Jpn. J. Cancer Res. **93**, 1164–1174, October 2002

Phosphorylation of Fas-associated Death Domain Contributes to Enhancement of Etoposide-induced Apoptosis in Prostate Cancer Cells

Keiji Shimada,[1] Mitsutoshi Nakamura,[1] Eiwa Ishida,[1] Munehiro Kishi,[1] Shin Yonehara[2] and Noboru Konishi[1, 3]

[1]Second Department of Pathology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521 and [2]Institute for Virus Research, Kyoto University, Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507

Fas-associated death domain (FADD) plays an important role as an adapter molecule in Fas (CD95/APO-1)-mediated apoptosis and contributes to anticancer drug-induced cytotoxicity. We treated three human prostate cancer cell lines with etoposide, a toposiomerase II inhibitor with activity against various tumors including prostate cancer. We found that the overexpression of FADD sensitizes etoposide-induced apoptosis through a rapid activation of c-Jun NH_2-terminal kinase (JNK) and, subsequently, of caspase 3. In addition, phosphorylation of FADD at serine 194 coincided with this sensitization. Treatment with the caspase 3 inhibitor, N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), or overexpression of either mitogen-activated protein kinase kinase (MKK) 7 or Bcl-xL canceled FADD-mediated sensitization to etoposide-induced apoptosis. Moreover, treatment with the caspase 8 inhibitor, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-IETD-fmk), or overexpression of viral FLICE/caspase-8-inhibitory protein (FLIP) from equine herpesvirus type 2 E8 also had an inhibitory effect, supporting a major involvement of a caspase 8-dependent mitochondrial pathway. Interestingly, FADD was phosphorylated, and etoposide-induced JNK/caspase activation and apoptosis were enhanced in the cells arrested at G2/M transition, but not in those overexpressing mutant FADD, in which 194 serine was replaced by alanine. Our results demonstrate that phosphorylated FADD-dependent activation of the JNK/caspase pathway plays a pivotal role in sensitization to etoposide-induced apoptosis in prostate cancer cells.

Key words: FADD — Phosphorylation — Etoposide — Prostate cancer

Fas-associated death domain (FADD) was originally identified as an adapter molecule for Fas-mediated apoptosis. Upon Fas stimulation by Fas ligand[1–3] or exposure to agonistic anti-Fas antibody,[4] death-inducing signaling complex (DISC) is formed by recruitment of at least FADD and procaspase 8. Procaspase 8 is subsequently activated in DISC and activated caspase 8 further stimulates downstream caspases such as caspases 3, 6 and 7, which can initiate apoptosis. In addition, other reports indicate that a FADD-dependent pathway significantly influences the cellular response to cytotoxic drugs; FADD overexpression seems to enhance tumor cell sensitivity to the drugs, whereas exposure to an antisense construct decreased sensitivity.[5]

c-Jun NH_2-terminal kinase (JNK) is a mitogen-activated protein kinase that regulates a number of physiological and pathological processes. JNK is strongly activated by and always linked to cell death under stressful stimuli such as ultraviolet (UV), γ irradiataion and cytotoxic drugs.[6] The entire role of JNK in cell death under a variety of stressful stimuli remains controversial. Tournier *et al.*,[7] however, have recently demonstrated that, in $JNK1^{-/-}$ and $JNK2^{-/-}$ mouse embryonic fibroblasts, activation of JNK and the release of cytochrome *c* into the cytoplasm by JNK-mediated mitochondrial cell death signaling are required for UV radiation-induced apoptosis. In addition, JNK is required for the apoptotic response to the genotoxin methyl-methanesulfonate and the drug anisomycin. Chen and Lai[8] also showed that persistent JNK activation in Jurkat T cells induces apoptosis, which is dependent on FADD and casapse 8.

Prostate cancer is a common diagnosis in both American and Japanese men. Improved procedures for surgical intervention and radiation have significantly reduced the number of fatalities. However, this type of cancer is highly resistant to chemotherapy, and there is still no effective cure for patients with advanced disease, especially in hormone-independent cases.[9–14] Recent reports have demonstrated novel mechanisms pertaining to chemosensitivity in prostate cancer cells by targeting FADD or JNK.[15, 16] There is thus growing evidence suggesting that a FADD- or JNK-dependent cell death mechanism plays an important role in chemotherapeutic sensitivity in various tumors, including prostate cancer, but the interaction between these proteins remains elusive. Scaffidi *et al.*[17] showed that FADD is phosphorylated at serine 194, which correlates with cell cycling, but is not apparently essential for

[3] To whom correspondence should be addressed.
E-mail: nkonishi@naramed-u.ac.jp

Fas-mediated apoptosis. However, they did not exclude the possibility that phosphorylation of FADD is important for other types of cell death.

In the present study, we focused on the effects of FADD, especially the phosphorylation of FADD at serine 194, and JNK activation on etoposide-induced apoptosis in three human prostate cancer cell lines, PC3, DU145 and LNCaP.[18] Our results clearly show that JNK activation, mediated by phosphorylated FADD, sensitizes prostate cancer cells equally to etoposide-induced apoptosis through caspase 3 activation. This phosphorylated FADD-dependent enhancement of JNK activation and its subsequent execution of the caspase cascade may be critical for sensitivity to drug-induced apoptosis in prostate cancer cells.

MATERIALS AND METHODS

Cell culture, plasmids and chemicals We purchased the human prostate cancer cell lines, LNCaP, DU145 and PC3, from American Type Culture Collection (Manassas, VA) and cultured the lines in RPMI supplemented with 10% fetal bovine serum. Plasmid Myc-tagged dominant-negative MKK7[19] was a gift from Dr. Nishida (Kyoto University, Kyoto). The expression vectors GFP (pTRE-d2GFP), β-gal (pβgal-control), and hygromycin-resistance gene (*pTK-Hyg*) were obtained from Clontech Laboratories Japan, Ltd. (Tokyo). Etoposide was purchased from Oncogene Research Products (Boston, MA); N-acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) and benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (z-IETD-fmk) were from MBL, Inc. (Nagoya); anti-FLAG antibody was from Sigma-Aldrich Japan, Ltd. (Tokyo); anti-FADD antibody was from Transduction Laboratories (Lexington, KY); anti-H (his) A (hemagglutinin) and anti-c-Myc antibodies were from Clontech Laboratories Japan, Ltd.; anti-Bcl-xL was from Sigma-Aldrich Japan, Ltd.; anti-194S-p-FADD and anti-JNK1 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Preparation of constructs and clones FLAG-tagged human FADD cDNA, prepared by conventional RT-PCR, was cloned into the mammalian expression vector pME18S,[20] and FADD and two mutants of FADD, serine (S) 194 alanine (A) and S194 aspartic acid (D), were generated using a Quick-change Site-directed Mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used for preparing the resulting FADD, S194A and S194D: 5′-GGAGTGGGGCCATGGCCCCGATGTCATGGAAC-3′, and 5′-GGAGTGGGGCCATGGACCCGATGTCATGGAAC-3′, respectively. JNK1 or p53 cDNA was also prepared by conventional RT-PCR and cloned into pCMV-HA vector (Clontech Laboratories Japan, Ltd.). Plasmids of pME18S-FLAG-tagged viral FLIP E8, pME18S-FLAG-tagged Bcl-xL, and pME18S-FLAG-tagged MST1-KR were constructed as previously described.[21–23]

Preparation of cell lysates, immunoprecipitation, and immunoblotting analysis Cells were washed once with phosphate-buffered saline (PBS) and suspended in lysis buffer (40 m*M* Hepes (pH 7.4) with 10% glycerol, 1% Triton X-100, 0.5% Nonidet P-40, 150 m*M* NaCl, 50 m*M* NaF, 20 m*M* β-glycerol phosphate, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, and 0.1 m*M* vanadate) with protease inhibitor mixtures (1 μg/ml aprotinin, leupeptin, and pepstatin). Cell lysates were cleared by centrifugation at 15 000 rpm for 30 min. For immunoprecipitation, cell lysates were incubated with 2 μg of anti-HA polyclonal antibody for 1 h at 4°C and precipitated with protein A-Sepharose (Amersham Pharmacia Biotech, Japan, Ltd., Tokyo). Cell lysates and immunoprecipitates were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Ltd., Bedford, MA). The membranes were blocked in Tris-buffered saline-Tween 20 (TBST) buffer (20 m*M* Tris-HCl (pH 7.5) containing 150 m*M* NaCl and 0.1% Tween 20) with 5% skim milk at room temperature for 1 h. The membrane was then incubated with anti-FLAG, anti-HA, anti-FADD, anti-c-Myc, or anti-Bcl-xL for 1 h, washed with TBST, and incubated with horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech). After further washing with TBST, peroxidase activity was detected on X-ray films using an enhanced chemiluminescence detection system.

Transfection of expression vectors For transient transfection, cells were seeded at 5×10^5 cells per well in 6-well plates. The cells were cultured in fresh medium for 24 h and then transfected with various vectors (0.2 μg/each vector) by using LipofectAmine (Invitrogen Japan, K.K., Tokyo) in accordance with the manufacturer's protocol. To establish stable cell lines, the pTK-Hyg vector, harboring the hygromycin-resistance gene, was co-transfected with an expression vector encoding dominant-negative Myc-tagged MKK7. The transfectants were then grown and selected in the presence of hygromycin.

Cell cycle arrest DU145 cells were treated with 4 m*M* hydroxyurea (Sigma, St. Louis, MO) or 10 ng/ml nocodazole (Sigma) for 24 h. Cells were washed with PBS, then harvested for flow cytometric and protein analyses as described below.

Flow cytometry A total of 5×10^6 non-treated and hydroxyurea- or nocodazole-treated DU145 cells were incubated with propidium iodide, then DNA contents were measured by flow cytometric analysis.

Assay of etoposide-induced apoptosis by β-gal staining in the transfected cells Cells were transfected for 24 h with either 0.5 μg of various expression vectors or with 0.5 μg of β-gal expression vector (pβgal-control). Transfection was followed by stimulation with various concentrations of etoposide for 48 h; the cells were then fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS

for 5 min at 4°C. After washing with PBS, the cells were overlaid with X-gal staining solution (5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, 1 m*M* $MgCl_2$, 0.02% NP-40, and 1 mg/ml X-gal in PBS) for 1 h at 37°C in the dark. The numbers of blue β-gal-positive cells with characteristic viable (flat, adherent) and apoptotic morphology (shrunken, round or blebbing formation) were calculated. At least 500 β-gal-positive cells were scored for each transfection in triplicate, and the mean percentages±their standard deviations of viable blue and apoptotic cells before and after treatment with etoposide were calculated.

Quantitative assay of etoposide-induced apoptosis in transfected cells Cells were transiently transfected with an expression vector encoding green fluorescence protein (GFP) (pTRE-2EGFP) as previously described.[23, 24] After a 3 h incubation, they were re-seeded. Following a further 21 h of culture, the cells were exposed to either 25 μg/ml etoposide solution or vehicle control for 48 h. Following a wash with PBS and detachment from the plate, the transfected cells were mixed with human HeLa cells pre-fixed with formaldehyde (5×10^5 cells/well). After staining with propidium iodide (PI), viable PI-negative transfected cells expressing various amounts of GFP were quantified by flow cytometry. HeLa cells, which can be discriminated from the transfected cells by size in flow cytometric analysis, were utilized for normalizing the number of the transfected cells to be measured by flow cytometry. Counting of PI-negative viable cells was stopped when a count of 1×10^5 PI-positive control HeLa cells was reached. Cell viability was represented by the mean percentage of the number of viable cells after etoposide treatment versus that before etoposide treatment for each independent transfection (n=3).

Cell viability assay DU145 cells were treated with hydroxyurea or nocodazole for 24 h on 96-well plates, then stimulated with 25 μg/ml of etoposide for 48 h, after which 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega, Tokyo) was added. After a 3 h incubation period, optical absorbance at 490 nm was measured using a microplate reader. Cell viability was expressed as mean percentage±the standard deviation of absorbance before and after treatment with various reagents. All experiments were performed in triplicate.

DNA fragmentation assay DU145 cells were untreated or treated with hydroxyurea or nocodazole for 24 h, then cells (10^7) were prepared and stimulated with 25 μg/ml of etoposide for 48 h. The stimulated cells were placed in lysis buffer containing 10 m*M* Tris-HCl (pH 7.4), 10 m*M* EDTA (pH 8.0) and 0.5% Triton X-100 for 20 min at 4°C. The lysate was centrifuged at 15 000 rpm for 20 min and the resulting supernatant was incubated with 4 μl of Rnase A (10 mg/ml) for 1 h at 37°C, followed by incubation with 4 μl of proteinase K (10 mg/ml) again for 1 h at 37°C. DNA was then extracted using 20 μl of 5 *M* NaCl and 120 μl of isopropanol for 10 min at room temperature followed by centrifugation at 20 000 rpm for 20 min. The DNA pellet was washed with 70% ethanol, then resuspended and loaded onto a 2% agarose gel and photographed under UV light.

***In vivo* analysis of caspase 3 activation** Cells were transfected with various expression vectors, using pME18S-FLAG-tagged mammalian STE20-like kinase 1 (MST1)-KR as a substrate for caspase 3. Following 24 h cultivation, they were stimulated with 25 μg/ml etoposide for 48 h, lysed with lysis buffer and separated on 12% SDS-polyacrylamide gels. After immunoblotting with anti-FLAG antibody, caspase 3 activation was assessed by the detection of intact and cleaved MST1-KR.[23, 24]

***In vitro* JNK kinase assay** A standard aliquot of cells (5×10^5 cells) was transfected with various expression vectors incorporating the HA-tagged *JNK1* gene. After 24 h of transfection, the cells were stimulated with 25 μg/ml of etoposide for the indicated times. Alternatively, 10^7 non-treated or hydroxyurea- or nocodazole-treated DU145 cells were stimulated with 25 μg/ml of etoposide for 48 h. Then, the cells were washed with PBS, lysed with lysis buffer, and immunoprecipitated with 2 μg of anti-JNK1 antibody. Immunoprecipitates were further incubated with 2 μg of the N-terminal peptide of c-Jun (amino acids 1–79; Santa Cruz Biotechnology, Inc.) in 25 μl of kinase reaction buffer (40 m*M* Hepes (pH 7.5) with 20 m*M* $MgCl_2$, 20 m*M* β-glycerol phosphate, and 0.1 m*M* vanadate) containing 25 μ*M* adenosine triphosphate (ATP) and 2.5 μCi of [γ-^{32}P]ATP for 30 min at 30°C. Reactions were terminated by adding 7 μl of Laemmli's sample buffer and boiling for 5 min. A portion of the sample (20 μl) was separated on a 12% SDS-polyacrylamide gels and autoradiographed.

RESULTS

Effect of phosphorylation of FADD at serine 194 on etoposide-induced apoptosis We analyzed whether FADD with or without phosphorylation at serine 194 plays a role in sensitization to etoposide-induced apoptosis in prostate cancer cells by using various FADD mutants. Expression of endogenous FADD and various FADD mutants in three prostate cancer cell lines was determined by immunoblotting of a detergent-soluble fraction (Fig. 1A). Since the upper band of FADD was detected in DU145 overexpressing wild type but not in S194A mutant by immunoblotting using anti-phosphorylated FADD at 194S antibody, we confirmed that the upper band is derived from phosphorylation at 194S. It is very difficult to strictly evaluate endogenous levels of FADD phosphorylation by this commercially anti-phosphorylated FADD

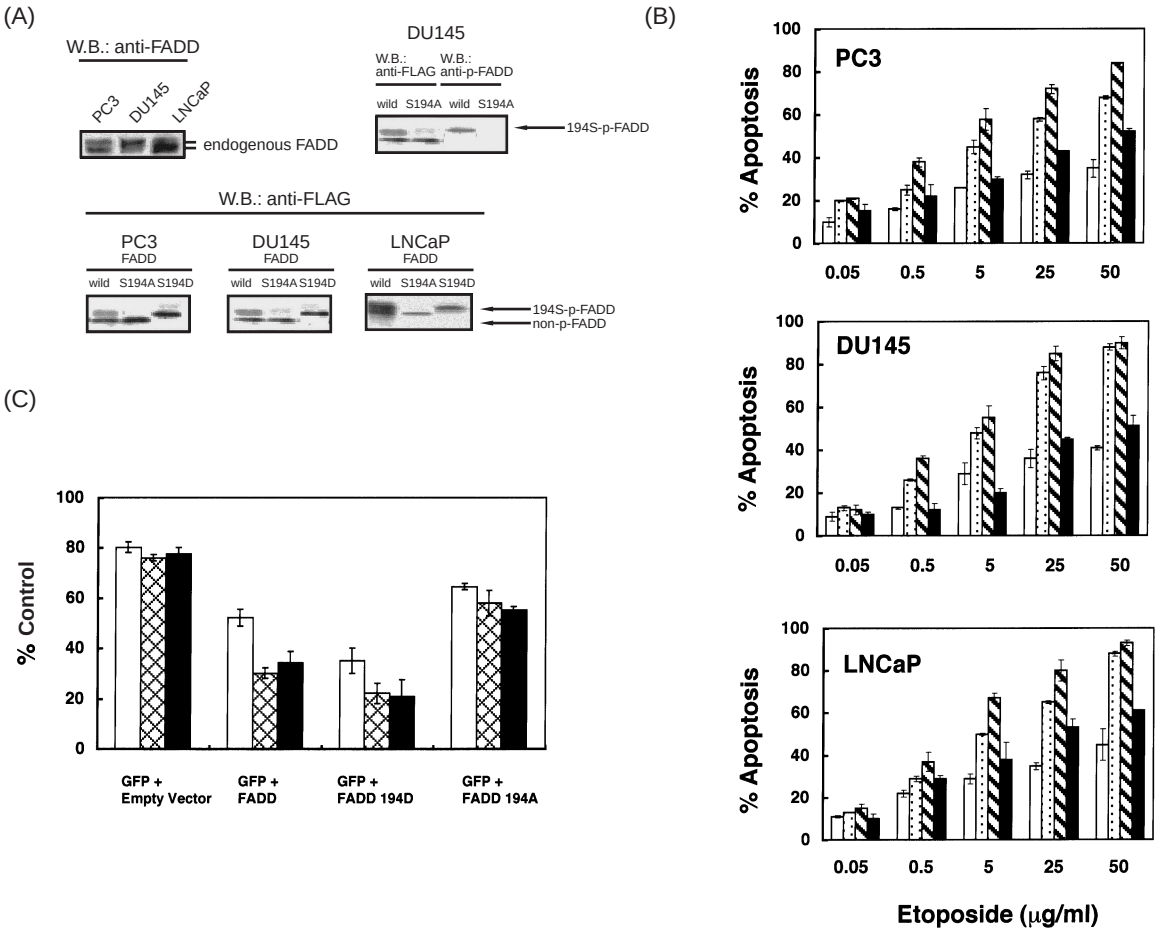

Fig. 1. Effects of transfected FADD and FADD mutants on etoposide-induced apoptosis in prostate cancer cell lines. (A) Expression of endogenous or transfected FADD and its mutants S194A and S194D in three prostate cancer cell lines, PC3, DU145 and LNCaP, was analyzed by immunoblotting with anti-FADD, anti-phosphorylated FADD (anti-p-FADD) at 194S or anti-FLAG antibody. (B) Cells were transfected with control empty vector, FLAG-tagged FADD, FADD S194A or FADD S194D together with a β-gal expression vector. After cultivation for the times indicated, cells were stimulated with various concentrations of etoposide for 48 h. The mean percentages (and standard error) of the number of apoptotic blue cells before and after treatment with etoposide were calculated in a blinded fashion as described in "Materials and Methods" (□ empty vector, ▩ FADD, ▨ S194D FADD, ■ S194A FADD). (C) Cells were co-transfected with control empty vector or various FADD mutants and a GFP expression vector. After cultivation for the time indicated, cells were stimulated with 25 μg/ml of etoposide or left unstimulated for 48 h. Viable transfected cells expressing various amounts of GFP were then quantified by flow cytometry. Numbers of GFP-positive (log GFP>1.0) cells were calculated, and the percentage (±standard error) of those stimulated with etoposide against those left unstimulated was calculated as indicated in the lower panel (□ PC3, ▩ DU145, ■ LNCaP). W.B.: western blotting.

antibody (data not shown). To investigate the effect of phosphorylated FADD on etoposide-induced cytotoxicity, we used two approaches—morphological quantification of apoptosis in a blinded fashion and flow cytometric quantification of cell viability. As shown in Fig. 1, B and C, the results obtained from these two assays correlate well. Transient transfection of FADD alone induced apoptosis in all prostate cancer cell lines, as did the various FADD mutants, including FADD S194A, in which serine 194 is replaced by alanine, which cannot be phosphorylated. The figures indicate the percentage of apoptotic cells (Fig. 1B) and the numbers of viable cells expressing each FADD mutant (Fig. 1C) before and after stimulation with etoposide. Transient transfection of FADD significantly enhanced etoposide-induced apoptosis in all prostate cancer cell lines, as did FADD S194D with an aspartic acid substitution at position 194; in fact, S194D transfection elicited sensitization even in a small number of GFP-expressing cells (Fig. 1C). In contrast, FADD S194A produced a comparatively low response. Another variant of

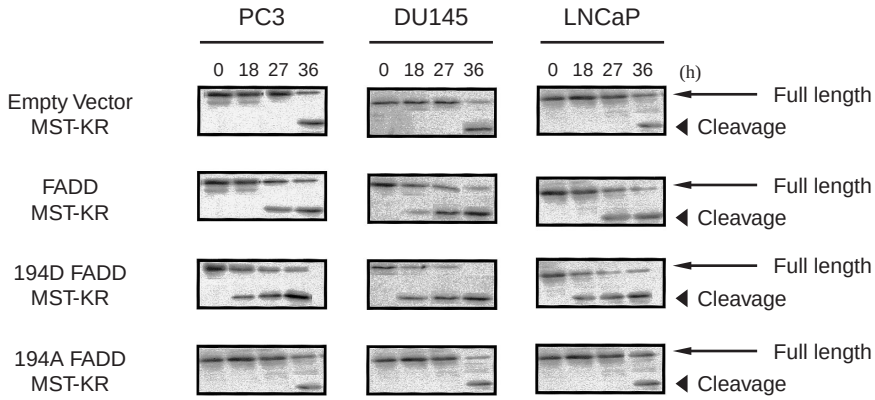

Fig. 2. The effect of overexpression of various FADD mutants on etoposide-induced caspase 3 activation. Cells were co-transfected with control empty vector or various FADD mutants and FLAG-tagged MST1-KR as a substrate for caspase 3. After cultivation for 24 h, cells were stimulated with 25 μg/ml of etoposide for the times indicated and the full-length and cleaved FLAG-tagged MST1-KR were analyzed by immunoblotting using anti-FLAG antibody.

FADD, in which glutamic acid was substituted for serine 194, gave the same results as FADD S194D (data not shown).

Phosphorylated FADD enhances etoposide-induced caspase 3 activation To investigate the role of serine 194 phosphorylation of FADD on etoposide-induced caspase 3 activation in prostate cancer cell lines, we transiently co-transfected the substituted FADD mutants with FLAG-tagged MST1-KR as a substrate for caspase 3 and analyzed the cleavage of MST1-KR after stimulation with etoposide. We first verified whether MST1-KR affects etoposide-induced apoptosis due to its dominant-negative effect. Overexpression of MST1-KR had no effect on etoposide-induced apoptosis in any of the prostate cancer cell lines examined (data not shown).

In both control cells and in cells transiently transfected with the F25Y FADD variant S194A, MST1-KR cleavage began 36 h after stimulation with etoposide (Fig. 2B). However, MST1-KR began to be cleaved by etoposide about 9 h earlier in cells transiently transfected with variant S194D, while in FADD-transfected cells, initial cleavage of MST1-KR was observed at 18 h after stimulation and was nearly complete at 36 h (Fig. 2B).

Inhibition of sensitization to etoposide-induced apoptosis by Bcl-xL and viral FLIP E8 As shown in Fig. 3, A and C, FADD-dependent enhancement of etoposide-induced apoptosis or MST1-KR cleavage was almost completely nullified by treatment with DEVD-CHO, a specific inhibitor of caspase 3. This suggests that caspase 3 activation is required for FADD-mediated sensitization to etoposide-induced apoptosis in prostate cancer cells. We then analyzed whether caspase 3 is activated via a so-called mitochondrial pathway or is dependent on caspase 8 activation.

As illustrated in Fig. 3, B and C, transient transfection with Bcl-xL significantly inhibited any enhancement by phosphorylated FADD of etoposide-induced apoptosis and caspase 3 activation in all prostate cancer cell lines. Transient transfection of FLIP E8 also suppressed FADD sensitization, as did treatment with the caspase 8 inhibitor, z-IETD-fmk (Fig. 3, A, B, and C). Expression of Bcl-xL or viral FLIP E8 in each cell line was confirmed by immunoblotting (Fig. 3D).

Activation of JNK contributes to phosphorylated FADD enhancement of etoposide-induced apoptosis In all cell lines transfected with either empty vector or FADD S194A, initial activation of JNK occurred 12 h after stimulation with etoposide and treatment with a pan-inhibitor of caspases, z-VAD-fmk, had no effect on such FADD-dependent JNK activation (data not shown). In contrast, JNK activation was observed at 6 h after stimulation in the cells expressing FADD S194D and at 9 h after in FADD-transfected cells (Fig. 4A). Interestingly, in all prostate cancer cell lines stably expressing the dominant-negative mutant of mitogen-activated protein kinase kinase (MKK) 7, phosphorylated FADD-dependent enhancement of etoposide-induced JNK activation, MST1-KR cleavage, and apoptosis were completely negated (Fig. 4, B and C). In addition, inhibition of etoposide-induced JNK activation by MKK7-KL had no significant effect on the phosphorylation status of co-transfected or endogenous FADD (data not shown), suggesting no major involvement of JNK activation in phosphorylation of FADD at 194S.

Cell cycle-dependent phosphorylation of FADD is well correlated with the sensitization to etoposide-induced apoptosis Scaffidi *et al.*[17] showed that phosphorylation of FADD at 194 serine was subject to the G2/M transition by 70 kDa cell cycle-regulating kinase. Here, we investigated

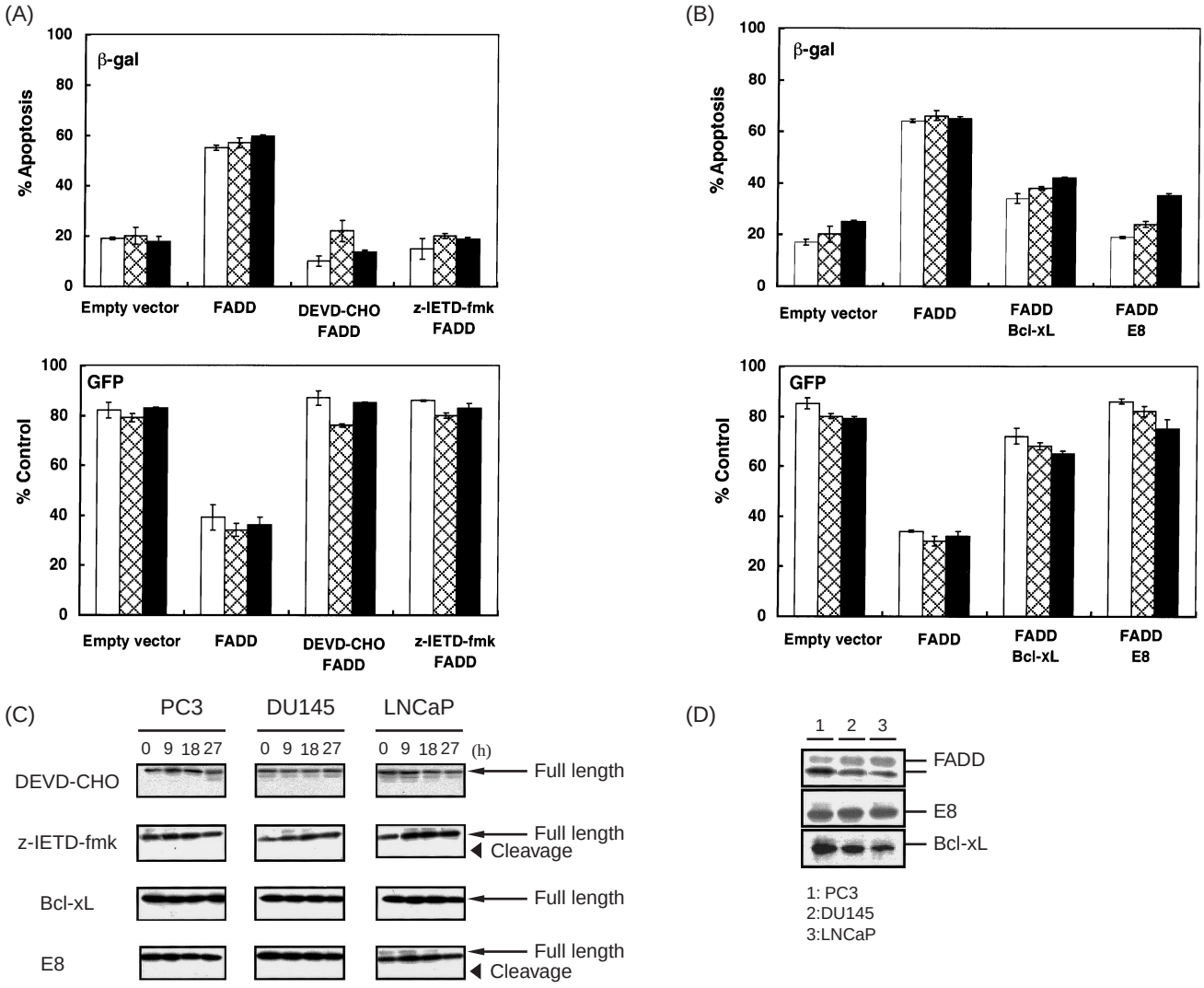

Fig. 3. Sensitization to etoposide-induced apoptosis by phosphorylated FADD is suppressed by viral FLIP E8 and Bcl-xL. (A) and (B) 5×10^5 cells from each of three prostate cancer cell lines, PC3, DU145, and LNCaP, were co-transfected with 0.2 μg of FADD and 0.4 μg of the GFP expression vector or a β-gal expression vector plus 0.4 μg of empty vector to adjust to a total transfected DNA volume of 1.0 μg. After cultivation for 24 h, cells were pretreated with 50 μM DEVD-CHO or z-IETD-fmk for 3 h. In addition, cells were co-transfected with 0.2 μg of FADD and 0.4 μg of GFP expression vector plus 0.4 μg of FLAG-tagged Bcl-xL or viral FLIP E8 and incubated for 24 h. Cells from (A) and (B) were then stimulated with 25 μg/ml of etoposide for 48 h. Morphological or flow cytometric analysis was performed and the mean percentages (±standard error) of apoptotic blue cells or viable GFP-positive cells were calculated before and after etoposide stimulation as described in "Materials and Methods" (□ PC3, ▨ DU145, ■ LNCaP). (C) 5×10^5 cells from each of the three prostate cancer cell lines were co-transfected with 0.2 μg each of FADD, FLAG-tagged MST1-KR, and empty vector to adjust the total transfected DNA volume to 1.0 μg (upper two lanes). After cultivation for 24 h, cells were treated with 250 μM DEVD-CHO or z-IETD-fmk for 3 h. The lower 2 lanes represent cells that were co-transfected with 0.4 μg each of F25Y FADD, FLAG-tagged MST1-KR plus FLAG-tagged Bcl-xL, or FLAG-tagged MST1-KR plus viral FLIP E8 and incubated for 24 h. These cells were then stimulated with 25 μg/ml etoposide for the time indicated and full-length and cleaved FLAG-tagged MST1-KR were analyzed by immunoblotting using anti-FLAG antibody. (D) The expression of FADD, viral FLIP E8 or Bcl-xL was analyzed by immunoblotting using anti-FLAG antibody.

whether etoposide-induced apoptosis in prostate cancer cells is affected by endogenous phosphorylation or unphosphorylation of FADD at the G2/M or G1/S. DU145 cells were arrested at the G1/S or G2/M boundary by treatment with hydroxyurea or nocodazole for 24 h. Cell cycle arrest was confirmed by flow cytometric analysis (Fig.

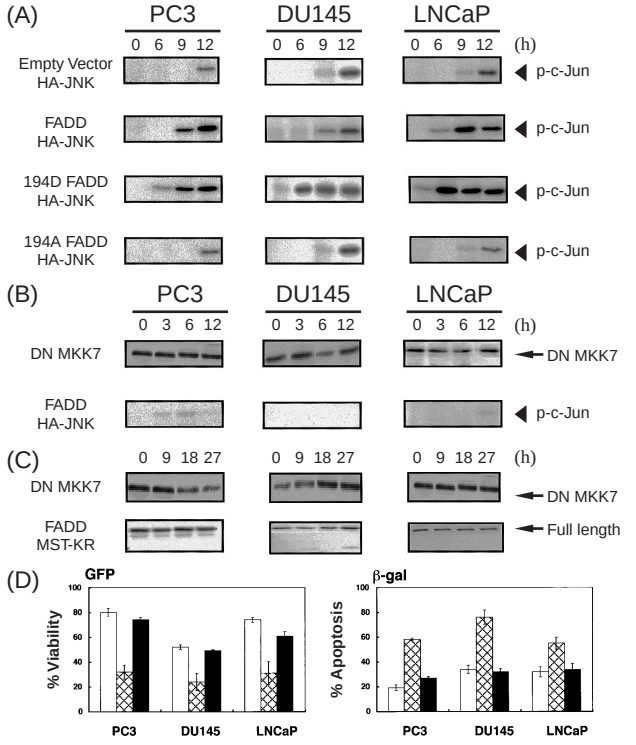

Fig. 4. Effect of JNK activation on the sensitization to etoposide-induced apoptosis by phosphorylated FADD. (A) 5×10^5 cells from each of the three prostate cancer cell lines were transfected with either 0.2 μg of control empty vector or with 0.2 μg each of one of the various FADD mutants plus HA-tagged JNK. After 24 h cultivation, cells were stimulated with 25 μg/ml of etoposide for the times indicated and immunoprecipitated with anti-HA antibody. An *in vitro* kinase assay of immunoprecipitated JNK was performed and phosphorylated GST-c-Jun (p-c-Jun) was quantified as described in "Materials and Methods." (B) Cultures of cells stably expressing Myc-tagged dominant-negative MKK7 were established and the expression of dominant-negative MKK7 was analyzed by immunoblotting with anti-Myc antibody. 5×10^5 cells were then co-transfected with 0.2 μg each of FADD and HA-tagged JNK. After 24 h cultivation, cells were stimulated with 25 μg/ml of etoposide for the times indicated and immunoprecipitated with anti-HA antibody. An *in vitro* kinase assay was performed as described above. (C) 5×10^5 cells stably expressing dominant-negative MKK7 were co-transfected with 0.2 μg each of FADD and FLAG-tagged MST1-KR. After 24 h cultivation, cells were stimulated with 25 μg/ml of etoposide for the times indicated and the full-length and cleaved FLAG-tagged MST1-KR were analyzed in total lysates by immunoblotting. (D) 0.2 μg of empty vector or 0.2 μg each of FADD and GFP or a β-gal expression vector were transfected into cells with or without stable expression of dominant-negative MKK7. After 24 h cultivation, cells were stimulated with 25 μg/ml of etoposide for 48 h, then cell viability and apoptosis were analyzed by morphological and flow cytometric analysis, respectively. The mean percentages (±standard error) of apoptotic blue cells or viable GFP-positive cells were calculated before and after etoposide stimulation as described in "Materials and Methods" (□ control, ▨ FADD, ■ FADD dominant-negative MKK7).

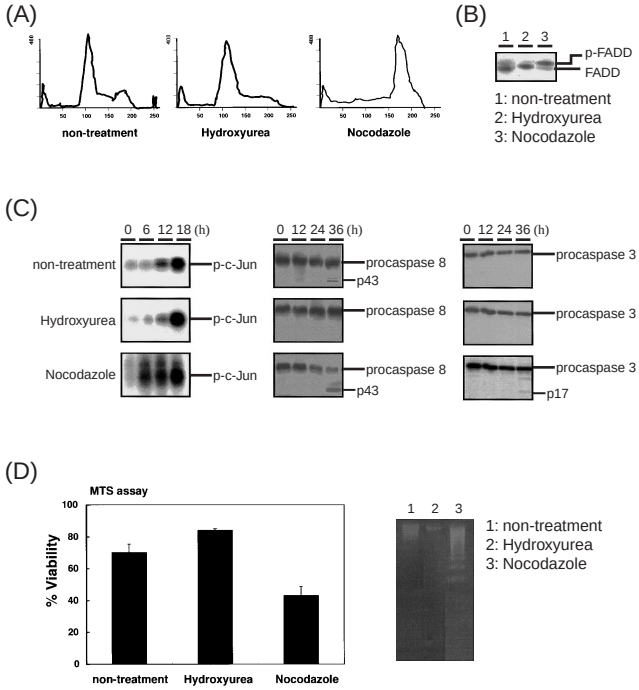

Fig. 5. Endogenous phosphorylation of FADD (p-FADD) dependent on the cell cycle affects the sensitization to etoposide-induced apoptosis. (A) Human prostate cancer cell line DU145 was left untreated or treated with hydroxyurea or nocodazole for 24 h. Then, DNA contents were measured by flow cytometric analysis as described in "Materials and Methods." (B) After non-treatment or treatment with hydroxyurea or nocodazole, cells were harvested and lysed with lysis buffer as described in "Materials and Methods." p-FADD was analyzed by immunoblotting using anti-FADD monoclonal antibody. (C) After incubation for 24 h as described above, 10^7 cells were treated with 25 μg/ml etoposide for the indicated times. Then, cells were harvested and whole cell lysates were immunoprecipitated with anti-JNK1 antibody and *in vitro* kinase assay for JNK was performed as described in "Materials and Methods." Alternatively, immunoblotting using anti-caspase 8 or 3 antibody was performed. (D) After non-treatment or treatment with hydroxyurea or nocodazole for 24 h, cells were stimulated with 25 μg/ml etoposide for 48 h. Then, cell viability was examined by MTS assay, and DNA fragmentation was analyzed as described in "Materials and Methods."

5A). As shown in Fig. 5B, cells arrested at G1/S predominantly contained unphosphorylated FADD, whereas FADD was quantitatively phosphorylated in cells arrested at G2/M. These results were consistent with the previous report by Scaffidi *et al.*[17] Interestingly, in cells arrested at G2/M, etoposide-induced activation of the JNK/caspase 8/3 pathway and apoptosis were enhanced, while such an enhancement was not found in those arrested at G1/S (Fig. 5C).

Overexpression of non-phosphorylable FADD blocked the sensitization at G2/M transition to etoposide-induced apoptosis To clarify whether cell cycle-dependent phosphorylation of FADD at serine 194 is essential for the sensitization to etoposide-induced apoptosis in the cells arrested at G2/M transition, we examined the effect of non-phosphorylable FADD (S194A FADD) overexpression on such sensitization using DU145. As shown in Fig. 6, A and B, in the cells overexpressing control vector, G2/M arrest induced by nocodazole resulted in enhancement of apoptosis and reduction of cell viability as measured by morphological and flow cytometric analysis, respectively. In contrast, in those overexpressing S194A FADD, such a G2/M arrest-related sensitization to etoposide-induced apoptosis was not seen. In addition, enhancement of etoposide-induced JNK activation at G2/M arrest was observed in control cells but not in S194A FADD-overexpressing cells (Fig. 6C).

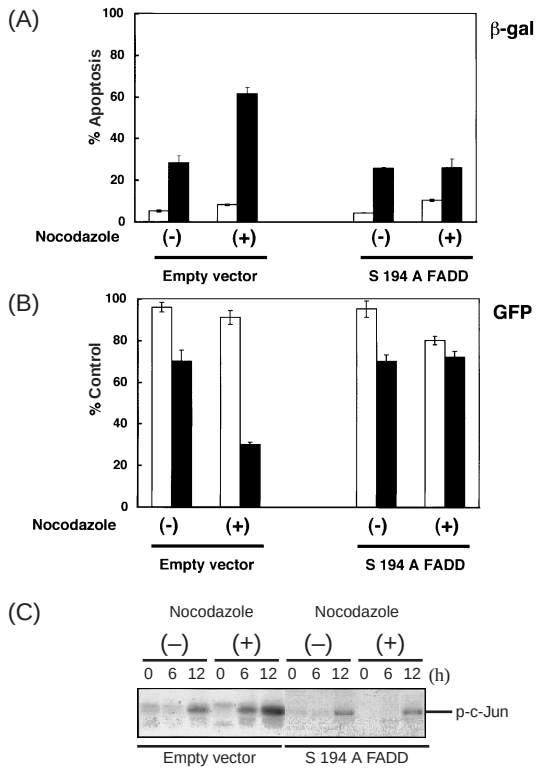

Fig. 6. Overexpression of S194A FADD canceled cell cycle-dependent sensitization to etoposide-induced apoptosis. (A) and (B) DU145 was transfected with control empty vector or mutant FADD, in which serine 194 was replaced by alanine plus a β-gal or GFP expression vector. After 24 h, cells were incubated with nocodazole for 24 h, and subsequently stimulated with 25 μg/ml etoposide for 48 h. The cell viability and apoptosis were analyzed by morphological and flow cytometric analysis, respectively. The mean percentages (±standard error) of apoptotic blue cells or viable GFP-positive cells were calculated before and after etoposide stimulation as described in "Materials and Methods" (□ etoposide (−), ■ etoposide (+)). (C) Cells were transfected with control empty vector or S194A FADD plus HA-tagged JNK1. After 24 h, cells were incubated with nocodazole for 24 h, and subsequently stimulated with 25 μg/ml etoposide for the indicated times. Then, the cells were lysed and immunoprecipitated with anti-HA antibody, and *in vitro* kinase assay was performed as described in "Materials and Methods."

DISCUSSION

Our work here demonstrates for the first time that serine 194 phosphorylation of FADD is correlated to enhanced sensitization to etoposide-induced apoptosis. However, a major problem had to be overcome to enable us to assess the physiological role of FADD in this particular system. We discovered that the FADD S194A variant, which cannot be phosphorylated at all, might not be the proper vehicle to evaluate the role of phosphorylation due to the presence of endogenous phosphorylated FADD within the cell. To compensate, we analyzed the amount of expression of both phosphorylated and unphosphorylated FADD by immunoblotting with an anti-phospho-serine 194 antibody. We found that, fortunately, all the cell lines used in the present experiment have relatively little phosphorylated FADD as compared to the unphosphorylated protein (data not shown). In addition, we compared the amount of cell death, MST1-KR cleavage, or JNK activation in the cells transfected with the FADD mutants with those transfected with empty vector. All of these approaches gave us a clearer picture of the role of phosphorylated FADD in etoposide-induced apoptosis.

With the exception of FADD S194A, overexpression of both endogenous and substituted mutant FADDs enhances etoposide-induced activation of JNK via MKK7 and MST1-KR cleavage. The kinase MST1 is directly activated by caspase 3 during apoptosis both *in vivo* and *in vitro*.[23] When co-transfected with MST1-KR, a mutant form of MST1, the cleavage of MST1-KR is similarly indicative of caspase 3 activation *in vivo*. Watabe *et al.* showed that a kinase mutant of MST1 can suppress a certain type of apoptosis due to its dominant-negative effect.[25] However, in our prostate cancer cell lines, we found that MST1-KR had no significant effect on apoptosis. This suggests that caspase 3 activation via MKK7 plays a major role in the etoposide sensitization cascade initiated by phosphorylated FADD.

JNK activation by etoposide was completely suppressed by overexpression of dominant-negative MKK7 (Fig. 4, B and C), but not by caspase inhibitors (data not shown), supporting the theory that JNK activation acts upstream of the caspase cascade. Recent reports have demonstrated that JNK can be activated via Fas in both caspase-depen-

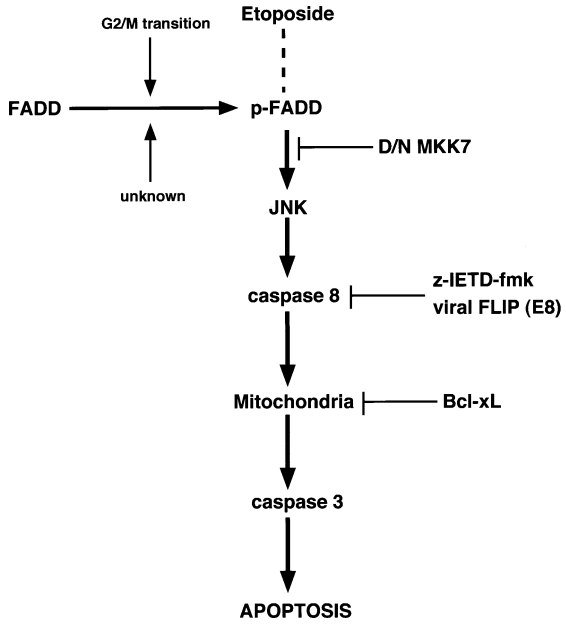

Fig. 7. Schematic presentation of sensitization to etoposide-induced apoptosis through FADD phosphorylated at 194S (p-FADD). When FADD is phosphorylated at 194S under certain conditions such as G2/M transition, etoposide-induced JNK activation and apoptosis are strongly enhanced. Overexpression of E8, Bcl-xL or treatment with various types of caspase inhibitors canceled this enhancement of apoptosis by etoposide, but did not affect JNK activation. Therefore, it is concluded that JNK activation can lead to caspase 8 activation followed by caspase 3 activation through the mitochondrial pathway. JNK is not associated with p-FADD. The detailed molecular mechanism, by which JNK is activated through p-FADD, or the key molecule related to FADD phosphorylation has not yet been determined or identified.

dent and -independent pathways.[26, 27] Since cytotoxic drugs such as etoposide, doxorubicin, and cisplatin are thought to induce Fas activation and subsequent interaction with FADD, it is possible that etoposide-induced Fas-FADD activation might, in turn, lead to acceleration of JNK activation in a caspase-independent manner, for which phosphorylation of FADD at serine 194 may be essential.

Bcl-xL, a member of the anti-apoptotic *Bcl-2* gene family, clearly prevents apoptosis by blocking cytochrome *c* release from mitochondria followed by caspase 9 and caspase 3 activation.[28] Bcl-xL is found in and profoundly associated with chemosensitivity in prostate cancer cell lines.[29–31] Viral FLIP E8 inhibits Fas-induced apoptosis without stimulating apoptosis itself, even though it consists of two repeated death-effector domains without a protease-like domain.[21, 32] We found that both Bcl-xL and viral FLIP E8 suppress FADD-mediated enhancement of etoposide-induced caspase 3 activation and apoptosis (Fig. 3B). A similar result was demonstrated upon treatment with relative specific inhibitors for caspase 8 or 3 (Fig. 3A). This result suggests a pathway following JNK activation involving caspase 3 activation via caspase 8 and mitochondrial cytochrome *c* release. Tournier *et al.* similarly proposed that stress-induced apoptosis follows a pre-apoptotic pathway initiated by JNK activation and progressing to cleavage of Bid, cytochrome *c* release from mitochondria, and caspase 3 activation of an apoptotic cascade[7]; Bid is cleaved and activated by caspase 8 and induces cytochrome *c* release.[33] As mentioned earlier, Chen and Lai showed that persistent JNK activation, mediated by overexpression of MKK7, induces apoptosis in Jurkat T cells and that this process is dependent on FADD and caspase 8.[8] Given these results, we propose that a rapid activation of JNK through phosphorylated FADD might activate caspase 8, thus triggering the execution of apoptosis via cytochrome *c* release and caspase 3 activation. In the present study, inhibition of caspase 8 suppressed enhancement of etoposide-induced caspase 3 activation and did so almost as effectively as overexpression of Bcl-xL, indicating that caspase 3 is mainly activated through caspase 8. Therefore, we conclude that a caspase 8-dependent mitochondrial pathway is mainly involved in a major cascade following rapid activation of JNK by phosphorylated FADD (Fig. 7), though we have little evidence regarding the detailed mechanism of JNK activation by phosphorylated FADD; this is an area that should be further evaluated. Extracellular stress-regulated kinase (ERK) or nuclear factor kappa B (NFκB) has been shown to affect JNK activation by various stressors such as anticancer drugs, UV exposure or irradiation. ERK and NFκB are constitutively activated in DU145, but not in LNCaP (Shimada *et al.*, submitted)[34]; however, FADD phosphorylation-dependent JNK activation in DU145 was not significantly different from that in LNCaP (Fig. 4A). In addition, a selective inhibitor of upstream kinase of ERK, PD98059, or an IκB kinase inhibitor, Bay117082, inhibited etoposide-induced ERK or NFκB activation but did not significantly affect FADD-dependent JNK activation (data not shown). These results clearly show that the ERK/NFκB pathway is not mainly involved in FADD-dependent enhancement of etoposide-induced JNK activation. Unphosphorylated FADD slightly sensitized prostate cancer cells to etoposide-induced apoptosis as shown in Fig. 1, although activation of both caspase 3 and JNK were not significantly modified.

In addition to the results from the overexpression experiment, we demonstrated that FADD is endogenously phosphorylated, by which the susceptibility to etoposide-induced apoptosis can be enhanced through acceleration of JNK/caspase activation, in DU145 arrested at G2/M tran-

sition. Interestingly, such G2/M arrest-related enhancement of etoposide-induced JNK activation or apoptosis was canceled by overexpression of non-phosphorylable FADD, probably through a dominant-negative effect. These results suggest that cell cycle-dependent phosphorylation of FADD at serine 194 may contribute to the susceptibility to etoposide-induced apoptosis, and raise the possibility that the phosphorylation of FADD/JNK/caspase pathway plays a critical role in the sensitization of prostate cancer cells to anticancer drug-induced apoptosis.

Our study indicates that phosphorylation of FADD at serine 194 accelerates the etoposide-induced cytotoxicity mechanism, in which JNK and caspases are sequentially activated dependently on the mitochondrial pathway (Fig. 7), and that regulation of FADD phosphorylation, or the kinase responsible for this phosphorylation, may provide a novel strategy for treatment of prostate cancer.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid (14770103) from the Ministry of Education Culture, Sports, Science and Technology, Japan.

(Received June 10, 2002/Revised July 29, 2002/Accepted August 1, 2002)

REFERENCES

1) Nagata, S. and Golstein, P. The Fas death factor. *Science*, **267**, 1449–1456 (1995).
2) Yonehara, S. Effects of anti-Fas antibodies on lymphocytes and other organs: preparation of original and new monoclonal antibodies and amelioration of systemic autoimmune disease. *Int. Rev. Immunol.*, **18**, 329–345 (1999).
3) Krammer, P. H. CD95's deadly mission in the immune system. *Nature*, **407**, 789–795 (2000).
4) Yonehara, S., Ishii, A. and Yonehara, M. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.*, **169**, 1747–1756 (1989).
5) Micheau, O., Solary, E., Hammann, A. and Dimanche-Boitrel, M. T. Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. *J. Biol. Chem.*, **274**, 7987–7992 (1999).
6) Lee, L. F., Li, G., Templeton, D. J. and Ting, J. P. Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). *J. Biol. Chem.*, **273**, 28253–28260 (1998).
7) Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A. and Davis, R. J. Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. *Science*, **288**, 870–874 (2000).
8) Chen, Y. and Lai, M. Z. c-Jun NH2-terminal kinase activation leads to a FADD-dependent but Fas ligand-independent cell death in Jurkat T cells. *J. Biol. Chem.*, **276**, 8350–8357 (2001).
9) Konishi, N., Hiasa, Y., Hayashi, I., Matsuda, H., Tsuzuki, T., Ming, T., Kitahori, Y., Shiraishi, T., Yatani, R. and Shimazaki, J. p53 mutations occur in clinical, but not latent, human prostate carcinoma. *Jpn. J. Cancer Res.*, **86**, 57–63 (1995).
10) Konishi, N., Cho, M., Yamamoto, K. and Hiasa, Y. Genetic changes in prostate cancer. *Pathol. Int.*, **47**, 735–747 (1997).
11) Matsushima, H., Kitamura, T., Goto, T., Hosaka, Y., Homma, Y. and Kawabe, K. Combined analysis with Bcl-2 and P53 immunostaining predicts poorer prognosis in prostatic carcinoma. *J. Urol.*, **158**, 2278–2283 (1997).
12) Theodorescu, D., Broder, S. R., Boyd, J. C., Mills, S. E. and Frierson, H. F., Jr. p53, bcl-2 and retinoblastoma proteins as long-term prognostic markers in localized carcinoma of the prostate. *J. Urol.*, **158**, 131–137 (1997).
13) Brewster, S. F., Oxley, J. D., Trivella, M., Abbott, C. D. and Gillatt, D. A. Preoperative p53, bcl-2, CD44 and E-cadherin immunohistochemistry as predictors of biochemical relapse after radical prostatectomy. *J. Urol.*, **161**, 1238–1243 (1999).
14) Stackhouse, G. B., Sesterhenn, I. A., Bauer, J. J., Mostofi, F. K., Connelly, R. R., Srivastava, S. K. and Moul, J. W. p53 and bcl-2 immunohistochemistry in pretreatment prostate needle biopsies to predict recurrence of prostate cancer after radical prostatectomy. *J. Urol.*, **162**, 2040–2045 (1999).
15) Huang, Y., He, Q., Hillman, M. J., Rong, R. and Sheikh, M. S. Sulindac sulfide-induced apoptosis involves death receptor 5 and the caspase 8-dependent pathway in human colon and prostate cancer cells. *Cancer Res.*, **61**, 6918–6924 (2001).
16) Chen, Y. R., Zhou, G. and Tan, T. H. c-Jun N-terminal kinase mediates apoptotic signaling induced by N-(4-hydroxyphenyl)retinamide. *Mol. Pharmacol.*, **56**, 1271–1279 (1999).
17) Scaffidi, C., Volkland, J., Blomberg, I., Hoffmann, I., Krammer, P. H. and Peter, M. E. Phosphorylation of FADD/MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. *J. Immunol.*, **164**, 1236–1242 (2000).
18) Rokhlin, O. W., Bishop, G. A., Hostager, B. S., Waldschmidt, T. J., Sidorenko, S. P., Pavloff, N., Kiefer, M. C., Umansky, S. R., Glover, R. A. and Cohen, M. B. Fas-mediated apoptosis in human prostatic carcinoma cell lines. *Cancer Res.*, **57**, 1758–1768 (1997).
19) Matsuda, S., Moriguchi, T., Koyasu, S. and Nishida, E. T lymphocyte activation signals for interleukin-2 production

involve activation of MKK6-p38 and MKK7-SAPK/JNK signaling pathways sensitive to cyclosporin A. *J. Biol. Chem.*, **273**, 12378–12382 (1998).
20) Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K. and Yonehara, S. The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis. *Nature*, **398**, 777–785 (1999).
21) OhYama, T., Tsukumo, S., Yajima, N., Sakamaki, K. and Yonehara, S. Reduction of thymocyte numbers in transgenic mice expressing viral FLICE-inhibitory protein in a Fas-independent manner. *Microbiol. Immunol.*, **44**, 289–297 (2000).
22) Watanabe, M., Shirayoshi, Y., Koshimizu, U., Hashimoto, S., Yonehara, S., Eguchi, Y., Tsujimoto, Y. and Nakatsuji, N. Gene transfection of mouse primordial germ cells *in vitro* and analysis of their survival and growth control. *Exp. Cell Res.*, **230**, 76–83 (1997).
23) Lee, K. K., Ohyama, T., Yajima, N., Tsubuki, S. and Yonehara, S. MST, a physiological caspase substrate, highly sensitizes apoptosis both upstream and downstream of caspase activation. *J. Biol. Chem.*, **276**, 19276–19285 (2001).
24) Kazama, H. and Yonehara, S. Oncogenic K-Ras and basic fibroblast growth factor prevent Fas-mediated apoptosis in fibroblasts through activation of mitogen-activated protein kinase. *J. Cell Biol.*, **148**, 557–566 (2000).
25) Watabe, M., Kakeya, H. and Osada, H. Requirement of protein kinase (Krs/MST) activation for MT-21-induced apoptosis. *Oncogene*, **18**, 5211–5220 (1999).
26) Villunger, A., Huang, D. C., Holler, N., Tschopp, J. and Strasser, A. Fas ligand-induced c-Jun kinase activation in lymphoid cells requires extensive receptor aggregation but is independent of DAXX, and Fas-mediated cell death does not involve DAXX, RIP, or RAIDD. *J. Immunol.*, **165**, 1337–1343 (2000).
27) Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H. and Baltimore, D. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science*, **281**, 1860–1863 (1998).
28) Tsujimoto, Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells*, **3**, 697–707 (1998).
29) Krajewski, S., Krajewska, M., Shabaik, A., Wang, H. G., Irie, S., Fong, L. and Reed, J. C. Immunohistochemical analysis of *in vivo* patterns of Bcl-X expression. *Cancer Res.*, **54**, 5501–5507 (1994).
30) McDonnell, T. J., Navone, N. M., Troncoso, P., Pisters, L. L., Conti, C., von Eschenbach, A. C., Brisbay, S. and Logothetis, C. J. Expression of bcl-2 oncoprotein and p53 protein accumulation in bone marrow metastases of androgen independent prostate cancer. *J. Urol.*, **157**, 569–574 (1997).
31) Lebedeva, I., Rando, R., Ojwang, J., Cossum, P. and Stein, C. A. Bcl-xL in prostate cancer cells: effects of overexpression and down-regulation on chemosensitivity. *Cancer Res.*, **60**, 6052–6060 (2000).
32) Bertin, J., Armstrong, R. C., Ottilie, S., Martin, D. A., Wang, Y., Banks, S., Wang, G. H., Senkevich, T. G., Alnemri, E. S., Moss, B., Lenardo, M. J., Tomaselli, K. J. and Cohen, J. I. Death effector domain-containing herpesvirus and poxvirus proteins inhibit both Fas- and TNFR1-induced apoptosis. *Proc. Natl. Acad. Sci. USA*, **94**, 1172–1176 (1997).
33) McDonnell, J. M., Fushman, D., Milliman, C. L., Korsmeyer, S. J. and Cowburn, D. Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. *Cell*, **96**, 625–634 (1999).
34) Mukhopadhyay, A., Bueso-Ramos, C., Chatterjee, D., Pantazis, P. and Aggarwal, B. B. Curcumin downregulates cell survival mechanisms in human prostate cancer cell lines. *Oncogene*, **20**, 7597–7609 (2001).