Articles

2004-04-23

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Recommended Citation

JNK Regulates HIPK3 Expression and Promotes Resistance to Fas-mediated Apoptosis in DU 145 Prostate Carcinoma Cells

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ABSTRACT

Elevated endogenous JNK activity and resistance to Fas receptor-mediated apoptosis have recently been implicated in progression of prostate cancer and can promote resistance to apoptosis in response to chemotherapeutic drugs. In addition, JNK has been demonstrated to promote transformation of epithelial cells by increasing both proliferation and survival. Although numerous studies have reported a role for JNK in promoting Fas receptor-mediated apoptosis, there is a paucity in the literature studying the antiapoptotic function of JNK during Fas receptor-mediated apoptosis. Consequently, we have used the recently described specific JNK inhibitor SP600125 and RNA interference to inhibit endogenous JNK activity in the prostate carcinoma cell line DU 145. We demonstrated that endogenous JNK activity increased the expression of a kinase, HIPK3, that has previously been implicated in multidrug resistance in a number of tumors. HIPK3 has also been reported to phosphorylate FADD. The interaction between FADD and caspase-8 was inhibited, but abrogation of JNK activity or HIPK3 expression was found to restore this interaction and increased the sensitivity of DU 145 cells to Fas receptor-mediated apoptosis. In conclusion, we present novel evidence that JNK regulates the expression of HIPK3 in prostate cancer cells, and this contributes to increased resistance to Fas receptor-mediated apoptosis by reducing the interaction between FADD and caspase-8.

INTRODUCTION

The Fas receptor is a member of the tumor necrosis factor superfamily of receptors and is expressed in many tissues (1). Ligation of Fas receptor with Fas ligand or Fas-activating antibodies results in Fas receptor clustering at the plasma membrane, recruitment, and activation of caspase-8 via the adapter protein FADD and subsequent cleavage of a number of downstream targets, culminating in apoptosis (2). Fas-mediated apoptosis is believed to be a mechanism by which the immune system destroys defective cells or cells expressing abnormal surface proteins (3). In addition, various anti-cancer drugs are dependent on Fas receptor activation in order to induce apoptosis (4, 5). As a result, there is a selective pressure on tumor cells to inhibit Fas receptor-mediated apoptosis, and resistance to Fas is a common event during cancer progression.

Prostate cancer is the most prevalent cancer in men and is a leading cause of death in elderly males (6). Current chemotherapeutic regimens do not increase long term survival in patients with prostate cancer (7), and this has been linked to an increased resistance to Fas receptor-mediated apoptosis (8). Hormone-refractory prostate cancer usually displays increased malignancy, proliferation, and metastatic potential over androgen-sensitive tumors and can survive in the absence of androgen. Transformation of prostate epithelial cells from a preneoplastic state into an intraepithelial neoplasm requires a number of sequential genetic alterations (9). Some of these aberrations in protein function increase the resistance of prostate cancer cells to apoptosis, whereas others decrease the dependence of prostate cancer cells on growth factors for survival and proliferation. One protein that has been implicated in transformation and progression in numerous tumors including prostate cancer (10), breast cancer (11), and lung cancer (12) is a stress-activated protein kinase called JNK.1 Overexpression of JNK conferred a partially transformed phenotype on fibroblasts by regulating the response of these cells to survival and proliferative signals (13). It also greatly enhanced the transformation potential of Ras (14) and BCR-Abl (15). Elevated endogenous JNK activity has been reported to correlate with drug resistance in cancer (16). Antisense strategies designed to reduce JNK expression have been shown to reduce prostate tumor burden and also sensitize these tumors to systemic therapy using chemotherapeutic drugs (17).

In this study, we investigate the role of endogenous JNK activity in Fas receptor-mediated apoptosis in prostate cancer. In contrast with our recent publications, where we show that prolonged overactivation of JNK is proapoptotic in prostate cancer cells (18, 19), we demonstrate here that endogenous JNK activity can promote survival in DU 145 prostate cancer cells. We show that inhibition of endogenous JNK activity decreases the expression of the Fas/FADD-interacting kinase HIPK3 (FIST/PKY/DYRK6). The interaction between FADD and caspase-8 is defective in DU 145 cells, but incubation with SP600125 restores the affinity of FADD for caspase-8 and restores the ability of DU 145 cells to undergo Fas receptor-mediated apoptosis.

MATERIALS AND METHODS

Cell Lines and Reagents—DU 145, PC-3, and Jurkat T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). PPC-1 and ALVA 31 cells were a gift of Gary and Heidi Miller (University of Colorado). DU 145 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum and 4 mM L-glutamine (all from Sigma). PC-3, PPC-1, and ALVA 31 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 4 mM L-glutamine. Jurkat T cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 4 mM L-glutamine, and 10 IU ml–1 penicillin/streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C and routinely subcultured every 2–3 days. Unless otherwise stated, cells were grown to 75% confluence before treatment with various drugs and inhibitors.

The probes used for the apoptosis assays were annexin V-FITC (IQ Products), propidium iodide (Sigma), and JC-1 (Molecular Probes). The primary antibodies used in this study were rabbit anti-c-Jun (Calbiochem); mouse anti-actin clone AC-15 (Sigma); mouse anti-phospho-JNK (Thr183/Tyr185) clone G9, mouse anti-caspase-8 clone IC12, and rabbit anti-caspase-3 (Cell Signaling Technology); rabbit anti-JNK and rabbit anti-protein kinase Cζ (anti-PKCζ) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-BID (BioSource International); mouse anti-poly(ADP-ribose) polymerase (PharMingen); mouse anti-FADD clone IF7 and rabbit anti-ERK2 (Upstate Biotechnology); and rabbit anti-rat HIPK3 (a gift of Jorma Palvimo, University of Helsinki, Finland). All peroxidase-conjugated secondary antibodies were from DAKO (Denmark). Rabbit anti-FADD (Upstate Biotechnology) was used for immunoprecipitations and protein G-agarose slurry was purchased from Pierce. The Fas-activating mouse anti-Fas IgM (clone CH11) was obtained from Upstate Biotechnology. The JNK inhibitor SP600125, the Mek1 inhibitor U0126, the p38 inhibitor SB203580, the PKCζ pseudosubstrate inhibitor, and the caspase inhibitors Z-IETD-fmk and Z-LEHD-fmk were from Calbiochem. Small interfering RNA oligonucleotides and control oligonucleotides were purchased from Dharmacon, and oligofectamine was purchased from Invitrogen. Primers used to amplify mRNA by reverse transcriptase-PCR were designed using GeneFisher software and were purchased from MWG. Other reagents required for reverse transcriptase-PCR were bought from Promega, λ-phosphatase was purchased from New England Biolabs, and all other chemicals were purchased from Sigma.

Apoptosis Assays—Annexin V-FITC and propidium iodide were used to detect apoptosis by flow cytometry as described previously (20). Unless otherwise indicated, cells were incubated with 200 ng ml^{-1} anti-Fas IgM for 24 h. Cells were preincubated for 1 h with 0.5% Me2SO in the presence or absence of SP600125. Caspase inhibitors were added to cells 10 min prior to treating with anti-Fas IgM. Cells were analyzed on a FACScan flow cytometer (BD Biosciences). Cell Quest software (BD Biosciences) was used to analyze the data. Mitochondrial membrane depolarization can be measured in intact cells using the fluorescent cationic cell-permeable probe JC-1 (21). DU 145 cells were incubated for 24 h with SP600125 and anti-Fas IgM as described above and incubated with JC-1 probe as described previously (21). Cells were analyzed by flow cytometry, and a decrease in FL-2 fluorescence is indicative of mitochondrial membrane depolarization.

SDS-PAGE and Western Blot Analysis—Cells were treated as described in the figure legends. The cells were then harvested and lysed in radioimmune precipitation buffer (50 mM Tris, pH 7.4; 150 mM NaCl; 1 mM each NaF, NaVO4, and EGTA; 1% Nonidet P-40; 0.25% sodium deoxycholate; 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride; 1 μg ml^{-1} each antipain, aprotinin, and chymostatin; 0.1 μg ml^{-1} leupeptin; 4 μg ml^{-1} pepstatin) for 20 min on ice. The lysates were centrifuged at 20,000 × g for 15 min to remove insoluble debris, and protein concentrations were determined. At least 30 μg of protein was loaded into each lane of an SDS-polyacrylamide gel. Electrophoresis of the samples and transfer to a nitrocellulose membrane were carried out. Staining of the membrane with the various antibodies was performed using the manufacturer's recommended protocol.

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λ-Phosphatase Treatment of Cell Lysates—λ-Phosphatase treatment was carried out on DU 145 cell lysates as described previously (22). Briefly, 30 µg of protein was diluted in 10 µl of 1× phosphatase buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM Na2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM MnCl2) with or without 1 µl of λ-phosphatase and incubated at 30 °C for 1 h. Samples were diluted in SDS-PAGE loading dye, and Western blot analysis was carried out as described earlier.

Cell Cycle Analysis—DU 145 cells were treated with 50 µM SP600125, 8 mM hydroxyurea, and 2 µg ml–1 nocodazole for 24 h. At least 200,000 DU 145 cells were washed in phosphate-buffered saline/EDTA and fixed in ice-cold 70% ethanol overnight at –20 °C. Cells were resuspended in phosphate-buffered saline/EDTA with 40 µg ml–1 propidium iodide and 200 µgml–1 DNase-free RNase A (Sigma) in the dark for 30 min, and DNA content of cells was analyzed on a FACScan flow cytometer.

RNA Interference—Cells were transfected with target or control siRNA (see Table I for sequences) using oligofectamine following the manufacturer's protocol exactly. Expression of HIPK3 was determined every day after transfection, and 4–5 days after transfection was found to be optimal for silencing HIPK3 in DU 145 cells. RNA and protein were extracted, taken on day 4, and apoptosis assays were begun on day 4 and completed by day 5.

TABLE I

Target sequences for RNA interference

Name	Sequence
Control	5′-AAA AAU UUC CAC CCC CCG GGC-3′
HIPK3	5′-AAU ACU UAC GAA GUC CUU CAU-3′
JNK1	5′-AAG GAA TAG TAT GCG CAG CTT-3′ 5′-GAAGCTCCACCACCAAAGATC-3′
JNK2	5′-GACTCAACCTTCACTGTCCTA-3′ 5′-CAGCGTGCACTAACTTCATGA-3′
c-Jun	SMARTPOOL™

Reverse Transcriptase-PCR—RNA was extracted from 100,000 DU 145 cells using Trizol reagent, and 0.5 µg was converted to cDNA using Moloney murine leukemia virus reverse transcriptase. Primers were subsequently used to amplify target sequences (Table II) using PCR, and DNA bands were visualized using agarose gel electrophoresis.

TABLE II

Primers for PCR

Name	Sequence
GAPDH	
Forward	5'-ACC ACA GTC CAT GCC ATC AC-3'
Reverse	5'-TCC ACC ACC CTG TTG CTG TA-3'
HIPK3	
Forward	5'-ACA TTG GAA GAG CAT GAG GCA GAG A-3'
Reverse	5'-CTG CTG AAA AGC ATC ACC ACA ACC A-3'
HIPK3/ FIST	
Forward	5'-GCT GTG CAG CCA CTA CAG A-3'
Reverse	5'-GCC TCT CCT TCT GAG TTC TGA-3'
JNK1	
Forward	5'-GCA GAA GCA AGC GTG ACA-3'
Reverse	5'-CTG GCC AGA CCG AAG TCA-3'
JNK2	
Forward	5'-ATA TTG CAG GAC GCT GCA-3'
Reverse	5'-CTT CAG GGT GCA GTC TGA-3'

Immunoprecipitation of FADD—A minimum of 500 µg of protein was used per sample. DU 145 cells were treated and harvested as described in the figure legends. The cells were lysed gently using lysis buffer (10 mM Tris, pH 7.5; 50 mM NaCl; 10 mM sodium pyrophosphate; 50 mM NaF; 1 mM NaVO4; 1% Nonidet P-40; 0.2 mM phenylmethylsulfonyl fluoride; 5 µg ml–1 each antipain, aprotinin, and chymostatin; 0.5 µg ml–1 leupeptin; 20 µg ml–1 pepstatin) and centrifuged at 20,000 × g for 15 min to remove insoluble material. Total cell protein was diluted to 1 µg ml–1 in phosphate-buffered saline and incubated with 10 µg µl–1 rabbit anti-FADD overnight at 4 °C. Protein G-agarose-conjugated beads (Pierce) were prepared according to the manufacturer's recommended instructions and incubated with the samples for a further 2 h at 4 °C. FADD was immunoprecipitated by centrifugation at 1,000 × g for 3 min. The beads were washed four times in phosphate-buffered saline and boiled in SDS-PAGE loading buffer for 5 min. The agarose beads were precipitated out of solution by centrifugation at 20,000 × g for 2 min, and the sample was loaded onto an SDS-polyacrylamide gel and analyzed by Western blot.

RESULTS

SP600125 Inhibits Endogenous JNK Activity in DU 145 Prostate Carcinoma Cells—Increased JNK activity, in particular JNK2, has been implicated in promoting proliferation and drug resistance in prostate cancer (10). Administering antisense directed against JNK1 and JNK2 inhibited prostate tumor growth significantly by increasing the susceptibility of cells to apoptosis (16) and was found to sensitize prostate cancer cells to cytotoxic drugs (23). JNK can activate the transcription factor c-Jun by phosphorylating two key residues, Ser63 and Ser73. Once phosphorylated, active c-Jun can alter the expression of a number of genes, including itself. We used the specific JNK inhibitor SP600125 (24) to inhibit JNK activity in DU 145 prostate carcinoma cells. JNK activity was assessed by analyzing the extent of normal c-Jun expression and phosphorylation in DU 145 cells incubated with and without 50 µM SP600125. In addition, the effects of SP600125 on the high levels of JNK activity that accompanies incubation with anisomycin were also analyzed. We found that c-Jun expression was reduced when incubated with 50 µM SP600125 for 4 h. Furthermore, anisomycin-mediated

phosphorylation of c-Jun was partially inhibited in cells pretreated with 50 µM SP600125 for 4 h. These data suggested that 50 µM SP600125 inhibits endogenous JNK activity in resting DU 145 cells and can also reduce the activity of JNK following treatment with anisomycin (Fig. 1A). The upstream JNK kinase MKK4 can be inhibited when high concentrations of SP600125 are used (24). We investigated whether MKK4 was significantly inhibited in DU 145 cells using 50 µM SP600125 and found that phosphorylation of JNK at Thr183 and Tyr185 by anisomycin was not affected in cells co-incubated with 50 µM SP600125. This suggests that inhibition of the JNK signaling pathway by SP600125 in DU 145 cells is due entirely to inhibition of JNK activity and not through inhibiting other upstream kinases in the JNK cascade (Fig. 1B).

SP600125 Sensitizes DU 145 Prostate Carcinoma Cells to Fas-mediated Apoptosis—We used 50 µM SP600125 to decrease the endogenous activity of JNK in DU 145 prostate carcinoma cells and observed a concomitant increase in the sensitivity of these cells to Fas receptor-mediated apoptosis (Fig. 1C). This was also evident for PC-3 prostate carcinoma cells (data not shown). This suggests that JNK increases the resistance to Fas receptor-mediated apoptosis in resting prostate carcinoma cells. Treatment with the caspase-8-specific inhibitor Z-IETD-fmk completely abrogated apoptosis, demonstrating that apoptosis in response to SP600125 and anti-Fas IgM was entirely dependent on caspase-8 activity (Fig. 1C). This result agrees with the current theory that caspase-8 is the apical caspase in the Fas receptor pathway and is absolutely required for the subsequent caspase cascade and apoptosis following Fas receptor engagement with Fas ligand. In contrast, inhibition of caspase-9 activity with the specific inhibitor Z-LEHD-fmk did not completely abrogate apoptosis in response to treatment with SP600125 and anti-Fas IgM. Therefore, mitochondrial amplification of caspase-9 activity may accelerate, but it is not absolutely required for apoptosis in response to SP600125 and anti-Fas IgM (Fig. 1C).

It has been reported that lower concentrations of SP600125 can inhibit JNK activity in some cells (24). We incubated DU 145 cells with 25 and 50 µM SP600125 and analyzed JNK activity through c-Jun expression. Although 50 µM SP600125 effectively reduces c-Jun expression, we found that 25 µM SP600125 was insufficient for decreasing JNK activity in DU 145 cells (Fig. 1D). In addition, 25 µM SP600125 did not significantly increase sensitivity of cells to Fas receptor-mediated apoptosis (Fig. 1E). These data suggest that 50 µM is the minimum concentration of SP600125 that inhibits JNK activity in DU 145 prostate cancer cells.

JNK Activity Inversely Correlates with Sensitivity to Fas Receptor-mediated Apoptosis in Prostate Cancer Cell Lines— Antisense strategies against JNK have proved effective in animal models of prostate cancer, suggesting that JNK activity plays a role in prostate tumor progression (17). We analyzed levels of phosphorylated JNK in four prostate cancer cell lines; DU 145 cells and PC3 cells were originally isolated from prostate cancer metastases, whereas PPC-1 and ALVA 31 cells originated from primary tumors. We found that DU 145 cells, PC3 cells, and PPC-1 cells all express high levels of phosphorylated JNK in comparison with ALVA 31 cells. Total JNK levels were similar in all cell lines studied (Fig. 1A). Although DU 145, PC3, and PPC-1 cells all express significantly more c-

Jun than ALVA 31 cells, this is most striking in DU 145 cells (Fig. 2B). When we compared these data with the sensitivity of cells to Fas receptor-mediated apoptosis, we found that elevated endogenous JNK activity correlated with resistance to Fas receptor-mediated apoptosis in prostate carcinoma cell lines (Fig. 2C). Only ALVA 31 cells were found to be sensitive to apoptosis induced by anti-Fas IgM.

Endogenous JNK Activity Increases the Resistance of DU 145 Cells to Fas-mediated Apoptosis by Inhibiting Caspase-8 Activation by Fas Receptor—Our data suggest that elevated JNK activity prevents Fas receptor-mediated apoptosis in prostate cancer cell lines. In order to gain further insight into this novel antiapoptotic function of JNK, we analyzed the major events that occur following incubation with SP600125 and anti-Fas IgM in DU 145 cells. We found that caspase-8 is only cleaved and activated when FADD and caspase-8 are recruited together to the death-inducing signaling complex (DISC) following Fas receptor engagement with anti-Fas IgM. Detectable cleavage products of caspase-8 were only evident in cells coincubated with both SP600125 and anti-Fas IgM (Fig. 3A). As a result, the recruitment and cleavage of caspase-8 in the DISC is defective in resting DU 145 cells, and inhibition of JNK activity can increase the cleavage of caspase-8 in response to anti-Fas IgM. We also analyzed events downstream of caspase-8 activation during Fas receptor-mediated apoptosis and observed cleavage and activation of Bid only in DU 145 cells incubated with both SP600125 and anti-Fas IgM (Fig. 3B). In addition, we only detected mitochondrial membrane depolarization and caspase-3 cleavage products in response to anti-Fas IgM when endogenous JNK activity had been decreased (Fig. 3, C and D). These results suggested that inhibition of JNK using SP600125 facilitated DU 145 cells to undergo Fas-mediated apoptosis primarily by enhancing either recruitment of or subsequent cleavage of caspase-8 at the DISC.

FADD Phosphorylation Is Regulated by Endogenous JNK in DU 145 Cells—A number of proteins are recruited to the cytoplasmic domain of Fas receptor oligomers. These include FADD, RIP, RAIDD, and DAXX, which function primarily as adapter proteins, and caspase-2, caspase-8, caspase-10, FLIP, and ASK1, which initiate various proteolytic and kinase cascades in cells (25). We did not observe any significant alterations in expression of Fas receptor, Fas ligand, FADD, caspase-8, DAXX, or FLIP in response to SP600125 treatment (data not shown). However, although FADD migrated as a double band in untreated and anti-Fas IgM-treated cells, incubation with SP600125 reduced the expression of the upper band (Fig. 4A). In addition, we found that SP600125 could reduce this double band in the prostate carcinoma cell lines PC-3, ALVA 31, and PPC-1 (data not shown). Incubation of protein extracts with λ-phosphatase reduced the expression of the upper band, confirming that this mobility shift was due to dephosphorylation of FADD (Fig. 4B).

Recently, it has been demonstrated that ERK can regulate FADD phosphorylation in leukemia cells. The authors demonstrated that inhibition of ERK activity using CI-1040 reduced FADD phosphorylation over a 72-h period and concomitantly increased the sensitivity of these cells to Fas receptor-mediated apoptosis (26). We also observed a decrease in FADD phosphorylation and a concomitant increase in the sensitivity of cells to Fas receptor-mediated apoptosis. However, induction of apoptosis is more rapid in our system and is evident after 24 h. We analyzed the extent

of FADD phosphorylation after 1-, 8-, and 24-h incubation with SP600125. We found that although FADD dephosphorylation was not immediate, it preceded induction of apoptosis and began to decrease significantly after 8 h (Fig. 4C). We were concerned that SP600125 may also inhibit ERK activity and consequently reduce the extent of FADD phosphorylation in DU 145 cells. Therefore, we inhibited ERK activity using the Mek inhibitor U0126 and p38 activity using the p38 inhibitor SB203580. We found that FADD phosphorylation in DU 145 cells was not dependent on either ERK activity or p38 activity (Fig. 4D). Next we wished to determine whether increased JNK activity could also increase the levels of FADD phosphorylation. However, 250 ng ml–1 anisomycin did not increase the levels of FADD phosphorylation in DU 145 cells (Fig. 4E). This suggests that either JNK-dependent FADD phosphorylation is already at a maximum level or that different signal transduction pathways are activated during endogenous JNK activity and anisomycin-induced JNK activity.

JNK Does Not Regulate Cell Cycle Progression in DU 145 Cells—FADD phosphorylation has previously been described and correlates with cell cycle progression. Cells arrested in the G1 phase of the cell cycle display predominantly unphosphorylated FADD, whereas cells arrested during mitosis display predominantly phosphorylated FADD (27). In addition, JNK has previously been implicated in cell cycle progression during DNA synthesis (28), and it was possible that JNK regulated FADD phosphorylation by arresting DU 145 cells in the G1 phase or S phase. We confirmed that phosphorylation of FADD is regulated by cell cycle-dependent kinase in DU 145 cells using 8 mM hydroxyurea to arrest cells during G1 phase and 2 μg ml–1 nocodazole to arrest cells during mitosis (Fig. 5A). However, incubation with SP600125 did not arrest DU 145 cells in the S phase of the cell cycle (Fig. 5, B and C). In addition, the rate of proliferation of cells incubated with SP600125 was not significantly altered compared with untreated DU 145 cells (data not shown). These data suggest that JNK does not regulate FADD phosphorylation in DU 145 cells by regulating cell cycle progression.

PKCζ Is Not Responsible for FADD Phosphorylation in DU 145 Cells—Our data suggested that JNK directly regulated the activity of a kinase responsible for phosphorylating FADD. A number of FADD-interacting kinases have been identified (29), and one kinase that has been shown to associate with and phosphorylate FADD in vivo is PKCζ, an atypical member of the PKC family (30). We observed that the expression of PKCζ was increased in the Fas-resistant prostate cancer cell lines DU 145 and PC-3 when compared with the Fas-sensitive PPC-1 and ALVA 31 prostate cancer cell lines (Fig. 6A). Moreover, we noted that FADD phosphorylation was more extensive in DU 145 cells and PC-3 cells than in PPC-1 and ALVA 31 cells (data not shown). However, the extent of FADD phosphorylation was not found to change when DU 145 cells were incubated with a pseudosubstrate inhibitor of PKCζ (Fig. 6B). Phosphorylation of ERK in response to PKCζ activity was significantly decreased using 15 and 20 μM PKCζ pseudosubstrate inhibitor (Fig. 6C), confirming that effective concentrations of the inhibitor were used. We also incubated cells with the general PKC inhibitors chelerythrine chloride (Fig. 6D) and Ro-32-0432 (Fig. 6E), but again we did not observe any decrease in FADD phosphorylation. Together, these data suggest that PKC isoforms are not required for FADD phosphorylation in DU 145 cells.

HIPK3 Phosphorylates FADD and Increases the Resistance of DU 145 Cells to Fas-mediated Apoptosis—The other protein kinase reported to phosphorylate FADD is HIPK3, a 170-kDa kinase that can regulate DISC formation in vivo (31). The expression of HIPK3 was found to be elevated in Fas-resistant DU 145 and PC-3 cells in comparison with more sensitive PPC-1 and ALVA 31 prostate carcinoma cells (Fig. 7A). Little is known about the function of HIPK3 in cells, and we used RNA interference to reduce the expression of HIPK3 in DU 145 cells as outlined under "Materials and Methods" (Fig. 7B). The extent of FADD phosphorylation was significantly reduced in cells incubated with HIPK3 RNA antisense oligonucleotides when compared with control RNA (Fig. 7C). In addition, we found that inhibition of HIPK3 expression increased the sensitivity of DU 145 cells to Fas receptor-mediated apoptosis when compared with control RNA (Fig. 7D).

SP600125 Decreases Expression of HIPK3 by Regulating Transcription of HIPK3 mRNA—Our data suggested that HIPK3 was responsible for phosphorylating FADD in DU 145 cells, and elevated expression of HIPK3 was associated with increased resistance to Fas receptor-mediated apoptosis. We also demonstrated that JNK activity was necessary for phosphorylation of FADD by HIPK3. However, the mechanism by which JNK regulates HIPK3 activity was not clear. JNK has been demonstrated to regulate activity of proteins through direct phosphorylation and regulating gene expression. We found that incubation of DU 145 cells with 50 µM SP600125 decreased the expression of HIPK3 mRNA (Fig. 8A) and also decreased the expression of HIPK3 protein (Fig. 8B). Therefore, JNK can regulate the activity of HIPK3 by altering the rate of transcription at the HIPK3 gene locus.

JNK Activity Inhibits the Interaction between FADD and Caspase-8 in DU 145 Cells—In order to understand the mechanism employed by HIPK3 to inhibit Fas receptor-mediated apoptosis, we immunoprecipitated FADD from DU 145 cell lysates and probed for caspase-8 expression. Although caspase-8 is believed to associate with FADD in unstimulated cells, we did not detect any interaction between FADD and caspase-8 in resting DU 145 cells. No interaction between FADD and caspase-8 was evident even after 24-h stimulation with 200 ng ml^{-1} anti-Fas IgM, and co-incubation with SP600125 and anti-Fas IgM for 24 h was required for caspase-8 association with FADD (Fig. 8C). This suggests that JNK interferes with FADD and caspase-8 binding in DU 145 cells by up-regulating the expression of HIPK3.

JNK RNAi Reduces FIST and HIPK3 Expression and Decreases the Extent of FADD Phosphorylation in DU 145 Cells— Several reports have suggested that targets of JNK are often phosphorylated preferentially by either JNK1 or JNK2. We used RNAi to selectively inhibit JNK1 or JNK2 expression in DU 145 cells, and siRNA sequences outlined in Table I were found to inhibit expression of JNK1 and JNK2 by 70–90% (Fig. 9A). New primers were designed to discriminate between HIPK3 and FIST by reverse transcriptase-PCR. Incubation of DU 145 cells with both JNK1 and JNK2 siRNA significantly reduced the expression of both HIPK3 and FIST mRNA (Fig. 9B). We did not observe any decrease in HIPK3 or FIST expression when cells were incubated with either JNK1 or JNK2 RNAi alone, suggesting that JNK1 and JNK2 have redundant roles in regulating HIPK3 expression (data not shown). FADD

phosphorylation was also dramatically reduced in cells incubated with JNK1 and JNK2 siRNA, but not when incubated with either JNK1 or JNK2 siRNA alone, further underscoring the redundant roles played by JNK1 and JNK2 in regulating HIPK3 expression (Fig. 9C).

c-Jun Is Required for HIPK3 Expression and FADD Phosphorylation in DU 145 Cells—To date, two splice variants of HIPK3 have been identified, HIPK3 and FIST. They share >95% homology with each other and only differ by the presence of an extra exon in HIPK3. However, transcription is regulated at different promoters in humans located 50,000 bases apart on chromosome 11. The principle transcription factor for JNK, c-Jun, is an integral part of the AP-1 transcription factor complex. We identified two putative AP-1 binding sites close to the TATA box at each promoter region. Therefore, we hypothesized that HIPK3 expression is regulated by JNK through the transcription factor c-Jun. We used RNAi to reduce c-Jun expression in DU 145 cells (Fig. 10A) and observed a concomitant decrease in expression of both HIPK3 and FIST (Fig. 10B) and FADD phosphorylation (Fig. 10C) when compared with control oligonucleotides.

DISCUSSION

The role of JNK in both survival and apoptosis has been well documented (32, 33). Recent studies have demonstrated that JNK activation in individual cells is usually an "all or none" response to initial signals. In contrast, when analyzing JNK activation in a population of cells such as by Western blot, a graded response is usually observed. This simply represents the percentage of cells expressing active JNK at the time of lysis, and it appears that the length of time that JNK is activated in a particular cell is the most important factor that determines the fate of that cell (34, 35). However, much work is required to identify the exact mechanisms employed by JNK in response to different stimuli.

In this study, we focus on the relationship between endogenous JNK activity and Fas receptor-mediated apoptosis. We have identified a previously undescribed role of endogenous JNK activity in preventing Fas receptor-mediated apoptosis. Inhibition of JNK activity using SP600125 enhanced the rate of Fas receptor-mediated apoptosis in DU 145 cells. We have shown that DU 145 cells, PC3 cells, and PPC-1 cells have elevated endogenous JNK activity and are resistant to Fas receptor-mediated apoptosis. Endogenous JNK activity is much lower in ALVA 31 cells, a prostate cancer cell line originally isolated from a well differentiated tumor. Unlike the other prostate cancer cell lines, ALVA 31 cells were found to be sensitive to Fas receptor-mediated apoptosis, and inhibition of JNK activity using SP600125 could increase the sensitivity of DU 145 cells and PC3 cells to Fas receptor-mediated apoptosis.

In previous studies, we found that inhibition of JNK expression using antisense actually inhibited Fas receptor-mediated apoptosis over 24 h when DU 145 cells were treated with both camptothecin and anti-Fas IgM (18). These results are contradictory to our current observations; however, this is believed to be as a direct result of the sensitivity of the apoptosis assays used in both studies. In our previous study, we used propidium iodide uptake and morphology as indicators of apoptosis. These assays are generally less sensitive than currently available assays and only detect cells undergoing

final events in the apoptosis program. When we used these assays as indicators of apoptosis following incubation with SP600125 and anti-Fas IgM, we did not observe any increase in apoptosis after 24 h, and significant increases only began after 48 h post-treatment (data not shown). In contrast, annexin V-FITC and JC1 are used to determine the extent of Fas receptor-mediated apoptosis in this study. These assays detect early events during Fas receptor-mediated apoptosis, and we detected significant increases in apoptosis after 24 h post-treatment using these assays. Therefore, it appears that the kinetics of Fas receptor-mediated apoptosis are different whether cells are sensitized with camptothecin or with SP600125.

We discovered that FADD phosphorylation was dependent on JNK activity in prostate cancer cells, and inhibition of JNK using SP600125 or RNAi prevented FADD phosphorylation. Two kinases that have been demonstrated to inhibit FADD phosphorylation and formation of the DISC following Fas receptor engagement with Fas ligand are PKCζ and HIPK3. PKCζ interacts with and phosphorylates FADD in hematopoietic cells, and it has been found that overexpression of PKCζ abrogates Fas receptor-mediated apoptosis by interfering with effective DISC formation (30). Overexpression of PAR-4, the cellular inhibitor of PKCζ activity, has been shown to sensitize prostate cancer cell lines to Fas-mediated apoptosis, and this may implicate PKCζ in the resistance of DU 145 cells to treatment with anti-Fas IgM (36). JNK1 activity has been reported to decrease the expression of PAR-4 in epithelial cells (37), and this may lead to an increase in the activity of PKCζ. In addition, increases in PKCζ expression have been reported during prostate cancer progression (38). Although we confirmed that the expression of PKCζ is elevated in DU 145 cells, we did not observe any decrease in the phosphorylation of FADD when we incubated cells with the PKCζ pseudosubstrate inhibitor. In addition, no increase in the sensitivity of cells to anti-Fas IgM was detected, and these data suggest that PKCζ does not phosphorylate FADD and regulate Fas receptor-mediated apoptosis in DU 145 cells.

A second FADD-interacting kinase called HIPK3 was first identified as a putative multidrug-resistant protein from studies of cancer cells (39, 40). The gene coding for HIPK3 was cloned in 1997 and was found to share significant homology with two other kinases, HIPK1 and HIPK2 (39). HIPK1 phosphorylates and promotes DAXX redistribution within the nucleus (41). HIPK2 has been reported to interact with TRADD, an adapter protein for a subset of the tumor necrosis factor receptor superfamily (42). Overexpression studies demonstrated that a splice variant of HIPK3 called FIST interacts with and phosphorylates FADD, another adapter protein for a subset of the tumor necrosis factor receptor superfamily (31). Common death receptor targets suggest that a principle role of this kinase family is in regulating various aspects of death receptor signaling.

We found that HIPK3 was expressed at higher levels in the Fas-resistant prostate cancer cell lines DU 145 and PC-3. RNA interference reduced the expression of HIPK3 in DU 145 cells, and this was accompanied by a decrease in the extent of FADD phosphorylation. This resulted in an increase in the sensitivity of cells to Fas-mediated apoptosis. HIPK3 was significantly reduced in cells treated with SP600125 and also RNAi targeted against JNK1 and JNK2. These novel findings suggest that

increased endogenous JNK activity during prostate cancer progression causes an increase in the expression of HIPK3 and appears to be a key event in increasing the resistance of prostate cancer cells to Fas receptor-mediated apoptosis.

Two splice variants of HIPK3 have been described, HIPK3 and FIST, and these are under the control of two different promoters. We identified two putative AP-1 binding sites on each promoter within 200 bases of the TATA box in humans. The major component of the AP-1 transcription factor complex, c-Jun, is regulated by JNK. We found that inhibition of c-Jun expression reduced the expression of HIPK3 and FIST in DU 145 cells and also reduced FADD phosphorylation. Therefore, our data suggest that endogenous JNK activity increases expression of HIPK3 and FIST and that this is dependent on c-Jun expression. Furthermore, we have shown that RNAi targeting HIPK3 and FIST reduces the extent of FADD phosphorylation and increases the sensitivity of these cells to Fas receptor-mediated apoptosis.

The association of FADD and caspase-8 was found to be defective in normal DU 145 cells, and interaction between these two proteins was only restored after co-incubation with SP600125 and anti-Fas IgM. Previous reports have implicated FADD phosphorylation with sensitivity to Fas receptor-mediated apoptosis. Overexpression of the FADD kinase PKCζ can inhibit the interaction between FADD and caspase-8 (30). Inhibition of ERK activity has been demonstrated to reduce FADD phosphorylation and increase the sensitivity of cells to Fas receptor-mediated apoptosis (26). In addition, FADD phosphorylation and Fas receptor-mediated apoptosis appear to be regulated by cell cycle progression in certain cell lines (43, 44). Although it has previously been reported that FADD phosphorylation does not affect Fas receptor-mediated apoptosis (27), these studies were performed using cell lines overexpressing wild type and mutant FADD and thus may not correlate with endogenous expression of FADD in cells.

Targets of JNK have previously been identified that either promote or inhibit Fas-mediated apoptosis at two key stages (Fig. 11). JNK can phosphorylate and alter the activity of a number of Bcl-2 family members, and this in turn modulates the sensitivity of the mitochondrion to apoptotic signals. JNK may also modulate early events during Fas-mediated apoptosis such as decreasing PAR-4 expression (36, 37) and enhancing the clustering of Fas receptor in response to Fas ligand (45). Our results have illuminated another target of JNK in Fas receptor-mediated apoptosis. By regulating HIPK3 expression and FADD phosphorylation through the transcription factor c-Jun, JNK can regulate the interaction between FADD and caspase-8 and increases the threshold of Fas receptor activation required to promote apoptosis in prostate cancer cells. However, HIPK3 is not the sole mechanism that increases resistance of DU 145 cells to Fas-mediated apoptosis. Bcl-2 family members and HSP27 may regulate other components of the Fas apoptotic pathway. These mechanisms are semiredundant and act independently to increase the threshold of Fas receptor activation required for apoptosis induction. As a consequence, it is likely that any therapy directed against Fas receptor for prostate cancer will target multiple inhibitory effects in order to maximize apoptosis and reduce the tumor burden in patients.

FIGURE LEGENDS

FIG. 1.

Inhibition of endogenous JNK activity with SP600125 sensitizes DU 145 prostate carcinoma cells to Fas-mediated apoptosis. A, JNK was activated with 250 ng ml–1 anisomycin in the presence or absence of 50 µM SP600125. Western blotting was used to determine the expression and phosphorylation of c-Jun in DU 145 cell lysates after 4 h of treatment. β-Actin was also probed to demonstrate equal protein loading in the lanes. B, JNK phosphorylation at Thr183 and Tyr185 was assessed by Western blot in cells incubated with 250 ng ml–1 anisomycin in the presence and absence of 50 µM SP600125 for 4 h. Total JNK expression was also analyzed to determine equal protein loading. C, DU 145 cells were incubated for 1 h with 50 µM SP600125 as outlined under "Materials and Methods" before the addition of 200 ng ml–1 α-Fas IgM agonistic antibody. Cells were stained 24 h after adding α-Fas IgM with annexin V-FITC and propidium iodide to visualize the extent of apoptosis. 25 µM caspase-8 inhibitor Z-IETD-fmk and caspase-9 inhibitor Z-LEHD-fmk were added 10 min before α-Fas IgM where indicated, and the error bars represent the S.D. of three independent experiments. D, DU 145 cells were incubated with 25 or 50 µM SP600125 or a Me2SO control for 4 h. Expression of c-Jun was determined by Western blot, and β-actin was also probed to demonstrate equal protein loading. E, DU 145 cells were incubated for 1 h with 25 or 50 µM SP600125 and subsequently treated with 200 ng ml–1 α-Fas IgM for 24 h. Apoptosis of annexin V-FITC and propidium iodide-stained cells was determined by flow cytometry.

FIG. 2.

Endogenous JNK activity correlates with resistance to Fas receptor-mediated apoptosis in DU 145 cells. A, the expression of JNK phosphorylated at Thr183 and Tyr185 was determined in DU 145 cells, PC-3 cells, PPC-1 cells, and ALVA 31 cells. Total JNK expression was also analyzed to ensure equal protein loading. B, total c-Jun expression was analyzed in the four prostate cancer cell lines: DU 145, PC-3, PPC-1, and ALVA 31. β-Actin was also probed to verify equal protein loading. C, DU 145, PC-3, PPC-1, and ALVA 31 cells were incubated with 200 or 400 ng ml–1 Fas or left untreated. Cells were stained with annexin V-FITC and propidium iodide after 24 h, and apoptosis was determined using flow cytometry.

FIG. 3.

SP600125 sensitizes DU 145 cells to Fas-induced apoptosis upstream of caspase-8 activation. A, expression of procaspase-8 (55/57 kDa) and the large caspase-8 active subunit (18 kDa) were analyzed by Western blot in lysates from DU 145 cells treated with 50 µM SP600125 and 200 ng ml–1 anti-Fas IgM for 24 h. Untreated and Fas-treated Jurkat cells were used as a positive control for caspase-8 cleavage products, and actin was also probed to demonstrate equal protein loading. B, the cleavage of BID (22 kDa) into the proapoptotic tBID (15 kDa) was analyzed in DU 145 cells treated with 50 µM SP600125 and 200 ng ml–1 anti-Fas IgM for 24 h. Untreated and Fas-treated Jurkat T cells were used as a positive control for BID cleavage following Fas receptor activation. β-Actin was probed for equal protein loading. C, mitochondrial membrane depolarization was assessed in DU 145 cells treated for 24 h with 50 µM SP600125 and 200 ng ml–1 anti-Fas IgM using the cationic

probe JC-1. A decrease in FL-2 fluorescence indicates depolarization of the mitochondrial membrane in the cells. Percentages refer to the population of cells with depolarized mitochondria in each sample, and data are representative of three independent experiments. D, Western blot analysis of procaspase 3 expression and cleavage in DU 145 cells treated for 24 h with 50 µM SP600125 and 200 ng ml–1 anti-Fas IgM. Fas-treated Jurkat cells were used as a positive control for procaspase 3 cleavage products. Procaspase 3 (35 kDa) and various cleavage products including active caspase subunits are evident. β-Actin was also probed to ensure equal protein loading.

FIG. 4.

SP600125 inhibits FADD phosphorylation in DU 145 cells. A, Western blot analysis of FADD in DU 145 cell lysates following treatment for 24 h with SP600125 and anti-Fas IgM as described earlier. The doublet band present in untreated and anti-Fas IgM-treated cells represents unphosphorylated and Ser194-phosphorylated FADD. Actin was also probed to ensure equal protein loading. B, λ-phosphatase treatment of DU 145 cells was performed as described under "Materials and Methods." The extent of FADD phosphorylation in untreated and λ-phosphatase-treated cell lysates was subsequently analyzed by Western blot. Actin was also probed to demonstrate equal protein loading. C, FADD phosphorylation was determined in DU 145 cells incubated with 50 µM SP600125 for 1, 8, and 24 h as indicated. β-Actin was also probed to determine equal protein loading. D, DU 145 cells were incubated with 50 µM SP600125, 10 µM U0126, 10 µM SB203580, or an Me2SO control for 8 h. Protein was extracted from cells, and Western blot analysis was used to determine the expression of c-Jun and the extent of FADD phosphorylation. β-Actin expression was also determined to verify equal protein loading. E, DU 145 cells were incubated with 250 ng ml–1 anisomycin for 24 h, and protein extracts were taken to determine the extent of FADD phosphorylation. β-Actin expression confirms equal protein loading.

FIG. 5.

SP600125 does not arrest cell cycle progression in DU 145 cells. A, effects of the cell cycle inhibitors hydroxyurea and nocodazole on FADD phosphorylation are investigated in DU 145 cells. Actin was probed to verify equal protein loading. B, cell cycle analysis in DU 145 cells incubated with 50 µM SP600125, 8 mM hydroxyurea, and 2 µg ml–1 nocodazole for 24 h. Propidium iodide was used to assess the DNA content of cells by flow cytometry as described under "Materials and Methods." C, bar chart representing DNA content and cell cycle phase for DU 145 cells incubated with SP600125, hydroxyurea, and nocodazole. Error bars, S.D. after three independent experiments.

FIG. 6.

PKCζ does not phosphorylate FADD in DU 145 cells. A, total expression of PKCζ was investigated in four prostate cancer cell lines by Western blot. Equal loading was verified by probing for poly-(ADP-ribose) polymerase. B, DU 145 cells were incubated with 20, 15, and 10 µM PKCζ pseudosubstrate inhibitor for 24 h, and the extent of FADD phosphorylation was subsequently determined by Western blot. Cells were also incubated with 50 µM SP600125 for comparative purposes, and actin demonstrated equal protein loading. C, expression of phosphorylated ERK was determined for the cell lysates used above to verify that effective concentrations of PKCζ pseudosubstrate inhibitor were used. Equal protein loading was verified by analyzing total expression of ERK2. D, DU 145 cells were incubated with 20 µM PKC inhibitor chelerythrine chloride or 50 µM SP600125 for 24 h. FADD

phosphorylation was subsequently determined in protein extracts by Western blot. β-Actin was used as a control to ensure equal protein loading. E, DU 145 cells were incubated with 10, 5, or 1 μM Ro-32-0432 or left untreated where indicated. FADD phosphorylation was determined by Western blot, and β-actin was used to determine equal protein loading. Cells treated with 50 μM SP600125 were also probed for comparative purposes.

FIG. 7.

HIPK3 phosphorylates FADD and increases the resistance of DU 145 cells to Fas-mediated apoptosis. A, total expression of HIPK3 was determined in the four prostate cancer cell lines. Expression of HIPK3 was found to be highest in DU 145 cells. Actin was also probed to verify equal protein loading. RNA interference was used to decrease the expression of HIPK3 mRNA over 4 days (B), and a decrease in FADD phosphorylation was also observed in DU 145 cells in comparison with control oligonucleotides (C). GAPDH (B) and actin (C) were also probed to demonstrate equal loading. D, DU 145 cells were incubated with control or HIPK3 small interfering RNA for 4 days and subsequently treated with 200 ng ml–1 anti-Fas IgM for 24 h. Apoptosis was measured using annexin V-FITC and propidium iodide staining as described under "Materials and Methods." The error bars represent the S.D. for three independent experiments.

FIG. 8.

SP600125 decreases expression of HIPK3 in DU 145 cells and increases the affinity of FADD for caspase-8. A, reverse transcriptase-PCR analysis of HIPK3 expression in DU 145 cells incubated with or without 50 μM SP600125 for 24 h. Expression of GAPDH was also determined to demonstrate equal loading of cDNA. B, Western blot analysis of HIPK3 expression in DU 145 cells following 24-h incubation with SP600125. Actin was also probed for equal protein loading. C, immunoprecipitation of FADD from DU 145 cell lysates incubated with 50 μM SP600125 for 24 h and 200 ng ml–1 for 1, 6, and 24 h where indicated. Caspase-8 and FADD expression were subsequently analyzed by Western blot.

FIG. 9.

RNAi inhibition of JNK expression reduces HIPK3 expression and FADD phosphorylation in DU 145 cells. A, DU 145 cells were inhibited with siRNA targeting JNK1, JNK2, both JNK1 and JNK2, or control siRNA for 48 h. Expression of JNK1 and JNK2 was subsequently determined using reverse transcriptase-PCR. GAPDH was used as an equal loading control. B, HIPK3 and FIST expression were determined in DU 145 cells incubated with JNK1 and JNK2 siRNA oligonucleotides or control oligonucleotides for 48 h. GAPDH expression was also analyzed to verify equal loading of samples. C, FADD phosphorylation was determined after incubation with JNK1, JNK2, JNK1 and JNK2, or control siRNA oligonucleotides for 48 h. Cells incubated with 50 μM SP600125 for 24 h were used for comparative purposes. β-Actin was used to verify equal protein loading between samples.

FIG. 10.

Inhibition of c-Jun expression using RNAi reduces HIPK3 expression and FADD phosphorylation in DU 145 cells. A, c-Jun expression was determined in DU 145 cells incubated with SMARTPool™ c-Jun siRNA or control siRNA for 24 and 48 h. β-Actin was also probed to verify equal protein loading. B, HIPK3 and FIST expression was determined by reverse transcriptase PCR in DU 145 cells incubated

with c-Jun or control siRNA where indicated. GAPDH was also probed to visualize equal loading. C, FADD phosphorylation was determined in DU 145 cells incubated with c-Jun or control siRNA for 24 and 48 h by Western blot. β-Actin was used to determine equal protein loading.

FIG. 11.

Targets of JNK during Fas receptor-mediated apoptosis. A, endogenous JNK activation promotes survival by phosphorylation of Bcl-2 family members and altering the activity of PKCζ and HIPK3. B, stress signals induce prolonged JNK activation and proapoptotic targets of JNK include increased processing of Bid; increased expression of Bim, DP5, Fas receptor, and Fas ligand; and tyrosine phosphorylation of Fas receptor by epidermal growth factor receptor (45).

Footnotes

1 The abbreviations used are: JNK, c-Jun N-terminal kinase; PKC, protein kinase C; Z-, benzyloxycarbonyl-; fmk, fluoromethyl ketone; FITC, fluorescein isothiocyanate; DISC, death-inducing signaling complex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; ERK, extracellular signal-regulated kinase.

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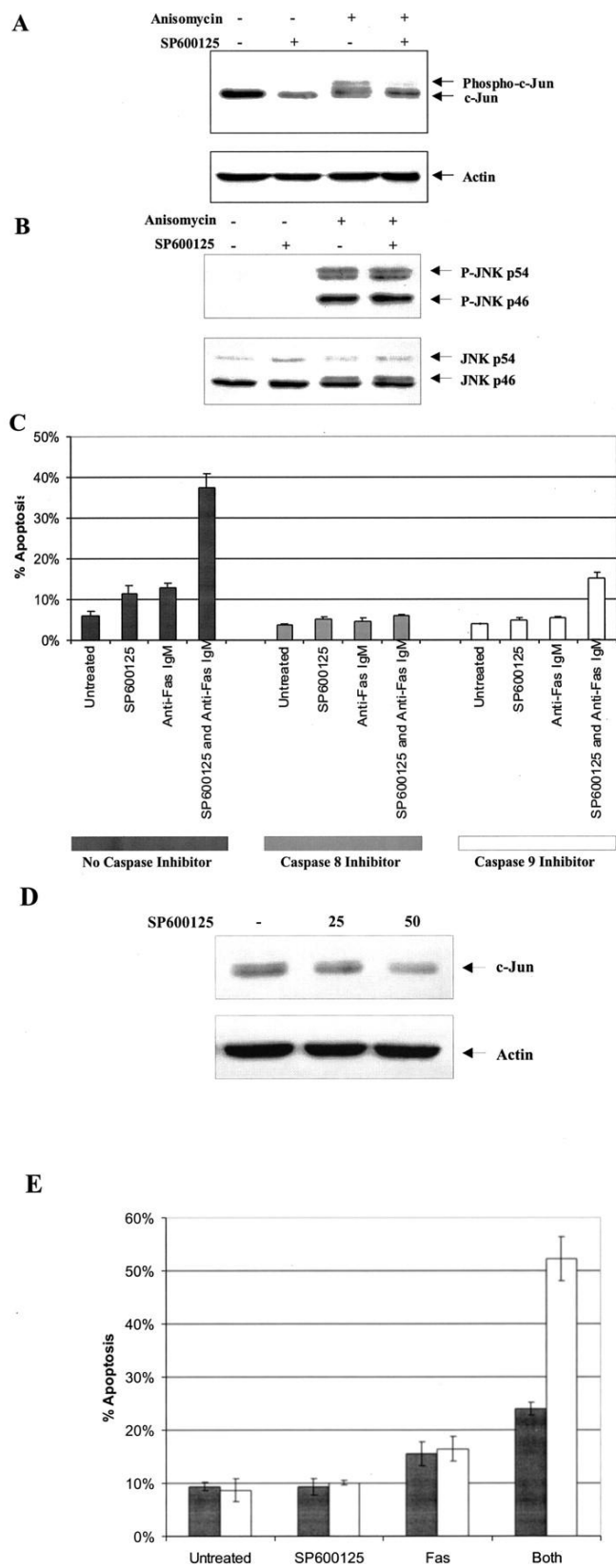45. Reinehr, R., Schliess, F., and Haussinger, D. (2003) FASEB J. 17, 731–733
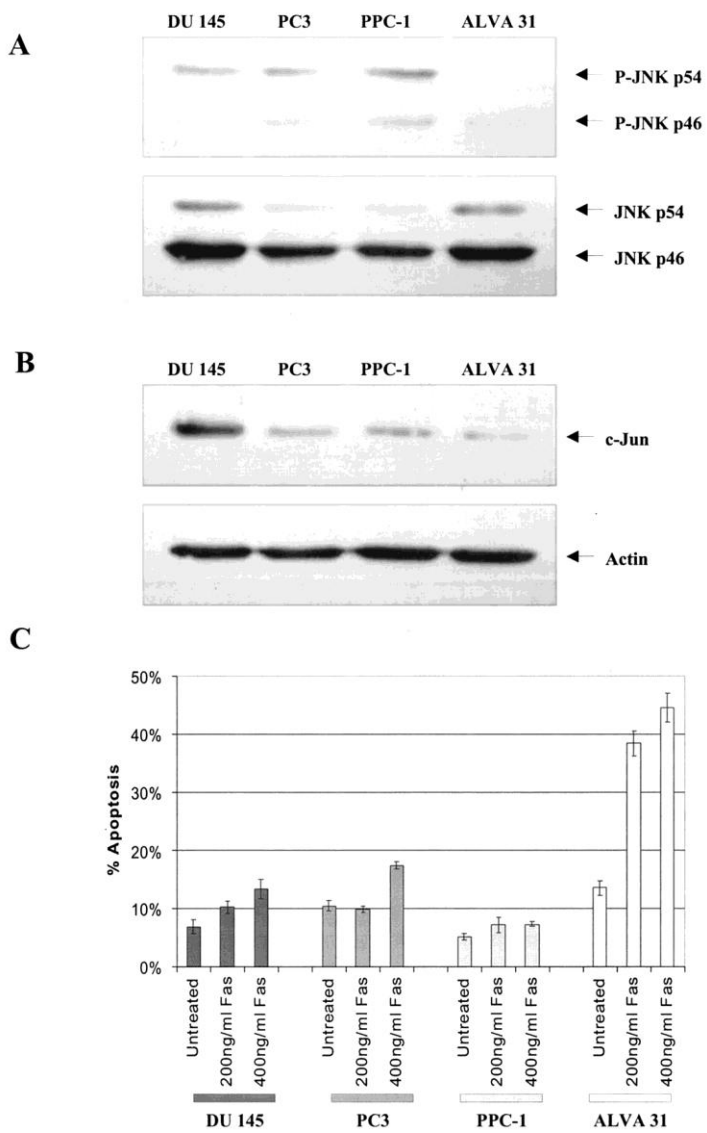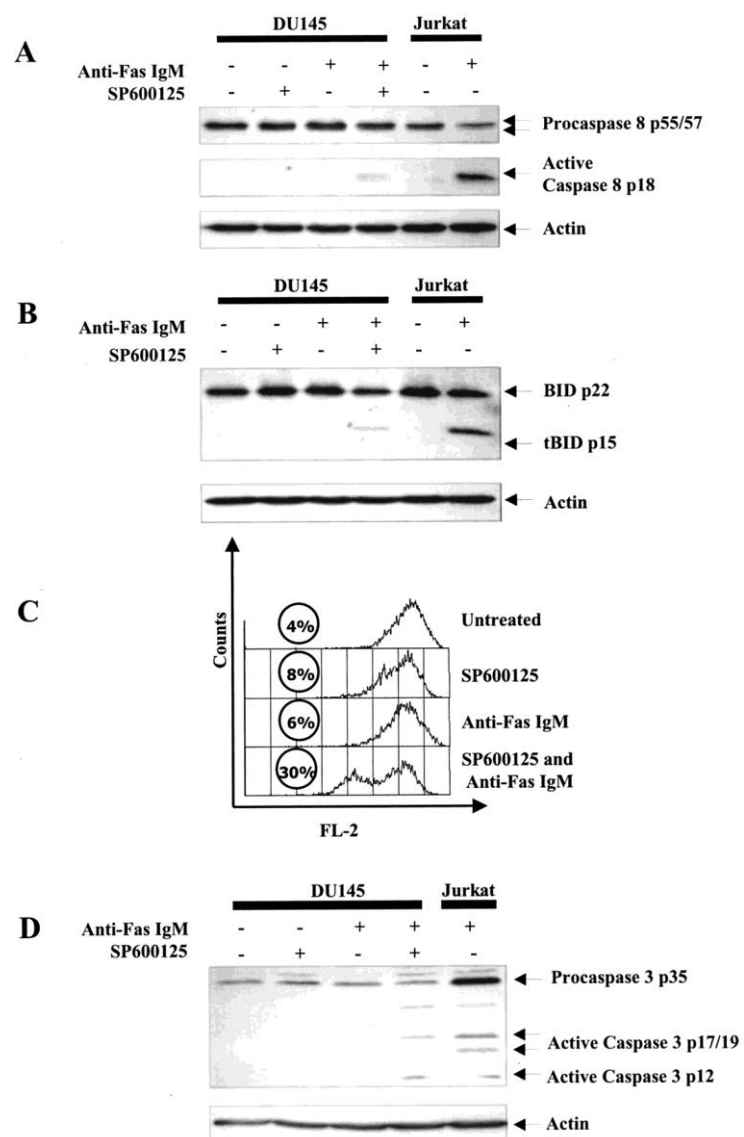
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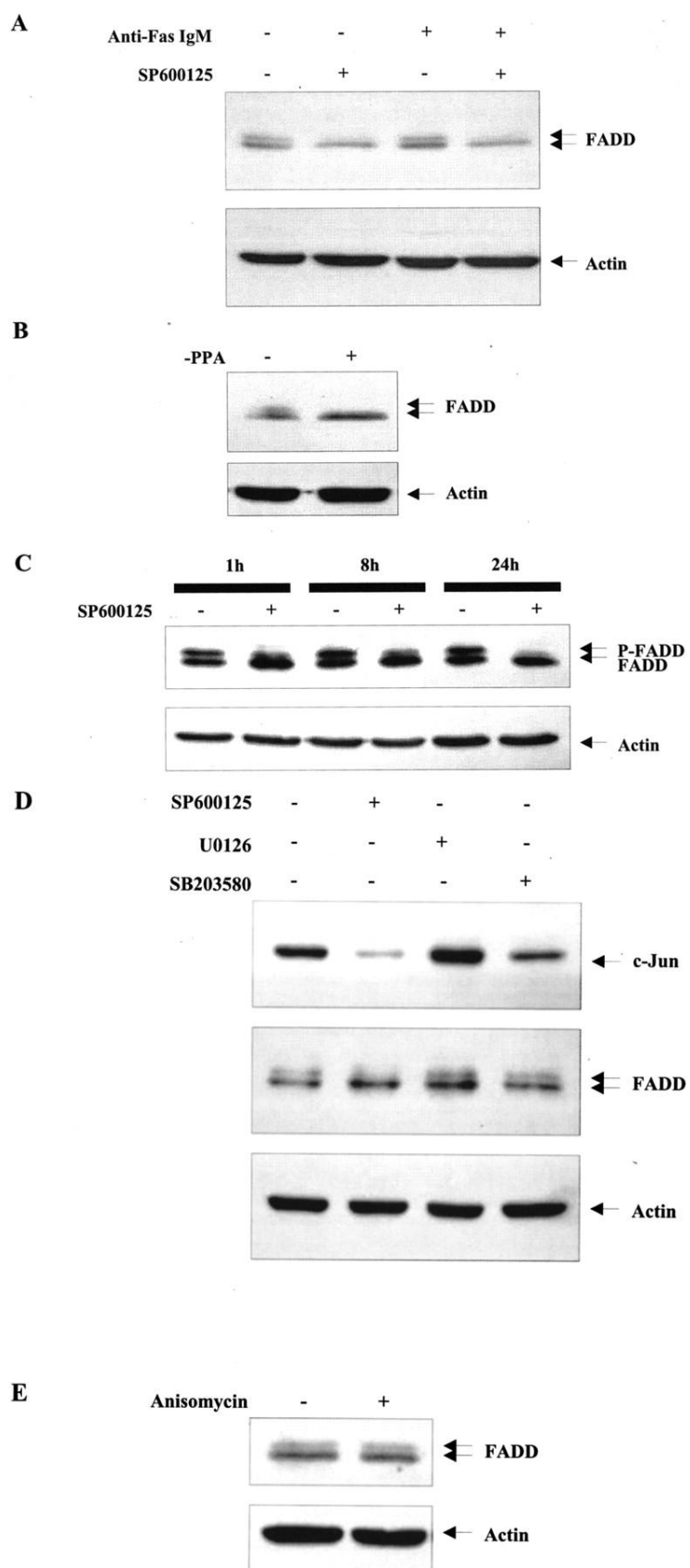
Figure 5.

Figure 6.

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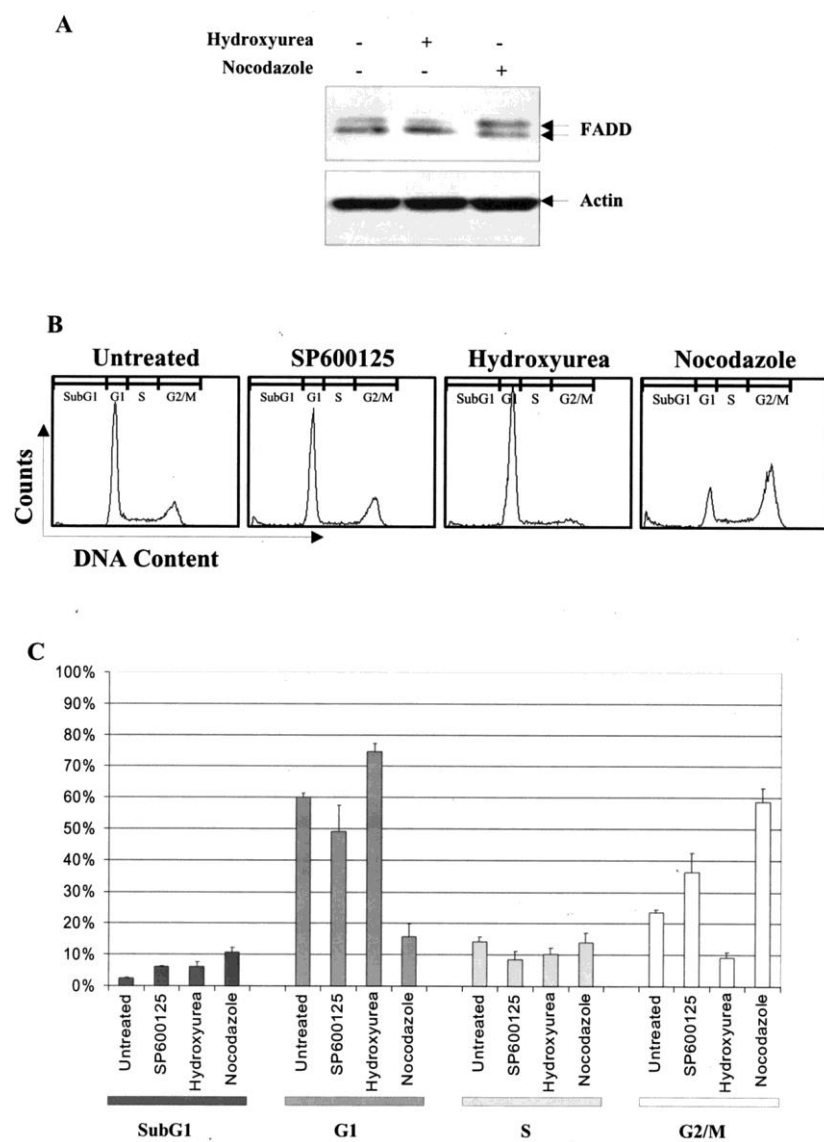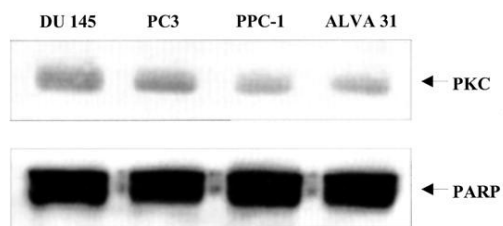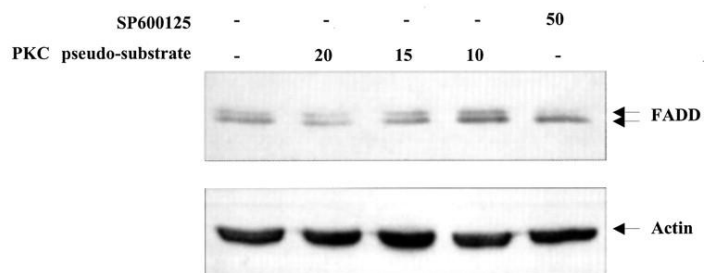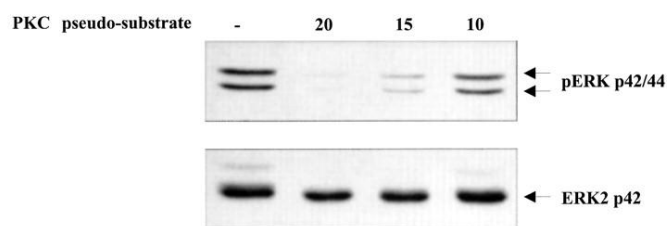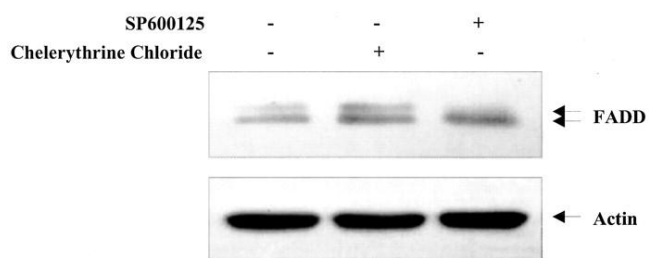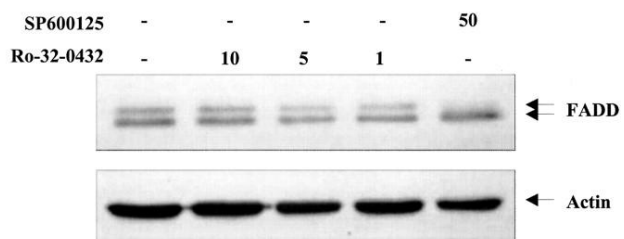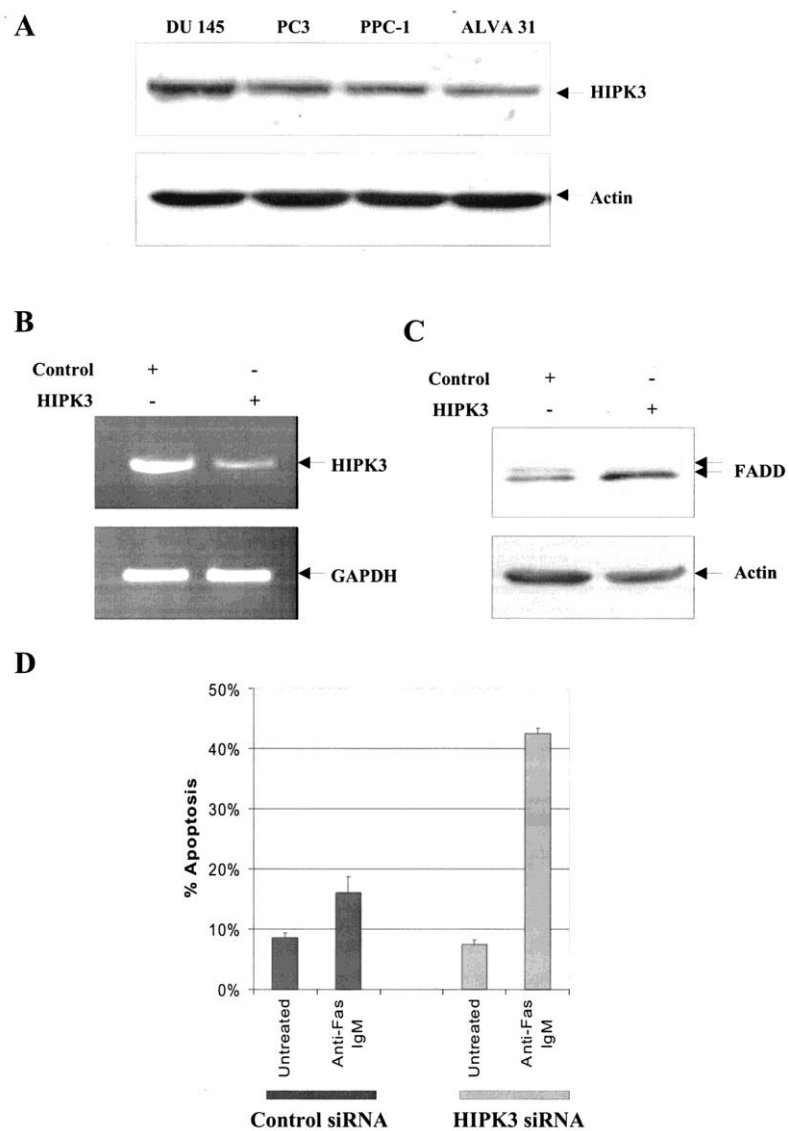
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Figure 8.

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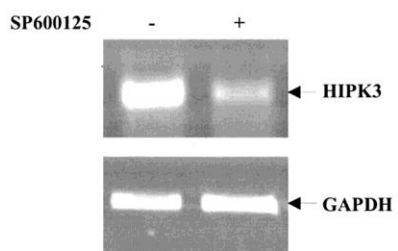
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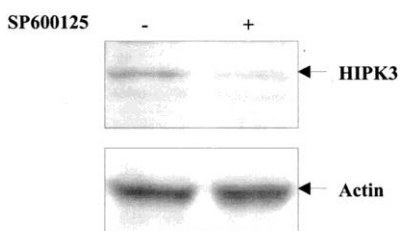
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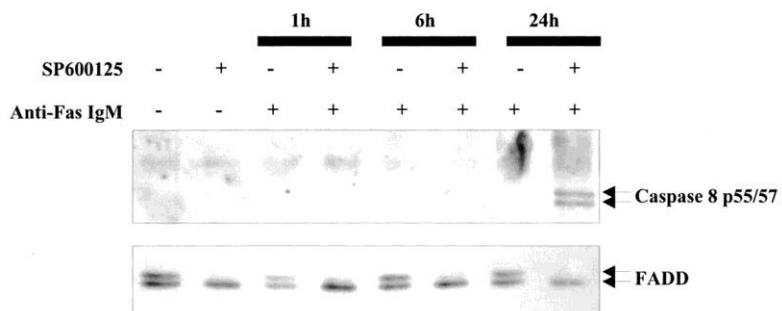
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Figure 10.

Figure 11.