-induced apoptosis than LF1210. By 36 h after Fas ligation, 80% of LF<sup>+</sup> cells were dead. In contrast, the viability of LF<sup>-</sup> cells was not affected by Fas ligation, demonstrating a requirement for cell surface expression of Fas antigen in Fas-mediated apoptosis.

To determine the susceptibility of these cell lines to a different apoptotic stimulus, the cells were exposed to 500 nM of PMA for 24 h to down-regulate endogenous PKC activity. Peristent, high-dose PMA treatment has been demonstrated to inhibit or suppress PKC activity in human and murine lymphocytes, and consequently to induce PH1 cells or mouse fibroblasts transfected with v-K-ras to undergo apoptosis (16, 30). Viable cells were enumerated at daily intervals (Fig. 1c). Down-regulation of endogenous PKC activity by persistent, high-dose PMA treatment resulted in cessation of proliferation in parental Jurkat cells, with recovery after 2 days. The number of PH1 cells, after down-regulation of PKC, decreased over 4 days, and no obvious recovery or regrowth was observed. After down-regulation of PKC activity by continuous PMA treatment, LF<sup>+</sup> and LF<sup>-</sup> cells behaved like Jurkat cells in that they ceased proliferation for 2 days and then regained normal growth profiles. DNA fragmentation assays showed that 35% of PH1 cells contained a hypodiploid DNA content 48 h after down-regulation of PKC activity, whereas DNA fragmentation in Jurkat, LF<sup>+<sup>/</sup>-</sup> and LF<sup>-</sup> cells did not increase (data not shown). Because the level of cell surface expression of Fas is one factor that can modulate the sensitivity of a cell to Fas-induced apoptosis, the effect of down-regulation of PKC activity on Fas expression was examined in the different cell types (Fig. 1a, lower panel). PH1 cells, as well as control Jurkat and LF<sup>+</sup> cells, under normal growth conditions or after 500 nM of PMA treatment for 24 h, were surface-stained with anti-Fas antibody. The profile of Fas staining after down-regulation of PKC was almost identical to that measured under normal growth conditions in all three cell lines. Thus, the increase in sensitiv-
FIG. 1. Fas expression and cell kinetics after PKC down-regulation or Fas ligation. a, under normal growth conditions (upper panel) or after 500 nM of PMA treatment for 24 h (lower panel), cells (1 x 10^6) were incubated with the anti-Fas antibody, followed by staining with a fluorescein-conjugated secondary antibody. The surface staining profiles of Fas expression (open profiles) are shown relative to the control (filled) profiles, stained with secondary antibody alone. Ctrl (lower panel) represents the superimposed profile of the same cells under normal growth conditions, for purposes of comparison. b and c, five replicate plates for each cell line were cultured either with the anti-Fas antibody or following down-regulation of PKC activity by 24 h of treatment with 500 nM of PMA. Viable cells were enumerated at the time points indicated in the figures. Error bars represent the standard deviation over five independent experiments. d, Jurkat, PH1, and LF(+) cells were cultured either under normal growth conditions, after addition of 500 nM of PMA for 24 h, or in the presence of anti-Fas Ab. Subsequently, the flow cytometric DNA profiles of the cells were analyzed. Different treatments are indicated at the bottom of the figure. e, cell lysates of Jurkat, PH1, LF(+), and LF(−) cells were prepared under normal growth conditions, following persistent (36 h), high-dose (500 nM) PMA exposure, and after addition of anti-human (1.5 μg/ml) or anti-mouse (5 μg/ml) anti-Fas Abs for 5 h (PH1 cells), 7 h (Jurkat cells), or 14 h (LF(+) and LF(−)). Subsequently, immunoblotting was performed by using anti-cyclin D, E, and A antibodies.
ity to Fas-mediated apoptosis conferred by activated p21<sup>ras</sup> was not the result of modulation of Fas receptor expression.

It has been demonstrated that apoptotic processes triggered by different effectors can be cell cycle-dependent or cell cycle-independent (54, 60, 61). To determine the cell cycle dependence of PKC/Ras-mediated or Fas-induced apoptosis, DNA profiles of the same populations of the four cell lines studied in Fig. 1, b and c, were analyzed (Fig. 1d). After down-regulation of PKC activity, the majority of Jurkat (94.8%), LF(+) (95.4%), and LF(-) (95.8%) cells accumulated in the G<sub>1</sub> phase. In comparison, no such G<sub>1</sub> cell cycle arrest occurred in PH1 cells, and a significant proportion of these cells (35%) remained distributed throughout the cell cycle. Taken together with the data from Fig. 1c, in which the numbers of PH1 cells steadily decreased after suppression of PKC, these results suggest that the operation of the PKC/Ras-related apoptotic program may require cell cycle progression. After addition of anti-human Fas antibody, when the death program had become apparent (5 h for PH1 and 7 h for Jurkat cells), there was no G<sub>1</sub> arrest of cell cycle. A significant proportion of either Jurkat (26%) or PH1 cells (26.2%) were still in S and G<sub>2</sub>/M phases following Fas ligation. Similarly, murine LF(+)- and LF(-) cells did not alter their DNA profiles 12 h after Fas ligation. It appears that Fas signaling had no impact on the cell cycle progression. To further confirm the results of the DNA profile analyses, the expression of certain G<sub>i</sub>/S cell cycle regulators, cyclins D, E, and A, were assayed in Jurkat, PH1, LF(+), and LF(-) cells, in response to Fas ligation or chronic, high-dose PMA treatment (Fig. 1e). An immunoblot of cyclin D showed that the protein was overexpressed in PH1 cells (about 3–4-fold) under normal growth conditions, which is in agreement with other reports that oncogenic ras can cause increased expression of cyclin D (14). After 36 h of high-dose PMA treatment or following 5 h of Fas ligation, the level of cyclin D expression did not change in PH1 cells. Cyclin D protein was expressed at a minimal level under normal growth conditions in unsynchronized Jurkat, LF(+), and LF(-) cells and increased to moderate levels after 36 h of down-regulation of PKC activity. After 7 h (for Jurkat cells) or 14 h (for LF(+)- and LF(-) cells) of Fas ligation, the expression of cyclin D did not change. Cyclins E and A were constitutively expressed in all cell lines under normal growth conditions, because the cell populations were unsynchronized. In Jurkat, LF(+)-, and LF(-) cells, the expression of cyclins E and A was undetectable following 500 nM of PMA for 36 h, consistent with an arrest in G<sub>1</sub> phase. However, in PH1 cells, the levels of cyclin E and A remained high after exposure to high-dose PMA. This result is in a good agreement with the data from DNA profile analyses, in which PH1 cells remained distributed throughout the cell cycle after down-regulation of PKC. There was no change in the levels of the cyclin proteins in any of the cells in response to Fas ligation.

Fas has been reported to activate JNK (49–52), and p21<sup>ras</sup> is involved in certain cellular responses leading to activation of JNK (e.g., the UV response) (62). To compare JNK induction in our experimental settings, the activity of JNK and JNK protein expression were analyzed (Figs. 2). The cells were cultured under normal growth conditions, after chronic, high-dose PMA treatment, or in the presence of anti-Fas Ab for 60 min. Subsequently, JNK activity was determined by its ability to phosphorylate an NH<sub>2</sub>-terminal (residues 1–79) c-Jun-GST fusion protein (Fig. 2a, upper panel). There was a detectable amount of phosphorylated c-Jun-GST in all control lanes, which may reflect basal JNK activity under normal growth conditions. After down-regulation of PKC, JNK activity was increased in PH1 cells about 4-fold. Fas-induced JNK activation was observed in all three cell lines and the magnitude of the induction ranged from 2-fold to 4-fold. This result therefore suggested that JNK activity may be required in, or at least related to, these two apoptotic processes. Immunoblot analysis showed that the level of JNK protein itself in the cells was not changed after induction of apoptosis by either chronic, high-dose PMA treatment or the presence of anti-Fas Ab (Fig. 2a, lower panel).

There are at least seven different isofoms of PKC in T lymphocytes (a, b, c, d, e, η, and θ). The isotypes of PKC are classified according to their structure and cofactor requirements for activation (63). PKC a, e, b, η, and θ activities are all dependent upon stimulation by diacylglycerol. In the presence of activated p21<sup>ras</sup>, the expression and the ratios of different PKC isotypes do not change in Jurkat cells (30, 64). Because p21<sup>ras</sup> is involved in PKC signaling during T cell activation and because p21<sup>ras</sup> activation has been suggested to play a role in Fas-mediated apoptosis (21, 35, 51), a potential requirement for PKC activity in the Fas-mediated apoptotic pathway was determined. A PKC-specific enzymatic assay was employed to measure the phosphotransferase activity of PKC in Jurkat cells after Fas ligation or treatment with 500 nM of PMA for 24 h (Fig. 2b). Induction of PKC activity was not observed either after Fas ligation or by re-stimulation of the cells with 50 nM of PMA following the chronic high-dose PMA treatment. This confirmed that chronic, high-dose PMA treatment did suppress PKC activity and suggested that PKC activation may not be directly involved in Fas signaling. In control experiments, PMA stimulation increased PKC activity, whereas chelerythrine, a PKC-specific inhibitor, suppressed PKC activity in the cells.

GAP negatively regulates p21<sup>ras</sup> activity by increasing the conversion of active GTP-bound Ras to the inactive GDP-bound state (55–57). To directly study a role for p21<sup>ras</sup> activity in the
two apoptotic pathways Fas-induced and PKC/Ras-mediated programmed cell death, GAP-specific antisense oligonucleotides were used in LF(+) cells to inhibit GAP expression so as to increase endogenous p21ras activity. The concentration of the antisense oligonucleotides required to suppress GAP expression was determined to be 20 μM by dose titration experiments (data not shown). LF(+) cells were exposed to 20 μM of either GAP-specific sense or antisense oligonucleotides for 48 h, and immunoblotting was then performed using an anti-GAP monoclonal antibody. The antisense, but not the sense, oligonucleotides suppressed the expression of GAP protein (Fig. 3a).

Subsequently, p21ras proteins were immunoprecipitated, and p21ras-associated guanine nucleotides were separated on a thin-layer chromatogram. c and d, LF1210 and LF(+) cells, untreated or treated with 20 μM of GAP antisense oligonucleotides for 48 h, were cultured with the anti-Fas antibody or cultured following down-regulation of PKC activity by 24 h of treatment with 500 nM PMA. Viable cells were enumerated at the time points indicated. Error bars represent the S.E. over five independent experiments.

FIG. 3. Up-regulation of endogenous p21ras activity in LF(+) cells by GAP antisense oligonucleotides. a, cell lysates from LF(+) cells were prepared after the cells were cultured in 20 μM of either GAP-specific sense or antisense oligonucleotides for 48 h. Immunoblotting was performed to assay the expression of the GAP. GAP (lane 1), 1 μg of GAP control lysate as positive control; Ctrl (lanes 2 and 4), unstimulated controls; 20 μM, 20 μM of either sense (lane 3) or antisense (lane 5) oligonucleotides. b, after addition of anti-Fas Ab for 15 min or no addition, PH1, Jurkat, LF(+), and LF(+) cells treated with 20 μM of GAP antisense oligonucleotides for 48 h were metabolically labeled with [32P]orthophosphate and lysed. In the presence of 20 μM, 20 μM of either sense (lane 3) or antisense (lane 5) oligonucleotides.
high-dose PMA treatment, the numbers of LF(+)
and LF1210 cells, treated with the GAP antisense oligonucleotides, steadily
decayed over 4 consecutive days, and approximately 70% of the
cells had undergone apoptosis by day 4 (Fig. 3c). This result
demonstrates that susceptibility to apoptosis after suppression
of PKC activity can be conferred by activation of wild-type,
endogenous p21ras, as well as by a mutated, oncogenic p21ras,
and is therefore dependent upon the activation of p21ras. Fur-
thermore, it provides evidence that even the normal promito-
genic activity of wild-type cellular p21ras protein can be redi-
rected toward pathways leading to cell death.

The ICE-like family of cysteine proteases has been impli-
cated in Fas-induced apoptosis (65). To determine whether the
apoptosis mediated by down-regulation of PKC required the
activity of CED-3/ICE-family proteases, the viabilities of Jur-
kat and PH1 cells were examined in the presence or absence of
ICE-like protease inhibitor Z-VADfmk after Fas ligation or
following PKC down-regulation by chronic PMA treatment
(Fig. 4). After addition of anti-Fas Ab, PH1 cells began to
undergo apoptosis at 5 h and were almost entirely apoptotic by
24 h (Fig. 4a). By contrast, when the cells were treated with
Z-VADfmk prior to Fas ligation, they remained nearly com-
pletely viable at 7 h after the addition of anti-Fas Ab, and by
24 h only 10–15% of PH1 cells had undergone apoptosis. Jurkat
cells were also sensitive to Fas ligation, but the magnitude of
the death process was lower than PH1 cells. However, 24 h
after addition of Z-VADfmk, less than 8% of Jurkat cells had
undergone apoptosis, which indicated there may be another
apoptotic pathway in addition to CED-3/ICE pathway, and Ras
may be involved in this pathway because the inhibition of
Fas-induced apoptosis by Z-VADfmk was more prominent in
PH1 cells than Jurkat cells. The DNA profile and the DNA
fragmentation of Jurkat cells following Fas engagement in the
presence of the inhibitor were also examined (data not shown).
There was no $G_1$ cell cycle arrest of Jurkat cells, and only a slight increase in DNA fragmentation (8–10%) was observed under such condition. After down-regulation of PKC by chronic, high-dose PMA treatment (Fig. 4b), there was only a moderate blocking effect (15–20%) of the apoptotic process in PH1 cells by Z-VADfmk. These findings suggested that the execution of apoptotic program by p21<sup>ras</sup> in PH1 cells during suppression of PKC activity may be downstream to, or not dependent completely on, CED-3/ICE-family proteases. Because down-regulation of PKC under normal conditions is not an apoptotic stimulus, Jurkat cells ceased growing for 2 days and regained the proliferation afterward, following suppression of PKC in the presence or absence of the inhibitor.

In some cases, the apoptotic program is dependent upon new synthesis of proteins. To compare any requirement for new protein synthesis in PKC/Ras-mediated apoptosis and Fas-induced apoptosis, a cell kinetic assay was conducted in the presence of the protein synthesis inhibitor cycloheximide at a concentration that inhibited the incorporation of [35S]methionine and [35S]cysteine into new protein by >90% (data not shown). Cycloheximide was added to the cell cultures 18 h after treatment with 500 nM PMA or 15 min before addition of anti-Fas antibody. In the presence of cycloheximide, after down-regulation of PKC by chronic, high-dose PMA treatment, the apoptotic process in PH1 cells was inhibited in both rate and magnitude in comparison with the cells treated with PMA alone (Fig. 4c). This finding suggested that the apoptosis induced by p21<sup>ras</sup> during inhibition of PKC activity is at least partially dependent upon new protein synthesis. There was no effect of cycloheximide on Fas-mediated apoptosis in PH1 cells (Fig. 4d), indicating an independence from a requirement for new macromolecular synthesis in Fas-triggered apoptosis. Possible confounding toxic effects of cycloheximide (or of the antisense cyclin E oligonucleotides utilized below) were minimized by determining the minimal inhibitory concentration range of the reagent and selecting a concentration in which the viability of the cells was maintained at least for 2 days.

The observed differences in cell cycle kinetics during Fas-induced versus PKC/Ras-mediated apoptosis (Fig. 1d) raised the question of whether the expression of cyclins is required for the latter. Cyclin E-specific antisense oligonucleotides were used to determine whether the expression of cyclins is required in either PKC/Ras-mediated or Fas-induced apoptosis in PH1 cells. The concentration of the antisense oligonucleotides required to abrogate cyclin E protein expression was determined in PH1 cells by immunoblotting for cyclin E protein following 2 days of exposure to the antisense oligonucleotides at different concentrations (Fig. 5a). Cyclin E antisense oligonucleotides at 5 μM suppressed the expression of the cyclin E protein in PH1 cells. Cyclin E antisense oligonucleotides significantly reduced PKC/Ras-mediated apoptosis in PH1 cells (range, 40–50% reduction) (Fig. 5b). By contrast, the same concentration of cyclin E sense oligonucleotides had no blocking effect on the apoptotic process. After Fas ligation, neither cyclin E antisense nor cyclin E sense oligonucleotides blocked the apoptotic process in PH1 cells (Fig. 5c). Therefore, cyclin E expression and, likely, consequent cell cycle progression are required for the PKC/Ras-mediated apoptotic program to be executed, but not for the Fas-mediated apoptotic program.

**DISCUSSION**

We have previously demonstrated that activated p21<sup>ras</sup> can cause human lymphoid Jurkat cells or mouse fibroblasts to undergo apoptosis under the condition of inhibition of PKC activity by chronic, high-dose PMA treatment or PKC inhibitors. This apoptosis is specifically related to p21<sup>ras</sup> expression (16). Jurkat cells express Fas antigen on the cell surface, and p21<sup>ras</sup> activation has been demonstrated as a requirement in Fas-induced apoptosis (18, 35, 51). The present investigation was designed to compare the basic molecular mechanisms of two different programmed cell death pathways requiring p21<sup>ras</sup> activity, Fas-induced apoptosis, and PKC/Ras-related apoptosis.
p21\textsuperscript{ras} and one of its downstream effectors, the stress-activated kinase family (JNK/p38), participate in the regulation of the apoptotic process in many different cell types (5, 16, 51, 66). For example, withdrawal of nerve growth factor from PC12 cells results in increased JNK activity, followed by apoptosis (67). It has also been shown that ceramides, which are generated at cell membrane through the activation of sphingomyelinsases by Fas ligation, stimulate p21\textsuperscript{ras} activity and induce cell death in T lymphocytes (35). In other studies, JNK activity has been demonstrated to be activated in Fas-mediated apoptosis. It appears that there must be some uncharacterized selectivity regarding Fas activation of JNK that distinguishes it from other pathways activating JNK, because transcription factor AP-1, which can be activated via enhanced phosphorylation of the c-Jun NH\textsubscript{2}-terminal activation domain by JNK in response to UV light or certain stresses, is not induced by Fas ligation (50). Here, we demonstrate that PKC/Ras-related apoptosis is at least partially dependent on cell cycle progression, but Fas-induced apoptosis is independent of cell cycle progression. However, p21\textsuperscript{ras} activity is required in both apoptotic processes (16, 35). Although JNK activity was demonstrated in both death processes, it is still not clear whether the activation of the kinase is necessary for the execution of the death program, or is due, rather, to the stress caused by the apoptotic stimuli. Further investigation of this question using antisense or dominant-negative approaches to determine the role of JNK in these two apoptotic processes is underway.

Down-regulation of PKC activity generally results in a reversible G\textsubscript{1} arrest in most cell types, including Jurkat (Refs. 16 and 59 and Fig. 1). After transient suppression of PKC activity, PH1 cells did not arrest in the G\textsubscript{1} phase. The progressive concurrent decreases in the number of viable cells and the increasing numbers of cells with fragmented DNA (16), indicating ongoing apoptosis, suggested a relationship with, and a potential requirement for, cell cycle progression to achieve the apoptotic consequence in these cells. The coincident increase in the expression of cyclins E and A in PH1 cells during the inhibition of PKC activity further supported the idea that these cells undergoing apoptosis fail to synchronize at a cell cycle checkpoint. Therefore, the data suggest that during suppression of PKC activity, activated p21\textsuperscript{ras} may force cells through a G\textsubscript{1} checkpoint and that such aberrant cell cycle progression events may be mediated by asynchronously expressed G\textsubscript{1}/S cyclins, with these inappropriate signals subsequently initiating the death program existing in the nucleus. A requirement for cell cycle progression and cyclin expression was tested for in PKC/Ras-related apoptosis by employing antisense inhibition of cyclin E, which significantly, but incompletely (60\%), inhibited the apoptotic process. Inhibition of new protein synthesis also inhibited PKC/Ras-related apoptosis to a similar extent. Because inhibition of cell cycle progression or macromolecule synthesis only partially protected the cells, we conclude that there are at least two apoptotic death pathways initiated in PKC/Ras-related apoptosis. One major pathway is dependent on abnormal cell cycle events, wherein the cell cycle may be required for assembling or synthesizing the proteins needed for the execution of the death program. The second pathway is independent of the cell cycle. This apoptotic program may be executed in the cytoplasm, perhaps involving the activation of some CED-3/ICE family members, without the requirement of nuclear events.

Both LF(+) and Jurkat cells express moderate levels of Fas antigen on the cell surface and undergo apoptosis in response to Fas ligation to a similar degree. Although the onset of apoptosis in Jurkat cells is earlier than in LF(+) cells, this may reflect intrinsic physiological differences among different cell lines or cells from different species. It appears that the Fas signaling pathway does not directly involve PKC activity. In the presence of oncogenic p21\textsuperscript{ras} or under conditions in which endogenous p21\textsuperscript{ras} activity is up-regulated, the apoptotic process initiated by Fas ligation is accelerated. Taken together with data previously presented by ourselves and others (16, 18, 35, 51, 59), this finding further supports the involvement of p21\textsuperscript{ras} in the regulation of Fas-induced apoptosis. Antisense cyclin E oligonucleotides or cycloheximide did not block the apoptotic process initiated by addition of anti-Fas Ab to PH1 cells. In combination with the finding that Fas ligation did not alter cell cycle progression, these data suggest that the components required for the Fas-initiated apoptotic process, such as CED-3/ICE family members, pre-exist in the cell.

Experiments using the protease inhibitor peptide Z-VADfmk showed that Z-VADfmk prevented about 85\% of PH1 cells from undergoing Fas-induced apoptosis, and such protection became minor in the control Jurkat cells. However, the inhibitor only moderately protected PH1 cells (about 20\%) from the death after suppression of PKC activity (PKC/Ras-related apoptosis). Because p21\textsuperscript{ras} is clearly involved in both of these apoptotic processes, it is tempting to speculate that p21\textsuperscript{ras} may function differentially in these two death programs, perhaps due in part to the involvement of PKC, a known anti-apoptotic effector in a number of systems (16, 22, 26, 27, 68–74). Under normal growth conditions in cells expressing an activated p21\textsuperscript{ras}, PKC may cooperate with the signals generated by p21\textsuperscript{ras} to transmit growth or mitogenic signals, and at some time may also act in a protective fashion to suppress the activation of apoptotic components in the cells as a result of the unregulated mitogenic signals generated by the activated p21\textsuperscript{ras}. After inhibition of PKC activity, however, the same signals generated by the activated p21\textsuperscript{ras}, without modification by PKC, may trigger the nuclear apoptotic machinery through the temporally incorrect activation of cell cycle progression events (16). Because endogenous (c)-p21\textsuperscript{ras} appears to have a role in apoptotic programs initiated by agents such as Fas and tumor necrosis factor, it is also possible that activated p21\textsuperscript{ras}, perhaps through activation of JNK, may have a more direct action on death effector pathways, such as the CED-3/ICE family members. The only modest protection afforded by protease inhibitors in the setting of PKC/Ras-related apoptosis compared with Fas-mediated apoptosis, however, suggests that p21\textsuperscript{ras} activation alone, without Fas ligation, does not result in proper recruitment of death effectors such as caspases, nor in the full and efficient activation of the CED-3/ICE family. However, p21\textsuperscript{ras} activation initiated by Fas ligation may facilitate the full activation of the Fas death signal by activating and accelerating (in addition to the Fas-MORT1/FADD-CED-3/ICE pathway) a parallel p21\textsuperscript{ras}-effected pathway leading to apoptosis, which is relatively independent of CED-3/ICE family proteases.

In conclusion, p21\textsuperscript{ras}, as an apoptotic signal transducer, may be involved in the regulation of at least two different types of apoptotic mechanisms, Fas-induced and PKC/Ras-related apoptosis. The analysis of PKC/Ras-related apoptosis in comparison with Fas-mediated apoptosis suggests that these two apoptotic programs may, to some extent, share common elements, including the activation of p21\textsuperscript{ras} and the JNK/JUN signaling pathway. Endogenous or exogenously activated p21\textsuperscript{ras} can accelerate both types of programs. p21\textsuperscript{ras}, under apoptotic conditions, may be a key intermediate molecule linking different cell death processes, such as cell cycle-independent (possibly cytoplasmic) and cell cycle-dependent (possibly nuclear) machinery.

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