The Recruitment of Fas-associated Death Domain/Caspase-8 in Ras-induced Apoptosis

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Abstract

Oncogenic Ras induces cells to undergo apoptosis after inhibition of protein kinase C (PKC) activity. The integration of differential signaling pathways is required for full execution of apoptosis. In this study, we used Jurkat as well as Fas/FADD-defective cell lines expressing v-ras to determine the upstream elements required for activation of the caspase cascade in PKC/Ras-mediated apoptosis. During this Ras-induced apoptotic process, caspase-8 was activated, possibly through its binding to Fas-associated death domain (FADD), in Jurkat/ras and Jurkat/Fas<sub>r</sub>/ras cells but not in Jurkat/FADD<sub>r</sub>/ras cells. c-Jun NH<sub>2</sub>-terminal kinase (JNK) was activated in all three cell lines expressing ras in response to apoptotic stimulation. Suppression of JNK by dn-JNK1 blocked the interaction of FADD and caspase-8 and partially protected Jurkat/ras and Jurkat/Fas<sub>r</sub>/ras cells from apoptosis. However, dn-JNK1 had no effect on PKC/Ras-induced apoptosis in Jurkat/FADD<sub>r</sub>/ras cells. The results indicate that FADD/caspase-8 signaling is involved in PKC/Ras-mediated apoptosis, and JNK may be an upstream effector of caspase activation.

Introduction

The connection of aberrant Ras signaling to human cancer prompted the investigation of the downstream effector pathways governed by Ras. A pathway in which Ras upregulates MAPK<sup>3</sup> activity by signaling through Raf kinase was elucidated by simultaneous studies in Drosophila and Caenorhabditis elegans (1). Subsequently, ras was found to regulate multiple downstream effector pathways, including MAPK, p38MAPK, JNK, phosphatidylinositol 3'-kinase, and the Rho family GTPases (2). Dominant-activating mutations in Ras have been discovered in a variety of human tumors and are believed to play an important role in the multistep progression of tumorigenesis (3). It has also been demonstrated that Ras can promote PCD after down-regulation of PKC activity, Fas engagement, or tamoxifen treatment (4–10). Cellular PKC activity can protect cells against apoptosis in response to a variety of apoptotic stimuli (11–13). Interestingly, certain apoptotic stimuli that operate through Ras, such as Fas engagement, also simultaneously block endogenous PKC activation of human and mouse lymphocytes (14). Our recent investigation suggested that several discrete signaling pathways may be required for the efficient execution of the Ras-mediated apoptosis (9, 15). By using cyclin E, the antisense oligonucleotides, caspase inhibitors (Z-VADfmk), or reactive oxygen species inhibitors, we have demonstrated that cell cycle events, caspase activity, and free radicals are potential mediators of the Ras-mediated apoptosis. However, the precise mechanism(s) whereby ras mediates PCD is unknown.

In general, PCD consists of three stages: initiation, effector, and execution phases. Fas ligation triggers the activation of FLICE/MACH/caspase-8, interleukin-Iβ converting enzyme-like proteases, and ceramide/Ras (16–23). During the effector phase, which can still be modulated by various factors, these initial events cause the activation of BID and subsequent disruption of the mitochondrial membrane (16–25). The increase of the permeability of the mitochondria in turn liberates cytochrome c and apoptosis inducing factor. Massive activation of proteases is subsequently elicited. Thereafter, in the degradation phase, these proteases execute the death program in an irreversible fashion. Studies using a protease inhibitor Z-VADfmk have suggested that caspase activity is involved in PKC/Ras-mediated apoptosis. Other mechanisms involved in the effector stage are not yet clear, with some studies indicating a requirement for JNK/p38 kinase activation (4, 5, 9, 26–28).

Although our previous investigation has demonstrated that full execution of PKC/Ras-mediated apoptosis requires multiple signaling pathways, including caspase activity, it is still unclear which downstream effectors of Ras mediate signaling during PCD. In this study, we have examined the role of caspase family members in the regulation of the Ras-mediated apoptosis. Jurkat cells and mutant Jurkat cell lines containing either Fas or FADD mutations were used to test a potential role for Fas/FADD signaling in PKC/Ras-mediated apoptosis.

Z-VADfmk, Z-Val-Ala-Asp-fluoromethyl ketone; BID, BH3 interacting domain.
apoptosis. Jurkat/\(\text{ras}\) and Jurkat/\(\text{Fas}^{-/-}/\text{ras}\) cells underwent apoptosis, after PKC down-regulation. In these cell lines, the induction of apoptosis induced interaction of FADD and caspase-8, BID cleavage, and release of mitochondrial cytochrome c. In contrast, Jurkat/\(\text{FADD}^{-/-}/\text{ras}\) cells were less susceptible to apoptosis in response to PKC down-regulation, and caspase-8 was not activated. JNK activity was elicited in all three cell lines during initiation of PKC/Ras-mediated apoptosis. The introduction of a \(\text{dn-JNK1}\) blocked the recruitment of caspase-8 to FADD and further reduced the magnitude of PKC/Ras-mediated apoptosis in Jurkat/\(\text{ras}\) and Jurkat/\(\text{Fas}^{-/-}/\text{ras}\) cells but not in Jurkat/\(\text{FADD}^{-/-}/\text{ras}\) cells. Overall, the results suggest that FADD-mediated, Fas-independent activation of caspase family members is a critical Ras-regulated apoptotic pathway.

Results
PKC/Ras-mediated Apoptosis Was Partially Dependent upon FADD but not upon Fas. To study the molecular mechanism of PKC/Ras-mediated apoptosis, v-Ha-ras was inserted into a retroviral vector, murine stem cell virus (MSCV), and introduced into the lymphoblastoid T cell line Jurkat A3. The activated \(\text{ras}\) gene was also introduced into the mutant cell lines Jurkat/\(\text{Fas}^{-/-}\) or Jurkat/\(\text{FADD}^{-/-}\), which are derived from Jurkat A3 cells by random mutagenesis (23) and are functionally deficient in Fas Ag or in the death adaptor protein FADD, respectively. We selected clones I4.1 (Jurkat/\(\text{Fas}^{-/-}\)) and I2.1 (Jurkat/\(\text{FADD}^{-/-}\)) for use in this investigation, because these mutant cells have been well characterized in other studies (23). The transfectants were tested to ensure that v-\(\text{ras}\) expression was maintained (the resulting cell lines are designated as Jurkat/\(\text{ras}\), Jurkat/\(\text{Fas}^{-/-}/\text{ras}\), or Jurkat/\(\text{FADD}^{-/-}/\text{ras}\)). \(\text{ras}\) expression in all three cell lines was analyzed by RNA blot analysis (Fig. 1A, upper panel). Increased amounts of ras mRNA could be detected in all three cell lines infected with the \(\text{ras}\) vectors. The levels of ras transcripts expressed were similar in all cell lines when normalized for the expression of \(\text{actin}\) transcripts (Fig. 1A, lower panel). The barely detectable levels of ras transcripts in Jurkat cells reflect the low basal expression of endogenous ras.

We have demonstrated previously that cells with increased Ras activity are susceptible to apoptosis in response to inhibition of PKC activity (9). Ras activation has also been suggested to be involved in Fas-induced PCD (9). To examine whether Fas or FADD may be required for the Ras-mediated apoptosis, DNA fragmentation assays were conducted in the cell lines (Jurkat, Jurkat/\(\text{Fas}^{-/-}\), or Jurkat/\(\text{FADD}^{-/-}\)) without or with ectopic expression of v-Ha-ras (Fig. 1B, upper panel). DNA fragmentation assays was also performed in Jurkat cells expressing dn-FADD (which encodes a FADD product containing an intact death domain and mutated death-effector domain; in the absence or presence of ras, cell lines designated Jurkat/dnFADD and Jurkat/dnFADD/ras, respectively). Under normal growth conditions, very low percentages of cells contained fragmented DNA, irrespective of v-\(\text{ras}\) expression. Chronic, high-dose PMA treatment has been demonstrated to down-regulate PKC activity in both human and murine lymphocytes (5). Down-regulation of PKC activity, in the absence of v-\(\text{ras}\), did not cause DNA fragmentation in Jurkat, Jurkat/\(\text{Fas}^{-/-}\), Jurkat/\(\text{FADD}^{-/-}\), or Jurkat/dn-FADD cells. In contrast, 48 h after down-regulation of PKC activity (>40% of Jurkat/ras cells or 38% of Jurkat/\(\text{Fas}^{-/-}/\text{ras}\) cells contained a hypodiploid DNA content, whereas the percentage of DNA fragmentation in Jurkat/\(\text{FADD}^{-/-}/\text{ras}\) was only ~20%. Similar results were also obtained from Jurkat/dnFADD/ras cells. To ensure that suppression of the Ras-mediated apoptosis seen in the FADD mutants was not attributable to clonal variation, DNA fragmentation analysis was conducted using different clones of Jurkat/\(\text{Fas}^{-/-}\) (E2 and I6.5) or Jurkat/\(\text{FADD}^{-/-}\) (E1 and I6.2),
expressing or not expressing \textit{v-ras}, in response to PMA treatment (Fig. 1B, lower panel). The Ras-mediated apoptosis was partially blocked in all FADD mutant clones but not in the \textit{Fas}_{\text{m}} clones. These data indicate that the death adaptor FADD may participate in the Ras-mediated apoptotic process, whereas the Fas Ag plays no role in the process.

Association of Caspase-8 with FADD during PKC/Ras-mediated Apoptosis. Cross-linking of Fas Ag by Fas ligand or anti-Fas Ab results in the formation of so-called DISC, which then recruits the adaptor protein FADD/MORT-1 and caspase-8 (20, 21, 29–31). This early event can be detected in many cell types during Fas-induced PCD. The resulting DISC leads to cleavage of caspase-8 and initiation of the caspase cascade (24, 25). It has been reported that Jurkat/ \textit{Fas}_{\text{m}}, cells express a \textit{FADD} gene of the correct size, whereas the resulting protein product contains a mutation at the COOH-terminus (23). To verify that the COOH-terminus of FADD was mutated in the FADD mutant cells, immunoprecipitation and immunoblotting of FADD with an anti-FADD Ab directed against the COOH or NH\textsubscript{2} terminus of the protein (upper two panels). The same blots were reprobed with an anti-actin Ab for the determination of equal loading of samples (lower two panels). In B, after treatment with either high-dose PMA (500 nM for 24 h) or an anti-Fas Ab (for 30 min), lysates from Jurkat cells or mutant Jurkat cell lines without (panels 1 and 3 from the top) or with (panels 2 and 4) ectopic expression of \textit{v-ras} were prepared. Upper two panels, after normalization for protein concentration, the samples were immunoprecipitated with the anti-FADD Ab directed against the NH\textsubscript{2} terminus of the protein, and the immunocomplexes were separated on a SDS-PAGE gel. After transfer of the proteins to nitrocellulose, immunoblotting was performed using an anti-caspase-8 Ab. Lower two panels, the same blots were reprobed with the anti-FADD Ab for determination of even loading of samples.

![Fig. 2](image) Caspase-8 binding to FADD during induction of Fas- or Ras-mediated apoptosis. In A, lysates containing an equal amount of total proteins from Jurkat, Jurkat/ \textit{Fas}_{\text{m}}, (I4.1), or Jurkat/ \textit{FADD}_{\text{m}}, (I2.1), with or without expressing \textit{v-ras}, were immunoprecipitated and immunoblotted with an anti-FADD Ab directed against either the COOH or NH\textsubscript{2} terminus of the protein (upper two panels). The same blots were reprobed with an anti-actin Ab for the determination of equal loading of samples (lower two panels). In B, after treatment with either high-dose PMA (500 nM for 24 h) or an anti-Fas Ab (for 30 min), lysates from Jurkat cells or mutant Jurkat cell lines without (panels 1 and 3 from the top) or with (panels 2 and 4) ectopic expression of \textit{v-ras} were prepared. Upper two panels, after normalization for protein concentration, the samples were immunoprecipitated with the anti-FADD Ab directed against the NH\textsubscript{2} terminus of the protein, and the immunocomplexes were separated on a SDS-PAGE gel. After transfer of the proteins to nitrocellulose, immunoblotting was performed using an anti-caspase-8 Ab. Lower two panels, the same blots were reprobed with the anti-FADD Ab for determination of even loading of samples.

Jurkat/ \textit{FADD}_{\text{m}} cells, immunoblotting was also conducted using the anti-FADD Ab directed against the NH\textsubscript{2}-terminus (Fig. 2A, second panel). Similar amounts of FADD were detected in all cell lines. Equal loading of the samples was verified by reprobing the same blots with an anti-actin Ab (Fig. 2A, third and fourth panels). Immunoblotting for FADD was also conducted in the other FADD_{m} cell lines (E1 and I6.2), and identical results were obtained (data not shown). These findings corroborate a previous report that the COOH-terminus of FADD is mutated in Jurkat/ \textit{FADD}_{\text{m}} cells (23). We next investigated whether the formation of DISC occurs during Ras-mediated apoptosis in the FADD mutant cells. The ability of caspase-8 to be coimmunoprecipitated with an anti-FADD Ab directed against the NH\textsubscript{2}-terminus of the protein was examined in Jurkat, Jurkat/ \textit{Fas}_{\text{m}}, (I4.1 and I6.2), or Jurkat/ \textit{FADD}_{\text{m}}, (I2.1 and E.1) cells without (Fig. 2B, first and third panels) or with (Fig. 2B, second and fourth panels) ectopic expression of \textit{v-ras}, under normal growth conditions, either 30 min after Fas ligation or 24 h after down-regulation of PKC. Protein complexes coimmunoprecipitating with the anti-FADD Ab from all cell lines were immunoblotted by an anti-caspase-8 Ab directed against the NH\textsubscript{2}-terminus of the protein (Fig. 2B, panels 1 and 2). Under normal growth conditions, no interaction between FADD and caspase-8 was
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After either Fas ligation or chronic, high-dose PMA treatment, lysates containing equal amounts of total protein from Jurkat, Jurkat/Fasm, or Jurkat/FADDm cells without (upper two panels) or with (lower two panels) expression of v-ras were separated on a SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with the anti-caspase-8 Ab.

Full-length BID protein was detected in all untreated cells, as well as in Jurkat and Jurkat/FADDm/ras cells treated with PMA. In contrast, the levels of the full-length BID were dramatically reduced in Jurkat/ras and Jurkat/Fasm/ras cells, after PKC down-regulation and a new M, 15,000 fragment (p15) was detected by an anti-BID Ab in these cells. The data suggest that BID is cleaved during the Ras-mediated apoptosis. As a control, the same analysis was carried out in the same cell lines after Fas ligation, and cleavage of BID was observed in both Jurkat and Jurkat/ras cells, as expected (data not shown).

Loss of mitochondrial transmembrane potential, the opening of permeability transition pores, and the release of cytochrome c from the mitochondria to the cytosol accompany the Fas-initiated apoptotic process. These processes may be mediated by BID (21, 24, 34). Because BID was activated in response to PKC down-regulation in the presence of activated ras and because BID has been linked to mitochondrial function, we next tested for mitochondrial cytochrome c release. After down-regulation of PKC, cytosolic fractions from Jurkat, Jurkat/ras, Jurkat/Fasm/ras, or Jurkat/FADDm/ras cells were immunoblotted for cytochrome c (Fig. 4B). Cytochrome c was detectable in the cytosol fractions prepared from Jurkat or Jurkat/ras cells after Fas ligation (Fig. 4B, Lanes 2 of the upper and lower panels). Cytochrome c was also released from the mitochondria to the cytosol in Jurkat/ras (Fig. 4B, lane 3 of the lower panel) or Jurkat/Fasm/ras cells (Fig. 4B, Lane 6 of the lower panel), after PKC down-regulation. Cytosolic cytochrome c could not be detected in Jurkat/FADDm cells (irrespective of v-ras expression status) after down-regulation of PKC activity by PMA treatment or in response to Fas ligation (Fig. 4B, Lanes 8 and 9 of the upper and lower panels). The low basal levels of cytochrome c observed in the cytoplasmic fraction from untreated Jurkat/ras cells may result from contamination during the fractionation procedure. These findings indicate that mitochondrial permeability is increased during the course of Ras-mediated apoptosis, possibly in a caspase-dependent manner.
JNK Is Activated by Ras-mediated Apoptotic Signals in a FADD-independent Manner. It has been reported that Ras signaling mediates cellular stress responses, resulting in the activation of JNK (e.g., the UV response; Refs. 35–37). Fas engagement can also increase the activity of the stress kinases (JNK or p38) in a caspase-dependent fashion that requires DISC signaling (23, 26, 38). Because JNK activation has been demonstrated in the Ras-mediated apoptosis (17), we investigated potential links between Fas/FADD activity and JNK activation in this death process. Jurkat cells or the mutant cell lines (with or without ectopically expressed v-ras) were cultured in the absence or presence of high-dose PMA for 20 h. Subsequently, JNK activity was assayed by determining its ability to phosphorylate an in vitro substrate (NH2-terminal [residues 1–89] c-Jun-GST fusion protein) (Fig. 5A, upper panel). Basal JNK activity was detectable in all untreated cell lines (Fig 5A, Lanes 1, 3, 5, 7, 9, and 11). JNK activity was not induced by chronic high-dose PMA treatment in Jurkat, Jurkat/Fasm, or Jurkat/FADDm (Fig. 5A, Lanes 2, 6, and 10). However, down-regulation of PKC activity resulted in prominent JNK activation in all three cells expressing v-ras (Fig. 5A, Lanes 4, 8, and 12). c-Jun phosphorylation for each cell line with or without treatment was also quantified by phosphomager analysis (Fig. 5A, middle panel). The levels of JNK1 protein was determined by immuno blotting a portion of the lysates from the same samples shown in the upper panel with an anti-JNK1 Ab (Fig. 5A, lower panel). The same levels of JNK1 were detected in all cell lines, indicating that PMA treatment did not alter the expression of JNK1.

Because JNK activity was induced during the process of PKC/Ras-mediated apoptosis, we then tested the effect of JNK inhibition (using dn-JNK1) or of high-level JNK expression on the Ras-mediated apoptosis. The dn-JNK1 or JNK1 construct, together with GFP-spectrin (the product of which is a membrane-localized, green fluorescent-fusion protein) were cointroduced into Jurkat/ras, Jurkat/Fasm/ras, or Jurkat/FADDm/ras cells. GFP-positive cells were assumed to also express either the dn-JNK1 or JNK1, because the expression vectors for the latter were in excess (8:1). Forty-eight h after addition of PMA (500 nm), the cells were fixed and stained with propidium iodide. A subpopulation of the cells expressing GFP was subsequently identified and gated by flow cytometry and analyzed for DNA fragmentation (Fig. 5B). A large proportion of Jurkat/ras (>40%) or Jurkat/Fasm/ras (>38%) cells underwent apoptosis in response to PKC down-regulation, whereas a smaller fraction of Jurkat/FADDm/ras cells (~22%) died under the same conditions. After introduction of dn-JNK1, the percentage of DNA fragmentation in response to PKC down-regulation was decreased to ~20% in Jurkat/ras or Jurkat/Fasm/ras cells. As expected, inhibition of JNK by expression of dn-JNK1 did not change the percentage of DNA fragmentation in Jurkat/FADDm/ras cells in response to chronic high-dose PMA treatment. However, overexpression of JNK1 enhanced the magnitude of apoptosis in either Jurkat/ras or Jurkat/Fasm/ras cells after down-regulation of PKC, with >50% of these cells containing fragmented DNA. The magnitude of apoptosis induced in the Jurkat/FADDm/ras cells was unchanged, despite JNK1 overexpression. The same DNA fragmentation assay was also conducted in the cells transfected with the empty pGEX construct, and the vector itself had no effect on the apoptotic process. These results indicate that JNK activity is involved in the regulation of caspase-mediated signaling in the Ras-dependent apoptotic process, most likely at a level upstream of FADD activation.

DNA fragmentation analyses were also conducted in Jurkat/dn-FADD/ras cells after introduction of dn-JNK1 or JNK1 by transient transfection. We compared the percentages of apoptotic Jurkat/dn-FADD/ras cells (which contain an NH2-terminal mutation of FADD) with that occurring in Jurkat/FADDm/ras cells (in which FADD contains a COOH-terminal mutation). Jurkat/dn-FADD/ras cells expressing either dn-JNK1 or JNK1 had similar percentage (~20%) of DNA fragmentation, after PKC down-regulation. These values are comparable with those elicited in the cells with COOH-terminal mutation of FADD under the same experimental conditions. These data again indicate that the involvement of FADD in PKC/Ras-mediated apoptosis requires a FADD protein containing both intact death and death-effector domains.
We then examined the correlation between JNK activation and caspase activity. Cell lysates from Jurkat/ras cells transfected with dn-JNK1 or empty vector (pGEX), untreated or treated with high-dose PMA, were assayed for coimmunoprecipitation of FADD with caspase 8 (Fig. 6, upper panel), for caspase-8 cleavage (Fig. 6, middle panel) and for BID cleavage (Fig. 6, lower panel). After down-regulation of PKC, caspase-8 could be coimmunoprecipitated with FADD in Jurkat/ras/mock cells but not in Jurkat/ras/dn-JNK1 cells. Correspondingly, the cleaved fragments of caspase-8 (p18) could be detected in Jurkat/ras/mock cells after suppression of PKC activity and was evident after Fas engagement. In contrast, the active form of caspase-8 was evident after Fas ligation in Jurkat/ras/dn-JNK1 but not in response to PKC down-regulation. Immunoblotting using an anti-BID Ab also demonstrated that BID was not cleaved in response to down-regulation of PKC activity in Jurkat/ras/dn-JNK1 cells. These data again suggest that JNK activity may be involved in one arm of the Ras-mediated apoptotic pathway, which may modulate a downstream, FADD/caspase 8-related signal.

Discussion
We have demonstrated previously that oncogenic Ras can elicit programmed cell death once endogenous PKC activity is inhibited (5). Ras recruits specific death signal transducers from various apoptotic signaling pathway to efficiently execute the death program (9, 15). A requirement for multiple signals, such as caspase activity, DAXX, and ceramide/Ras activation, has also been elucidated in Fas-initiated cell death (4, 6, 8, 11, 27). JNK activation was observed in the course of PKC/Ras- or Fas-induced apoptotic program, but its role in the PKC/Ras-mediated cell death is still unclear. The addition of the caspase inhibitor Z-VADfmk partially suppressed PKC/Ras-mediated cell death, indicating that caspase family members may be involved in this process (9). It is therefore important to understand what upstream elements are required for caspase activity in this apoptotic process. In this report, by using Fas- or FADD-mutant cells, we have tested signaling pathways and the role of JNK in PKC/Ras-mediated cell death.

Analysis of lymphocytes from FADD-deficient mice revealed that the proteases of the caspase family are crucial factors in the execution of the PCD process elicited by various stimuli, including Fas-mediated apoptosis (23, 39). In addition, in Myc-induced apoptosis, Myc might sensitize mouse embryonic fibroblasts to, and subsequently recruit, the Fas apoptotic signal (40). We have reported previously that the caspase inhibitor Z-VADfmk, or suppression of cyclin E expression, or N-acetyl-l-cysteine (to block the pro-
duction of reactive oxygen species), all partially protected Jurkat cells expressing v-ras from PKC/Ras-mediated apoptosis. These data suggest that multiple pathways may be required for the full execution of this death process (9, 15). In this study, using Fas- and FADD-mutant cells, we demonstrated that PKC/Ras-mediated cell death was partially blocked in Jurkat/FADDm/ras cells, relative to Jurkat/ras or Jurkat/Fasm/ras cell lines. Furthermore, we have shown that this partial blockade is accompanied by failure to activate caspase-8/BID. JNK activation occurred in all ras-expressing cells (Jurkat/ras, Jurkat/Fasm/ras, or Jurkat/FADDm/ras) during the Ras-mediated apoptotic process. Blockade of JNK by dn-JNK1 partially suppressed cell death and abrogated the activation of caspase-8 in Jurkat/ras and Jurkat/Fasm/ras cells. Therefore, the activation of caspase family members may be at the level of FADD and is likely to occur in a Fas-independent fashion. Moreover, JNK may be upstream of caspase recruitment/activation in Ras-mediated apoptosis (Fig. 7).

The trimerization of Fas Ag mediated by Fas ligand, anti-Fas Ab, or UV irradiation triggers multiple Fas-related death pathways (37, 41–43). The main death pathway in response to Fas ligation involves the formation of DISC, in which FADD, as an adaptor, recruits caspases to the death domain of Fas Ag and subsequently initiates the caspase cascade. Lymphocytes from FADD−/− mice are resistant to Fas-mediated apoptosis, demonstrating the requirement of FADD in Fas signaling (23, 39). However, the involvement of FADD signaling in the other forms of apoptosis is still poorly understood and may be dependent upon cell type or stimuli. The cytotoxic drugs commonly used in anticancer therapy can induce tumor cell death by forming DISC and subsequently initiating the caspase cascade without primary engagement of Fas Ag (44–46). HT29 cells, a human colon carcinoma cell line, became more sensitive to drug-induced apoptosis when stably overexpressing FADD, whereas the introduction of an FADD-antisense construct desensitized the cells to anticancer drugs (46). During anoikis, a type of apoptosis induced through detachment of adherent cells from the extracellular matrix (47–50), caspase-8 and caspase-3 are strongly activated, and a dominant-negative form of FADD, but not soluble extracellular domains of Fas Ag or tumor necrosis factor-related receptors (DR4 or DR5), blocks this detachment-mediated cell death. These data indicate that other unknown intracellular receptor(s) may be involved in this process (49, 50). Our study demonstrates that FADD binding to caspase 8, and subsequent activation of caspase 8, occur during the PKC/Ras-mediated apoptotic process in Fas-deficient cells. Using a FADD mutant in which the C-terminus of FADD was mutated, we demonstrated that the FADD/caspase 8 association did not occur, and coincidentally, the extent of apoptosis in these cells was significantly reduced. These results suggest that, as in Fas-induced apoptosis, the initiation of FADD activation during the PKC/Ras-mediated cell death process requires an existence of an intact C-terminal death domain. Studies using an alternate FADD mutant, a dominant-negative form of FADD which contains an intact death domain and mutated N-terminal-death-effector domain, showed that the suppression of PKC/Ras-mediated cell death was similar to that observed in cells containing the C-terminal FADD mutation. Therefore, the death-effector domain of FADD is also required for the recruitment of caspase activity in this death process. Our data suggest the possible existence of intracellular death receptor/receptors which may, during the initiation phase of the PKC/Ras-mediated apoptotic process, recruit FADD and form a death complex through their death domain. This may
then allow the death effector domain of FADD to interact with caspase 8, leading to a downstream caspase cascade. However, we have not ruled out the potential involvement of other membrane-bound death receptors (such as TNF-related receptors) in this recruitment during PKC/Ras-mediated apoptosis. It is also possible that FADD/caspase 8 may trigger the caspase cascade in a ligand-independent, but BID-dependent, manner during PKC/Ras-mediated apoptosis.

Two types of Fas signaling pathways have been proposed in different cell lines (25). In the Fas type I pathway, a relatively large amount of caspase-8 binds to DISC in response to Fas ligation. A downstream protease cascade is elicited, and the mitochondrial transmembrane potential is disrupted. In type II signaling, a small amount of caspase-8 is activated, resulting in apoptosis through mitochondrial damage and subsequent cytochrome c release into the cytoplasm (25). In the PKC/Ras-mediated cell death process, the activation of caspase-8 was accompanied by the cleavage of BID and cytochrome c release. Overexpression of bcl-2 in these cells inhibits BID activation and protects the mitochondria from disruption (data not shown). The FADD/caspase-8-mediated pathway involved in the PKC/Ras-triggered cell death process thus appears similar to Fas type II signaling, although Fas is not involved. It is possible that, somewhat, the reduced FADD/caspase-8 association in PKC/Ras-induced apoptosis, in comparison with that occurring during Fas-triggered cell death, cannot initiate or amplify the full caspase cascade. Therefore, caspase signaling may account for only one pathway in the Ras-induced apoptosis.

The stress-activated kinase family (JNK/p38) participates in the regulation of the apoptotic process in different cell types, and its activity has been particularly implicated in cytochrome c-mediated apoptosis (9, 40, 51, 52). Withdrawal of nerve growth factor from PC12 cells results in the activation of JNK, leading to apoptosis (53). JNK activation has also been shown to occur in Fas-mediated apoptosis (23, 26, 38). Other studies have suggested that DAXX can bind to Fas and further elicit JNK activation in Fas-induced apoptosis (27). The activation of this stress-related kinase mediated by Fas ligation is defective in FADD-deficient mutants, but the kinase activity of JNK could still be induced by osmotic shock (23). Our current study demonstrates that down-regulation of PKC in ras-expressing cells resulted in JNK activation, followed by apoptosis. Dominant-negative JNK could suppress the activation of caspase-8 and BID and attenuate the magnitude and kinetics of cell death in those ras-expressing, FADD-intact cells. Conversely, overexpression of JNK enhanced the magnitude of this apoptotic process. Furthermore, JNK activity was initiated by down-regulation of PKC in the Fas-mutant (Jurkat/Fasm/ras) and FADD-mutant (Jurkat/FADDm/ras) cell lines. Therefore, JNK may act upstream of, or in parallel to, FADD/caspases during Ras-mediated apoptosis. This contrasts with JNK activation in Fas-initiated PCD, wherein JNK activation requires FADD (23). The higher levels of c-Jun phosphorylation by JNK observed in Jurkat/FADDm/ras cells may result from a blockade of a positive apoptotic feedback network involving phosphatase(s) activities. Alternatively, it is possible that the higher JNK activity under such conditions is a reflection of the delayed kinetics of the apoptotic process and of JNK activation, which may occur as a result of FADD deficiency. Determining precisely how JNK activity is involved in the regulation of the caspase-related arm of the PKC/Ras-mediated apoptotic signaling requires further investigation.

In summary, activated Ras, as an inducer of apoptosis, regulates PCD through multiple pathways. Caspase activity is involved in PKC/Ras-induced cell death. The initiation of caspase cascade in this process is FADD dependent and Fas independent. JNK may be a key intermediate molecule linking caspase activity to more upstream elements in PKC/Ras-mediated apoptosis.

Materials and Methods

Cell Lines

Mutagenesis. Jurkat A3 (1 × 10⁸ cells) were either left untreated or treated with ethyl methane sulfonate (200 μg/ml for 24 h; Sigma; Refs. 23 and 54). Cells were cultured in the normal growth medium for 5 days prior to selection with the anti-Fas Ab. Clones were generated by serial dilution in 96-well plates in the presence of the anti-Fas Ab. After 2–4 weeks, surviving clones were expanded and further characterized. ICR191 mutagenesis was also used as described (23, 55, 56). Briefly, the cells (1 × 10⁶) were untreated or treated with ICR191 at 2 μg/ml for 2 h. Cells were exposed to ICR191 for three cycles to increase the frequency of mutagenizing both alleles. After the initial treatment, the mutagen was washed out, and the cells were allowed to recover for 15 days. Cells were again treated with ICR191 and allowed to recover for 2 days before the final treatment. Clones were selected by serial dilution in 96-well plates in the presence of the anti-Fas Ab. Subsequently, surviving clones were expanded and further characterized by RNA and immunoblot analysis.

Transfection. Jurkat A3, and its Fasm, and FADDm mutants, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. The v-Ha-ras gene was inserted into EcoRI/Cl4 sites in frame with Neo (selection marker) in the murine stem cell virus retroviral vector. The v-Ha-ras retroviral expression vector was introduced into the amphotropic murine retroviral vector packaging line DAMP, and filtered supernatant containing the packaged vectors was used for infection. Subsequently, infected cells were selected in growth medium containing 0.7 μg/ml of G418. The dn-JNK1, JNK1, and dn-FADD were inserted into the expression vector pGEX (a generous gift of Dr. M. Greenberg, Harvard Medical School, Boston, MA).

RNA Blot Analysis of ras Expression

After infection with ras gene retroviral vector, total cellular RNA was isolated by guanidine thiocyanate/phenol RNA extraction, quantified, separated by electrophoresis on formaldehyde agarose gels, and transferred to nitrocellulose. Hybridization was performed with a ras probe, randomly labeled with [³²P]dCTP. Blots were rehybridized with a β-actin probe to assure equal loading of RNA.
**Immunoblotting and Coimmunoprecipitation**

Immunoprecipitation assays were carried out as follows. After treating the cells (20 × 10⁶) with 1.5 μg/ml of the anti-human-Fas Ab for 60 min or 500 nM PMA for 24 h, the lysates were prepared with lysis buffer [30 mM Tris (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, and 1% Triton X-114]. Subsequently, proteins were immunoprecipitated with an anti-FADD Ab directed against the NH₂-terminus of the protein for 4 h (Pharmingen), separated by electrophoresis, and were immunoblotted with an anti-caspase-8 Ab (Pharmingen). After determining the concentration of protein in the lysates, samples containing 100 μg of total proteins were separated on a SDS-PAGE gel, transferred to nitrocellulose, and blotted with the appropriate Abs [anti-FADD Abs, anti-caspase-8 Ab directed against cleaved p18, anti-BID Ab, or anti-JNK1 Ab (Santa Cruz Biotechnology, Inc.)].

**Preparation of Cytosol Fractions and Immunoblotting for Cytochrome c**

For the preparation of the cytosol fraction, cells (1 × 10⁷) were lysed and separated by centrifugation at 100,000 × g at 4°C (14). The supernatant was collected as a cytosol fraction. Samples containing equal amounts of total protein from the cytoplasmic fractions were separated on a 15% SDS-PAGE gel and immunoblotted with an anti-cytochrome c Ab (Pharmingen).

**JNK Activity Assay**

A GST-cJun (1–79) fusion protein coupled to glutathione beads (Santa Cruz Biotechnology, Inc.) was used as a substrate for the solid-phase JNK assay. Cells (1 × 10⁷ from each cell line) were treated with 500 nM PMA for 24 h and subsequently lysed in lysis buffer [25 mM HEPES (pH 7.5), 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na₃VO₄, 2 μg of leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride]. One hundred μg of protein from each lysate were mixed with glutathione-agarose beads to which 20 μg of GST-cJun were bound. The beads were incubated in kinase reaction buffer [12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM MgCl₂, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na₃VO₄, 20 μM ATP, and 3 mM DTT] plus 2.5 μCi of [γ-³²P]ATP at 30°C for 30 min. The phosphorylated proteins were resolved on a 10% SDS-PAGE gel and visualized by autoradiography.

**DNA Fragmentation Assay**

After the indicated treatments, cells (1 × 10⁶) were washed with 1× PBS twice and resuspended in 1 ml of 1% sodium citrate, 0.1% Triton X-100 and 50 μg of propidium iodide/ml, containing 100 μl of RNAse (at 1 mg/ml). The stained samples were kept in the dark at 4°C overnight before analysis. Assay of nuclear DNA content was performed with a FACScan (Becton Dickenson). Data analysis and display were performed using the Cell-Fit software program. Cell-Fit 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.

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**References**

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