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# Fas Activation of the p38 Mitogen-Activated Protein Kinase Signalling Pathway Requires ICE/CED-3 Family Proteases

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**The Fas receptor mediates a signalling cascade resulting in programmed cell death (apoptosis) within hours of receptor cross-linking. In this study Fas activated the stress-responsive mitogen-activated protein kinases, p38 and JNK, within 2 h in Jurkat T lymphocytes but not the mitogen-responsive kinase ERK1 or pp70$^{S6k}$. Fas activation of p38 correlated temporally with the onset of apoptosis, and transfection of constitutively active MKK3(glu), an upstream regulator of p38, potentiated Fas-induced cell death, suggesting a potential involvement of the MKK3/p38 activation pathway in Fas-mediated apoptosis. Fas has been shown to require ICE (interleukin-1β-converting enzyme) family proteases to induce apoptosis from studies utilizing the cowpox ICE inhibitor protein CrmA, the synthetic tetrapeptide ICE inhibitor YVAD-CMK, and the tripeptide pan-ICE inhibitor Z-VAD-FMK. In this study, *crmA* antagonized, and YVAD-CMK and Z-VAD-FMK completely inhibited, Fas activation of p38 kinase activity, demonstrating that Fas-dependent activation of p38 requires ICE/CED-3 family members and conversely that the MKK3/p38 activation cascade represents a downstream target for the ICE/CED-3 family proteases. Intriguingly, p38 activation by sorbitol and etoposide was resistant to YVAD-CMK and Z-VAD-FMK, suggesting the existence of an additional mechanism(s) of p38 regulation. The ICE/CED-3 family–p38 regulatory relationship described in the current work indicates that in addition to the previously described destructive cleavage of substrates such as poly(ADP ribose) polymerase, lamins, and topoisomerase, the apoptotic cysteine proteases also function to regulate stress kinase signalling cascades.**

Fas (APO-1/CD95) encodes a transmembrane type I receptor belonging to the tumor necrosis factor (TNF) receptor superfamily which includes TNF receptors 1 and 2 (TNFR1 and TNFR2), nerve growth factor (NGF) receptor, CD27, CD30, CD40, and OX40 (47, 67; reviewed in references 65 and 80). The cognate Fas ligand is a type II transmembrane protein belonging to the TNF family (83). The Fas receptor system has been extensively studied as a model of apoptosis, since cross-linking of the Fas receptor with Fas ligand or specific agonist antibodies results in rapid programmed cell death (47, 89, 97).

Fas-induced apoptosis plays an important role in T- and B-cell homeostasis in the immune system and participates in T-cell activation-induced cell death (5, 22, 48), the elimination of autoreactive B cells (71), and the maintenance of sites of immune privilege (1). *lpr* mice defective in Fas (93), *gld* mice defective in Fas ligand (84), and humans with mutations in Fas (34, 73) all develop an autoimmune, lymphoproliferative disorder, indicating that Fas-induced death plays an important role in the peripheral deletion of lymphocytes.

The essential signalling events linking Fas receptor cross-linking to apoptosis have been the subject of intense investigation. The intracellular domain of Fas contains an approximately 70-amino-acid "death domain" required for the induction of apoptosis (45). The death domain is conserved among other proteins implicated in apoptosis such as TNFR, TRADD, and FADD and appears to act as a heterodimerization interface (reviewed in reference 17). Several proteins, including the death domain-containing protein FADD/MORT-1 (4, 12), a putative kinase, RIP (82), the tyrosine phosphatase FAP-1/PTP-BAS (76), and a protein with no known homologs, FAF-1 (14), interact with the Fas intracellular domain in two-hybrid assays. In addition, the cysteine protease Mch5/FLICE/MACH has recently been shown to interact with the Fas receptor complex through FADD (3, 64) (see below). It has also been reported that ceramide levels increase within minutes after Fas receptor ligation and that ceramide may behave as a second messenger in Fas-induced apoptosis (16, 38, 39, 85).

Fas-induced apoptosis requires the action of interleukin-1β (IL-1β)-converting enzyme (ICE) or ICE/CED-3 family proteases, as demonstrated by studies utilizing tri- and tetrapeptide inhibitors of ICE or the cowpox viral protein CrmA, which is a natural ICE inhibitor (13, 27, 28, 59, 72, 86, 88). ICE was originally identified as a cysteine protease capable of cleaving pro-IL-1β into mature, biologically active IL-1β and was later identified as a homolog of the nematode *Caenorhabditis elegans* cell death gene, *ced-3* (7, 88, 99; reviewed in reference 98). The ICE/CED-3 protease superfamily encompasses a highly homologous group including mammalian ICE (7, 88), TX/ICH2/ICE rel-II (29, 49, 63), ICE-rel-III (63), Nedd-2/ICH1 (53, 91), CPP32/YAMA/apopain (33, 66, 87), Mch2 (31), ICE-LAP3/Mch-3/CMH-1 (24, 32, 58), ICE-LAP6 (25), Mch4 (30), and Mch5/FLICE/MACH (3, 30, 64). This protease superfamily shares a conserved active site pentapeptide QACXG (where X = R in all family members except ICE-LAP6, where X = G, and Mch4 and Mch5, where X = Q) and an absolute dependence on the central cysteine residue for catalytic activity (reviewed in references 40, 43, and 94). The recent identification of the protease Mch5/FLICE/MACH and its ability to

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interact with the death effector domain of the Fas receptor-associated protein FADD suggests a mechanism by which Fas receptor cross-linking might activate an ICE/CED-3 family protease cascade (3, 64). In *C. elegans*, genetic studies with the ICE-like *ced-3* locus have strongly implicated the cysteine proteases as downstream and requisite effectors of apoptosis (98). Similarly, in higher eukaryotes, ICE/CED-3 family proteases have been implicated as apoptotic effectors through the ability of CrmA to inhibit apoptosis induced by NGF withdrawal, Fas, TNF, ionizing irradiation, and chemotherapeutic agents (27, 36, 44, 59, 62, 86). More specifically, *crmA* is capable of directly inhibiting the proteases ICE (28, 62, 72), CPP32 (87), and granzyme B (68). In addition, specific tetrapeptide inhibitors of ICE and CPP32 and pan-ICE tripeptide inhibitors have been used to show the involvement of ICE/CED-3 family members in many forms of apoptosis (reviewed in references 40 and 94). Specifically, the irreversible tetrapeptide ICE protease inhibitor YVAD-CMK and the tripeptide pan-ICE inhibitor Z-VAD-FMK have been shown to block Fas-mediated apoptosis (13, 27, 28).

Recently, stress-activated mitogen-activated protein kinases (MAPKs) such as p38 and JNK (also known as stress-activated protein kinase) (20, 41, 54, 56) which are not responsive to classic mitogenic stimuli but are instead activated preferentially by a variety of extracellular stresses have been identified (reviewed in references 6, 19, and 92). Both p38 and JNK are activated by UV irradiation, osmotic shock, and TNF (20, 37, 41, 69, 79). JNK is also activated by $\gamma$ irradiation and cytosine arabinoside (11, 51). Notably, p38 and JNK are themselves phosphorylated and activated by upstream kinases. The p38 MAPK can be phosphorylated and activated by MKK3, MKK4, and MKK6, although the physiological relevance of each upstream regulator has not been clearly defined (21, 42, 57, 70). Once activated, the p38 MAPK can phosphorylate the transcription factors ATF2 and Elk-1 (69, 70) and is also capable of activating MAPKAP kinase 2, which can in turn phosphorylate hsp25 (35, 74). Similarly, JNK has been placed downstream of the MEKK1-MKK4 (also known as JNKK or SEK) pathway (21, 57, 61, 75, 96). Interestingly, many of the signals activating the stress-activated MAPKs induce apoptosis in parallel; JNK and, to a lesser extent, p38 have been implicated as potential mediators of the apoptotic program in response to NGF withdrawal and ceramide (90, 95).

In this study, we demonstrate the selective coupling of the Fas receptor to the stress-activated MAPKs, p38 and JNK, but not to mitogen-activated kinases such as ERK1 and p70 S6 kinase. The 2-h onset of p38 and JNK activation correlates well with the onset of apoptosis; moreover, transfection of cells with a constitutively active upstream regulator of p38, MKK3(glu), enhanced Fas-mediated death, suggesting a potential role for the MKK3/p38 activation pathway in transducing the Fas apoptotic signal. Fas stimulation of p38 activity was inhibited by CrmA, YVAD-CMK, and Z-VAD-FMK, demonstrating that Fas induction of p38 requires the action of ICE/CED-3 family proteases and, conversely, that these proteases can regulate stress kinase activity. Finally, we provide evidence for alternative, YVAD-CMK- and Z-VAD-FMK-resistant modes of p38 regulation, using sorbitol and the chemotherapeutic agent etoposide.

## MATERIALS AND METHODS

**Materials.** YVAD-CMK (Ac-Tyr-Val-Ala-Asp-chloromethylketone) was purchased from Bachem Biosciences Inc. (Philadelphia, Pa.). Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) was purchased from Enzyme Systems Products, Inc. (Dublin, Calif.). Anti-human Fas monoclonal antibody (Ab; CH11, murine) was purchased from Kamiya Biomedical Co. (Thousand Oaks, Calif.) and used at 100 ng/ml unless otherwise indicated. Anti-FLAG monoclonal Ab (M2, murine) was purchased from Eastman Kodak (New Haven, Conn.). The pRSV-MKK3(glu) expression vector and the parental vector pRSV have been previously described (70). pBabePuro-*crmA* and murine CD8 expression vectors were kindly provided by Junying Yuan and R. Bram, respectively. Antibodies to MAPKAP kinase 2 and hsp25 substrate were kindly provided by Matthias Gaestel. D-Sorbitol, etoposide (used at 10 μg/ml), and trypan blue dye were purchased from Sigma (St. Louis, Mo.). [$\gamma$-$^{32}$P]ATP was obtained from New England Nuclear (Boston, Mass.). SB 203580 and SB 106978 were kindly provided by John Lee (SmithKline Beecham).

**Cell culture.** Jurkat cells were generously provided by G. Crabtree. Jurkat clone A3 was isolated by limiting dilution after replica plating and several rounds of selection in Fas Ab in order to obtain a cell line that had a low spontaneous rate of resistance to Fas-mediated apoptosis [$1/(2 \times 10^7)$ cells]. Jurkat cells were cultured in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (HyClone). Cells were maintained in 5% $CO_2$ at 37°C. Twenty-four hours before an experiment, cells were seeded at $8 \times 10^5$/ml in complete medium.

**Protein kinase assays.** Jurkat cells ($2 \times 10^6$/ml were treated with Fas Ab (100 ng/ml) for 2 h. Cells ($3 \times 10^6$) were pelleted by pulse microcentrifugation, washed with cold 1× phosphate-buffered saline (PBS), and lysed in lysis buffer as previously described (8). Lysates were clarified by centrifugation at 13,000 × $g$ for 10 min at 4°C. Immune-complex kinase assays were performed as previously described (8), using rabbit polyclonal antisera to p38 (69) or monoclonal anti-FLAG Ab (Eastman Kodak) with glutathione *S*-transferase (GST)–ATF2 as the substrate (69), rabbit polyclonal antisera to JNK1 (69) and GST-Jun(1-79) as the substrate (20), rabbit polyclonal antisera to pp70$^{S6k}$ (15) or p90$^{rsk}$ (9) with GST-S6 as the substrate, rabbit polyclonal antisera to ERK1 (10) and myelin basic protein (MBP) (Sigma) as the substrate, and rabbit polyclonal antisera to MAPKAP kinase 2 and hsp25 as the substrate (74). Bacterial expression and purification of GST fusion proteins have been previously described (81). Kinase assays for p38 were incubated in a 30-μl reaction volume at 30°C for 15 min, stopped with 30 μl of 2× sample buffer as previously described (8), analyzed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, dried, and autoradiographed. $^{32}$P incorporation into substrate was quantitated with a Molecular Dynamics PhosphorImager using ImageQuant software.

**Immunoblot analyses.** Immunoblot analyses were performed as previously described (15). Twenty to 30 μg of total protein was analyzed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were incubated with either anti-FLAG Ab (1:1,000), anti-ERK1 (1:5,000), or anti-pp70$^{S6k}$ (1:2,500) and visualized with secondary Ab coupled to horseradish peroxidase (Boehringer Mannheim), using enhanced chemiluminescence (Amersham, Little Chalfont, England).

**Apoptosis assays.** Chromosomal DNA was isolated to assess the extent of oligonucleosomal cleavage, using a modification of a previously described protocol (46). Briefly, $10^6$ cells were treated with various apoptotic agents, pelleted in a microcentrifuge, washed once in cold 1× PBS, and then lysed in 300 μl of TENS-proteinase K (10 mM Tris-HCl [pH 7.4], 1 mM EDTA [pH 7.4], 150 mM NaCl, 1% SDS, 0.2 mg of proteinase K per ml). Digestion was allowed to proceed overnight at 37°C. Lysate was then extracted twice with phenol-chloroform and once with chloroform. DNA was precipitated, dried in a SpeedVac, and resuspended in 30 μl of TE-RNase A (10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8], 0.5 mg of RNase A per ml). Then 15 μl was analyzed on a 1% Tris-borate-EDTA agarose gel electrophoresed at 20 to 30 V.

For trypan blue dye exclusion assays, an aliquot of cells at $2 \times 10^6$/ml was removed and mixed with 0.1% dye at a 1:1 ratio. Live cells, which exclude trypan blue dye, were quantitated with a hemocytometer.

**Stable and transient transfection of cells.** Jurkat cells ($10^7$) were electroporated with 20 to 25 μg of DNA (250 V, 950 μF) in a Bio-Rad Gene Pulser II. Cells were allowed to recover for 48 h before treatment. Cells stably expressing *crmA* were generated by electroporating Jurkat A3 cells with *Sca*I (New England Biolabs)-linearized pBabePuro-*crmA*. Cells were grown in the presence of puromycin (0.25 μg/ml; Sigma) to select for stable integrants. Clonal cell lines were isolated and screened for expression of *crmA* by resistance to Fas-mediated apoptosis and reverse transcription-PCR. Three *crmA*-expressing clones, named A4, B4, and C9, were used in these studies.

**FACS analysis.** Jurkat cells were transfected by electroporation as described above. Cells were transfected with a 1:2 or 1:3 ratio of pActLyt-CD8 to target DNA to increase the probability that CD8-expressing cells were also cotransfected with experimental DNA; total DNA amounted to 20 to 25 μg. After 24 h, cells were loaded onto a Ficoll-Paque (Pharmacia) gradient to remove cells that died during the transfection procedure. Cells were pelleted, resuspended in 5 ml of 1× PBS, layered on top of 3 ml of Ficoll-Paque (Pharmacia), and spun at 2,000 rpm for 30 min. Live cells were collected at the interface and transferred into media. Approximately 40 to 48 h posttransfection, cells were treated with or without Fas Ab (100 ng/ml) for various times, pelleted at 2,400 rpm for 2 min, and washed once in fluorescence-activated cell sorting (FACS) wash buffer (PBS, 0.5% bovine serum albumin fraction V [Sigma]). Cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated murine anti-Lyt-2 (CD8) Ab (Collaborative Biomedical Products, Bedford, Mass.) at a concentration of 5 μg/ml for 30 min on ice; monoclonal anti-immunoglobulin G (IgG)-FITC antibodies (Pharmingen) were used as controls at 10 μg/ml. Cells were washed twice with

FACS wash buffer, resuspended in 0.5 ml of FACS wash buffer, and stored on ice in the dark until FACS analysis. Five minutes prior to flow cytometric analysis, propidium iodide (PI; Sigma) was added to each sample at 40 μg/ml. Cells were analyzed on a FACS Vantage flow cytometer (Becton Dickinson, San Jose, Calif.). A minimum of 2,000 to 5,000 CD8-FITC-positive cells were collected per file and analyzed with Cell Quest version 1.2 software (Becton Dickinson).

## RESULTS

**Fas selectively activates the p38 and JNK MAPKs.** Little is known about the downstream signalling events linking the Fas receptor to the execution of programmed cell death. We sought to determine whether one or more signalling systems, represented by the MAPKs p38, JNK or ERK1, or pp70$^{S6k}$, might play a role in transmitting death signals from the cell surface into the interior. The Jurkat T-cell leukemic cell line can be induced to undergo apoptosis by cross-linking the Fas receptor with either Fas ligand or specific Fas Ab, such as the anti-Fas IgM antibody CH11 (97). After several rounds of selection in Fas Ab, we isolated a clonal Jurkat cell line (A3) which was exquisitely sensitive to Fas-mediated apoptosis, with a spontaneous rate of resistance to Fas-induced death of only $1/(2 \times 10^7)$ cells (see Materials and Methods). Treatment of this Jurkat cell line with Fas Ab resulted in increased p38 and JNK kinase activities after 2 h (Fig. 1A); equivalent induction was also observed in the nonclonal parental cells (data not shown). Concentrations of Fas Ab higher than 100 ng/ml did not increase the level of p38 activation or the amount of cell death, indicating antibody excess (data not shown). Fas receptor ligation did not activate molecules in the mitogen-responsive MAPK pathway, such as ERK1 or pp90$^{rsk}$, nor was the mitogen-sensitive pp70$^{S6k}$ activated, indicating an unanticipated degree of specificity (Fig. 1B and data not shown). It should be noted that Fas-mediated activation of p38 was consistently 5- to 8-fold, whereas osmotic shock (0.4 M sorbitol) activation of p38 was typically 20- to 25-fold (data not shown).

To determine the temporal relationship between the induction of p38 kinase activity and cell death, trypan blue dye exclusion and DNA ladder formation were used to monitor cell death after treatment of Jurkat cells with Fas Ab. In most systems, cells undergoing apoptosis exhibit DNA cleavage into oligonucleosomal fragments, which appear as a DNA ladder on agarose gel electrophoresis. Treatment of Jurkat cells with Fas Ab resulted in cell death within 2 to 4 h (Fig. 2A), in parallel with DNA ladder formation (Fig. 2B) and p38 activation (Fig. 1), indicating a temporal correlation between p38 kinase activity and the onset of apoptosis. The decline of p38 kinase activity after 6 to 8 h of Fas Ab treatment probably reflects decreased cell yield from progressive apoptosis (Fig. 2).

Since Fas activation of p38 does not occur until about 2 h after treatment with Fas Ab, we investigated the possibility that transcription is required. The transcriptional inhibitor actinomycin D did not block Fas-mediated activation of p38 at 2 h (Fig. 3); rather, a slight enhancement was observed. Attempts to use anisomycin to block translation were confounded by the fact that anisomycin is itself a strong inducer of p38 kinase activity (data not shown).

**Constitutively active MKK3 enhances Fas-mediated death.** Previous studies have demonstrated that the MKK3 kinase behaves as a potent upstream activating kinase for p38 and that replacement of MKK3 Ser-180 and Thr-193 with glutamic acid to mimic phosphorylation leads both to constitutive activation of this kinase, MKK3(glu), and to subsequent p38 activation in cotransfection experiments (70). To assess the possible contribution of the MKK3/p38 pathway to Fas-mediated apoptosis, this activated MKK3(glu) allele was used in transfection studies. MKK3(glu) tagged with a FLAG epitope was then cotransfected into Jurkat cells along with FLAG-tagged p38, and cytoplasmic extracts were subjected to anti-FLAG immunoprecipitation. These anti-FLAG immunoprecipitates were then used in immune complex kinase assays for p38, utilizing GST-ATF2 (70) as the substrate; MKK3 does not phosphorylate ATF2 (70). Transfected MKK3(glu) strongly activated p38 in a Fas-independent manner, confirming that transfected MKK3(glu) was constitutively active (Fig. 4A; compare lanes 5 and 6). Under these conditions, the cotransfection of MKK3 and p38 produced an elevation of basal p38 kinase activity relative to the transfection of p38 alone (Fig. 4A; compare lanes 3 and 5). GST-ATF2 was not limiting under these conditions (data not shown). Uniform expression of transfected MKK3 and p38 was confirmed by Western blotting with anti-FLAG Ab (data not shown).

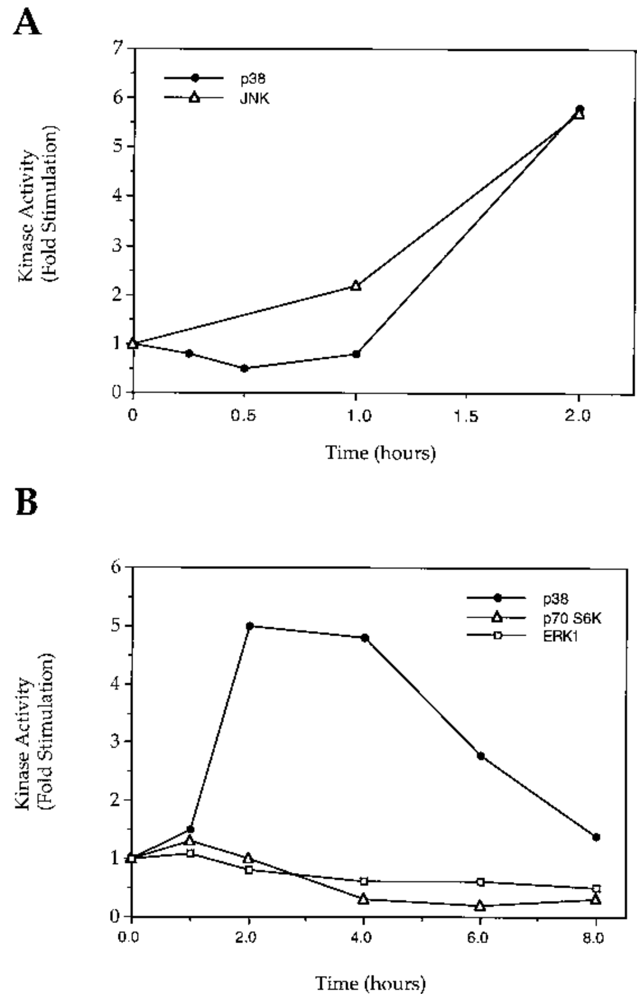
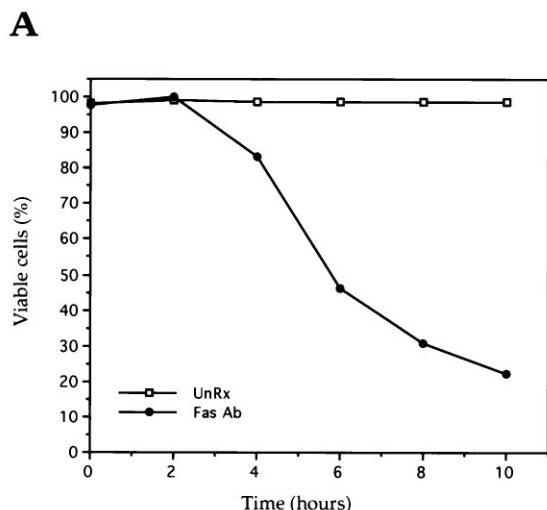



FIG. 1. Fas receptor cross-linking activates p38 and JNK. Jurkat cells ($2 \times 10^6$/ml) were treated with Fas Ab (CH11) at 100 ng/ml for various times. Cells were lysed, and p38 (A and B) and JNK (A) kinase activities were measured in an immune-complex kinase assay using GST-ATF2 and GST-Jun, respectively, as substrates. Results are representative of three experiments. (B) ERK1 and pp70$^{S6k}$ kinase activities were measured in an immune-complex kinase assay using MBP and GST-S6, respectively, as substrates. Equal numbers of cells were treated with Fas Ab for each time point. Kinase activities were not normalized for loss of cells due to apoptosis. Data are presented as fold stimulation over activity in untreated controls. Similar results were obtained in two independent experiments.

Transfection of this constitutively active MKK3(glu) into



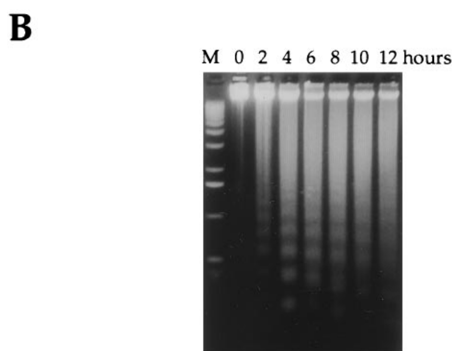




FIG. 2. The onset of Fas-induced apoptosis correlates temporally with p38 and JNK activity. (A) Jurkat cells ($2 \times 10^6$/ml) were treated with Fas Ab (100 ng/ml) or left untreated (UnRx). At various times, an aliquot was removed and stained with trypan blue dye (0.1%) to distinguish dead cells. Cells were quantitated with a hemocytometer; 100 to 200 cells were counted per time point. Data represent the average of two experiments. (B) Aliquots of Jurkat cells ($10^6$) were lysed at various times after treatment with Fas Ab and analyzed for DNA ladder formation on a 1% agarose gel. Results are representative of three independent experiments.

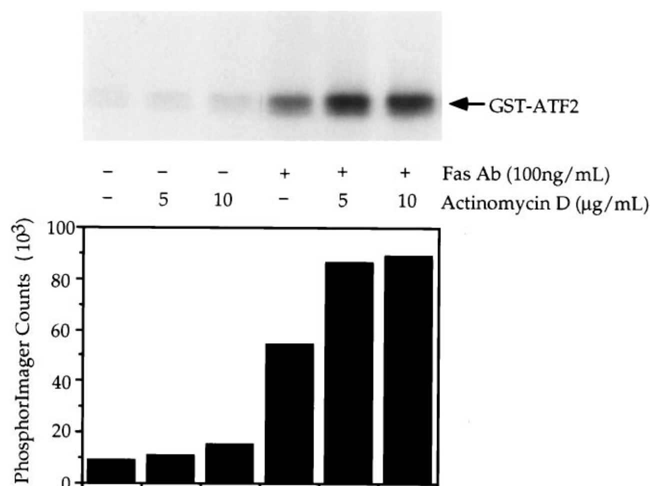



FIG. 3. Fas activation of p38 does not require transcription. Jurkat cells ($2 \times 10^6$/ml) were left untreated or treated with Fas Ab for 2 h either alone or in combination with 5 or 10 μg of actinomycin D per ml as indicated. p38 kinase activity was measured in an immune-complex kinase assay using GST-ATF2 as the substrate. Cells were treated with actinomycin D for 30 min prior to Fas Ab treatment. The lower panel shows the quantitation of $^{32}P$ incorporation as determined by a PhosphorImager. Results are representative of three independent experiments.

Jurkat cells resulted in an increase in basal cell death and an enhancement of Fas-induced death (Fig. 4B). Jurkat cells bear a T-helper $CD4^+$ phenotype and do not express surface CD8 (data not shown). This fact was exploited to cotransfect a murine CD8 expression plasmid with either MKK3(glu) or pRSV to provide a convenient surface marker for transfected cells. To help ensure that every cell expressing surface CD8 would also contain the cotransfected MKK3(glu) or pRSV control plasmid, CD8 was transfected at a 1:3 molar ratio with MKK3(glu) or pRSV. The transfected cells were then treated with Fas Ab or mock treated, followed by dual-color FACS analysis with simultaneous staining for (i) CD8 expression with FITC-conjugated anti-CD8 Ab and (ii) cell death status with PI (excluded by live cells but absorbed by dead cells) (52). This method routinely detected 2,000 to 5,000 transfected cells (CD8-FITC positive) and enabled simultaneous determination of whether the CD8-FITC-positive cells presumably cotransfected with MKK3(glu) or vector control were alive (PI negative) or dead (PI positive). Transfection of MKK3(glu) only slightly increased basal cell death (6 to 12%); however, MKK3(glu) enhanced the amount of Fas-induced death from 35 to 70% of the value for transfected cells after 6 h of treatment compared with the pRSV control (Fig. 4B).

To address the potential involvement of p38 in Fas-mediated apoptosis, a series of experiments was carried out with the specific p38 inhibitor SB 203580 (18, 56). This inhibitor has been shown to specifically inhibit p38 kinase activity in vitro, with no effect on a variety of kinases tested, including JNK, MAPKAP kinase 2, and ERK-1 (18). Jurkat cells were treated with Fas Ab for 2 h to activate p38 in the presence or absence of the p38 inhibitor SB 203580 or an inactive analog, SB 106978 (Fig. 5A). Since SB 203580 is a reversible inhibitor which would be washed out in an in vitro p38 immune-complex kinase assay, a downstream target kinase for p38, MAPKAP kinase 2, was assayed for immune-complex kinase activity as a reflection of in vivo p38 kinase activity (74), using hsp25 as the substrate. Fas stimulation for 2 h resulted in the activation of MAPKAP kinase 2 (Fig. 5A), an activation which was completely inhibited by 10 μM p38 inhibitor SB 203580 but unaffected by the inactive analog, SB 106978 (Fig. 5A). In parallel, aliquots of cells from this experiment were analyzed for cell death (Fig. 5B). Although SB 203580 completely inhibited p38 kinase activity in cells as measured by MAPKAP kinase 2 activation, the drug did not inhibit Fas-induced death (Fig. 5B). These results indicate that Fas activation of p38 and MAPKAP kinase 2 alone is not required for Fas-induced death and that the observed stimulation of Fas-induced death by MKK3(glu) may involve other, unidentified downstream targets. Alternatively, Fas activation of p38 and MAPKAP kinase 2 may function in a redundant cell death pathway.

**Fas induction of p38 kinase activity requires ICE/CED-3 family proteases.** Fas-mediated apoptosis requires ICE or ICE/CED-3 family proteases, based on the ability of the cowpox viral protein CrmA, which inhibits ICE proteases, and of specific tetrapeptide ICE inhibitors to inhibit Fas-induced death (27, 59, 86). Since ICE and p38 are both activated by Fas receptor cross-linking, we investigated a possible regulatory relationship between p38 and ICE/CED-3 family members during Fas signalling. Jurkat cells were cotransfected with *crmA* and FLAG-epitope tagged p38; these cells were subsequently treated with Fas Ab for 2 h to activate p38, and cyto-

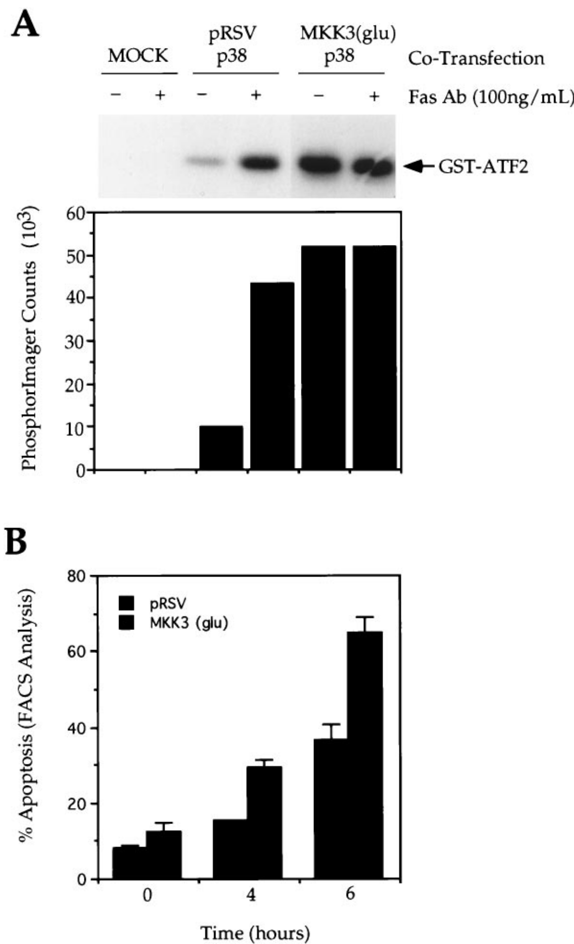



FIG. 4. Constitutively active MKK3(glu) enhances Fas-induced apoptosis. (A) Jurkat cells were either mock transfected or cotransfected with empty pRSV vector and epitope-tagged pCMV-p38 or with pRSV-MKK3(glu) and epitope-tagged pCMV-p38 as indicated. Forty-eight hours later, equal numbers of cells were either left untreated or treated with Fas Ab for 2 h. Transfected p38 activity was measured in an immune-complex kinase assay using anti-FLAG Ab and GST-ATF2 as the substrate. PhosphorImager counts are shown beneath the gel. Results are representative of seven experiments. (B) Jurkat cells were cotransfected with the surface marker pACTLyt-CD8 and either empty pRSV vector or pRSV-MKK3(glu) at a ratio of 1:3. Cells were left untreated or treated with Fas Ab for 4 or 6 h. Transfected cells were distinguished by FACS analysis measuring surface expression of CD8 (using anti-CD8-FITC Ab), and dead cells were distinguished by PI uptake. At least 2,000 CD8-positive cells were analyzed per time point. Data are presented as percent apoptotic cells (mean ± standard deviation [$n = 3$]). Similar results were observed in six experiments.

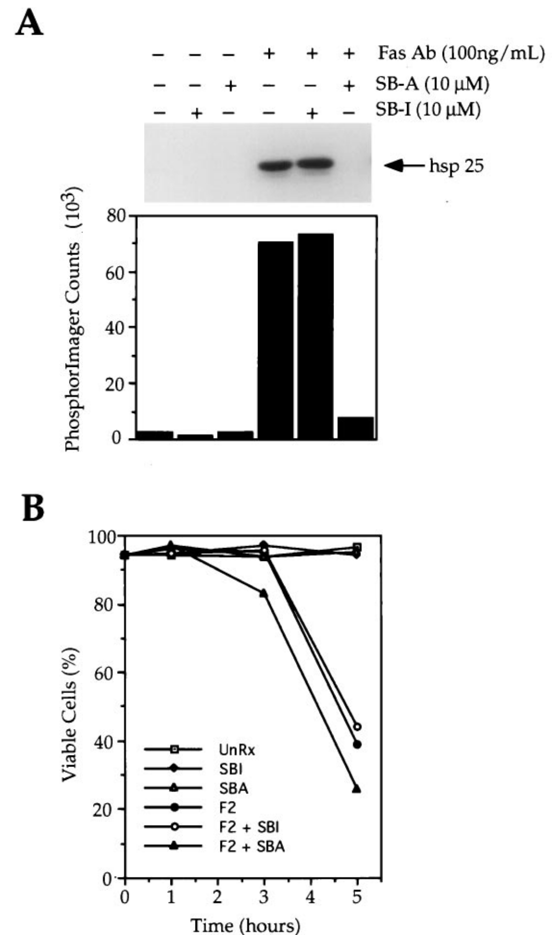



FIG. 5. Fas activation of p38 is not required for cell death. (A) Jurkat cells were left untreated (lane 1), treated with Fas Ab alone for 2 h (lane 4), treated with inactive SB 106978 (SB-I) or active SB 203580 (SB-A) either alone (lanes 2 and 3) or in combination with Fas Ab for 2 h (lanes 5 and 6). MAPKAP kinase 2 activity was measured against purified hsp25. Cells were preincubated with the SB drugs for 30 min prior to Fas Ab treatment. (B) Aliquots of the cells from the experiment in panel A were removed prior to cell lysis and monitored for cell death, using trypan blue dye exclusion. UnRx, untreated; F2, Fas Ab treatment for 2 h. Results in panel B are representative of six independent experiments, three of which were carried out by using FACS analysis of 10,000 cells per data point, using PI uptake as an indicator of cell death.

plasmic extracts were prepared. A p38 immune-complex kinase assay was then performed with an anti-FLAG antibody and GST-ATF2 as the substrate. Cotransfection of *crmA* and p38 inhibited Fas-mediated activation of p38 greater than 50% compared to transfection of p38 alone, and the extent of this antagonism increased with higher ratios of transfected *crmA* to p38 DNA (Fig. 6A). Western blot analysis with an anti-FLAG antibody confirmed equivalent levels of expression of transfected p38 (data not shown).

To confirm that transfected *crmA* functioned to block cell death, *crmA* and CD8 were cotransfected into Jurkat cells, and FACS analysis was used to identify 2,000 to 5,000 transfected cells (CD8-FITC positive) and to determine the relative amounts of dead (PI-positive) and live (PI-negative) cells within the transfected CD8-positive population. Under these conditions, *crmA* inhibited Fas-induced apoptosis greater than 50% at both 6 and 8 h (Fig. 6B). In agreement with these transient transfection studies, three clonal Jurkat cell lines stably expressing *crmA* displayed reduced activation of p38 and apoptosis in response to Fas compared to vector controls or the parental Jurkat cell line (data not shown). Taken together, these results indicate that biologically active *crmA* capable of blocking Fas-stimulated cell death also strongly blocks p38 activation and that a CrmA-inhibitable protease, probably a member of the ICE/CED-3 family, lies upstream of p38 in the Fas signalling pathway.

To further confirm the role of ICE/CED-3 family proteases as upstream regulators of p38 during Fas signalling, experiments were performed with the irreversible tetrapeptide ICE protease inhibitor YVAD-CMK or the irreversible tripeptide pan-ICE inhibitor Z-VAD-FMK, which both inhibit Fas-mediated apoptosis (13, 23, 27, 28). Jurkat cells were treated with a range of concentrations of YVAD-CMK either alone or together with Fas Ab for 2 h to activate p38, and an immune-

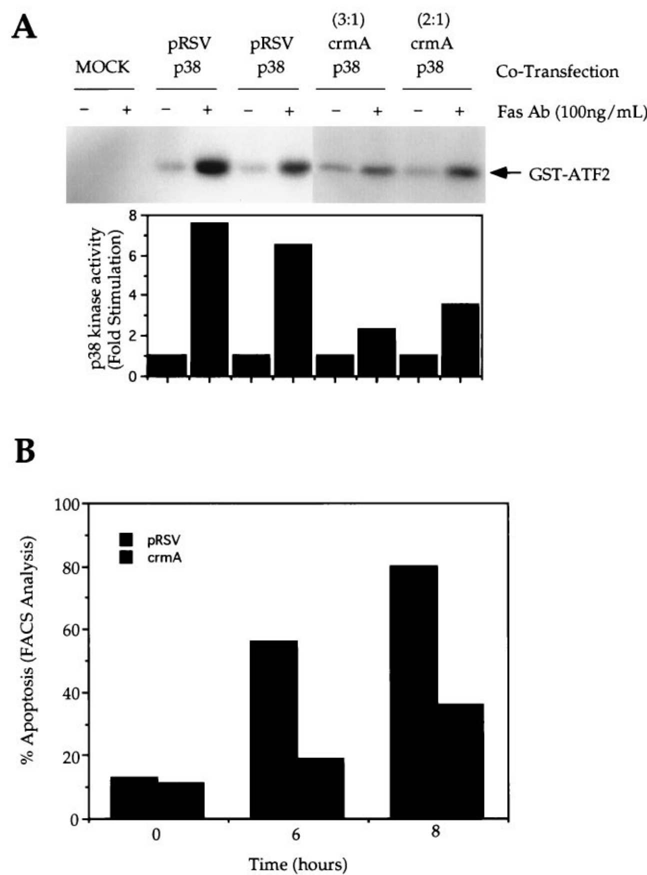



FIG. 6. Fas activation of p38 is antagonized by *crmA*. (A) Jurkat cells were either mock transfected, cotransfected with empty pRSV vector and epitope-tagged pCMV-p38, or cotransfected with *crmA* and epitope-tagged pCMV-p38 as indicated. The ratio of *crmA* to p38 DNA is indicated in parentheses. Forty-eight hours later, equal numbers of cells were left untreated or treated with Fas Ab for 2 h. Transfected p38 activity was measured as in Fig. 4. PhosphorImager counts are shown beneath the gel. Results are representative of five experiments. (B) Jurkat cells were cotransfected with surface marker pACTLyt-CD8 and either empty pRSV vector or *crmA*. Cells were left untreated or treated with Fas Ab (100 ng/ml) for 6 or 8 h. FACS analysis was used to quantitate the amount of cell death in the transfected population as in Fig. 4. At least 2,000 CD8-positive cells were analyzed per time point. Data are presented as percent apoptotic cells. Results are representative of two independent experiments.

complex kinase assay was performed with an anti-p38 antibody and GST-ATF2 as the substrate. Treatment of Jurkat cells with Fas Ab for 2 h activated p38 (Fig. 7A), consistent with earlier results (Fig. 1). Notably, Fas Ab treatment in combination with increasing doses of YVAD-CMK inhibited Fas-mediated p38 activation in a dose-dependent fashion, with 70% inhibition at 10 μM drug and complete inhibition at 120 μM drug (Fig. 7A). YVAD-CMK produced dose-dependent inhibition of Fas-induced death in parallel, with 10 μM drug inhibiting cell death by approximately 50% at 9 h and 120 μM drug completely (>95%) inhibiting cell death (Fig. 7B). As controls, untreated cells, vehicle-treated cells, or cells treated with various concentrations of YVAD-CMK (0.1 to 120 μM) alone did not cause apoptosis or activate p38 (Fig. 7). Consequently, increasing doses of YVAD-CMK inhibited both p38 activation and cell death with similar concentration dependence. The pan-ICE inhibitor Z-VAD-FMK completely inhibited Fas activation of p38 (Fig. 7C) and Fas-induced death (Fig. 7D), further supporting a role for ICE-CED-3 family proteases upstream of p38. YVAD-CMK (120 μM) or Z-VAD-FMK (200 μM) did not inhibit osmotic shock (sorbitol)-induced activation of p38 (Fig. 7A, right panel, and data not shown), suggesting that ICE/CED-3 family members play a specific role in Fas-mediated p38 activation. In addition, YVAD-CMK (120 μM) did not inhibit phorbol ester-induced activation of pp90$^{rsk}$, ERK1, or ERK2, further indicating the specificity of this inhibitor (data not shown).

**Etoposide activation of p38 does not require ICE/CED-3 family proteases.** We subsequently explored whether non-receptor-mediated apoptotic signals would activate p38 and whether this activation would require ICE/CED-3 family proteases. Etoposide is a DNA-damaging agent that interacts covalently with topoisomerase II and DNA, inducing DNA strand breaks and apoptosis in many systems (50, 78). Jurkat cells were treated with etoposide for various times, and the effects on p38 and JNK kinase activities were examined. p38 and JNK were activated in a biphasic pattern with maximal activation at 4 to 6 h after etoposide treatment, whereas ERK1 and pp70$^{S6k}$ were not affected (Fig. 8A). Cell death, as monitored by trypan blue dye exclusion and DNA ladder formation, commenced at 4 to 6 h after etoposide treatment (Fig. 8B and C). Hence, like Fas, etoposide preferentially activates the p38 and JNK kinases with time kinetics paralleling the onset of apoptosis.

The potential sensitivity of etoposide activation of p38 to the ICE inhibitor YVAD-CMK was also evaluated. Jurkat cells were treated with etoposide for 4 h, either alone or in combination with increasing concentrations of YVAD-CMK (1 to 120 μM), and endogenous p38 kinase activity was assayed. Surprisingly, YVAD-CMK did not inhibit etoposide activation of p38 even at a high concentration (120 μM) which completely inhibited Fas-induced p38 activation and cell death (Fig. 9A, 7C, and 7D). As 120 μM YVAD only partially inhibited cell death from etoposide (Fig. 9B), as opposed to its total inhibition of Fas-induced death (Fig. 7A), it was formally possible that the lack of YVAD sensitivity of etoposide activation of p38 resulted from incomplete YVAD blockade of the potentially numerous proteases involved in etoposide-mediated apoptosis. We therefore used the pan-ICE inhibitor Z-VAD-FMK to attempt a more encompassing blockade of cysteine proteases during etoposide treatment. Jurkat cells were exposed to etoposide for 4 h in the presence or absence of Z-VAD-FMK (100 to 200 μM) (Fig. 9C), and p38 kinase activity was assayed as described above. Consistent with a broader spectrum of protease inhibition with ZVAD, 100 μM Z-VAD-FMK completely inhibited etoposide-induced cell death, as judged by trypan blue dye exclusion (Fig. 9D) and FACS analysis using PI (data not shown). However, despite complete blockade of etoposide-induced death, 100 μM Z-VAD-FMK did not inhibit etoposide-induced p38 activity (Fig. 9C), consistent with results for YVAD (Fig. 9A). Thus, p38 activation correlates with the onset of etoposide-induced apoptosis, but in contrast to Fas-mediated activation of p38, etoposide induction of p38 is not mediated by ICE or ICE/CED-3 family proteases sensitive to YVAD-CMK or Z-VAD-FMK.

## DISCUSSION

We have shown that cross-linking of the Fas receptor by Fas Ab results in the preferential stimulation of the stress-activated kinases p38 and JNK without activation of mitogen-activated kinases such as ERK1, pp70$^{S6k}$, or pp90$^{rsk}$ (Fig. 1 and data not shown). This activation occurred after 2 h, concomitant with or slightly prior to the onset of Fas-induced apoptosis (Fig. 2), and did not require de novo transcription (Fig. 3), in agreement with previous results revealing that Fas-induced death

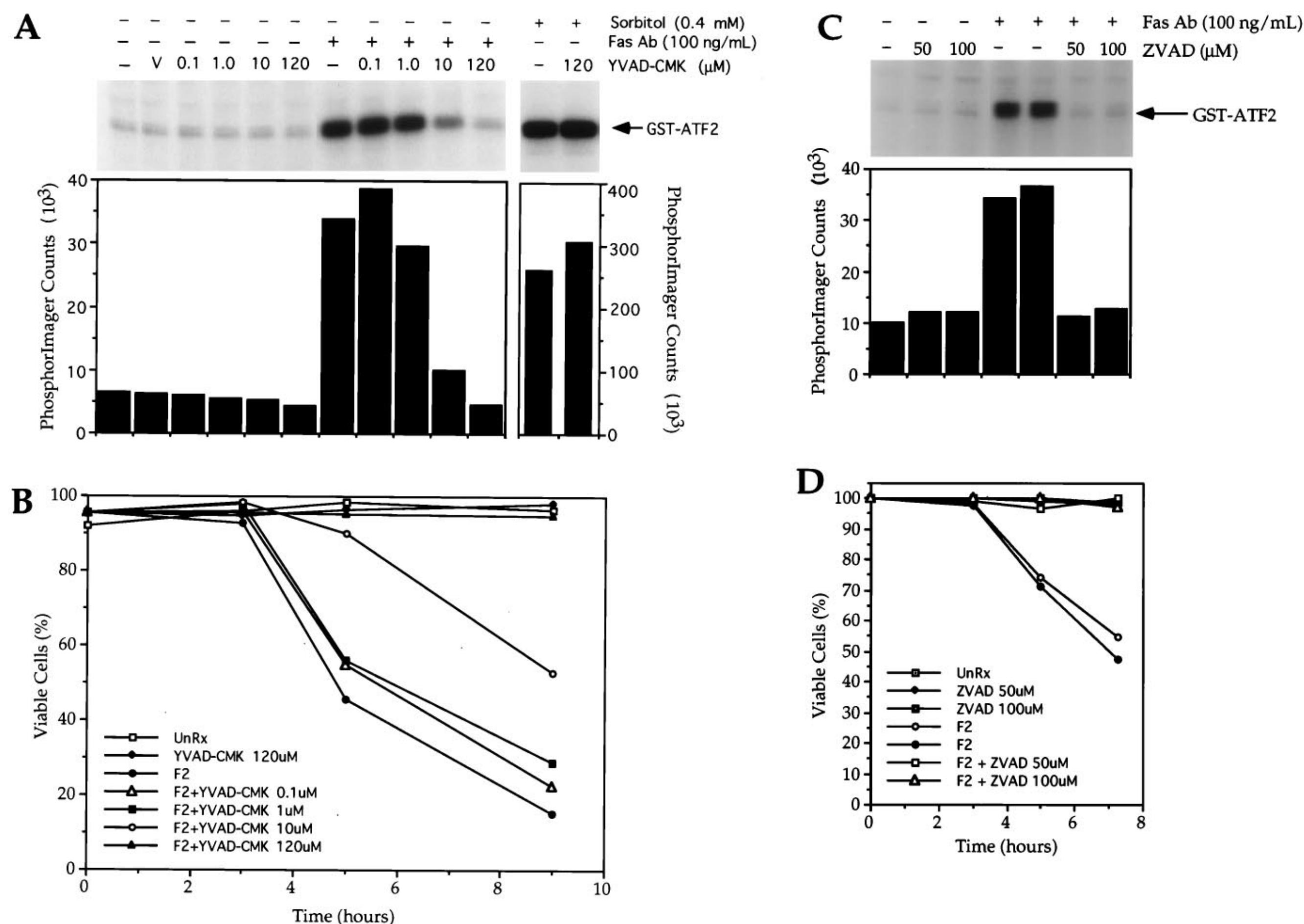



FIG. 7. Fas activation of p38 is mediated by ICE/CED-3 family proteases. (A) Jurkat cells were left untreated (lane 1), treated with vehicle alone (lane 2), treated with Fas Ab alone (lane 7), or treated with various concentrations of YVAD-CMK either alone (lanes 3 to 6) or in combination with Fas Ab (lanes 8 to 11) for 2 h. Cells were treated with sorbitol (0.4 M) either alone (lane 12) or in combination with YVAD-CMK (lane 13) for 15 min. Cells were preincubated with YVAD-CMK for 1 h prior to Fas Ab or sorbitol treatment. p38 kinase activity was measured in an immune-complex kinase assay using GST-ATF2 as the substrate. (B) Aliquots of the cells from the experiment in panel A were removed prior to cell lysis and monitored for cell death by using trypan blue dye exclusion. F2, Fas Ab treatment for 2 h. Cells left untreated (UnRx) or treated with vehicle alone or YVAD-CMK alone (0.1 to 10 μM) were completely viable throughout the time course. Results are representative of three experiments. Similar results were observed with FACS analysis. (C) Cells were either left untreated (lane 1), treated with Fas Ab alone for 2 h (lanes 4 and 5), or treated with various concentrations of Z-VAD-FMK either alone (lanes 2 and 3) or in combination with Fas Ab for 2 h (lanes 6 and 7). p38 kinase activity was measured as described above. (D) Aliquots of the cells from the experiment in panel C were removed prior to cell lysis and monitored for cell death, using trypan blue dye exclusion. UnRx, untreated; F2, Fas Ab treatment for 2 h. Results are representative of two independent experiments. Similar results were observed with FACS analysis.

does not require transcription or translation (47, 97). Compared with the rapid (<5 min) activation kinetics of the well-described mitogen-activated MAPK cascade, the delayed (2 h) time kinetics of p38 and JNK activation are consistent with a membrane-distal effector function as opposed to a role in membrane-proximal signal transduction.

Recent studies using *ced-3* mutants in *C. elegans*, and the CrmA and tri- or tetrapeptide ICE inhibitors in higher eukaryotes, have elegantly defined the central importance of the ICE/CED-3 superfamily of cysteine proteases to the apoptotic program (28, 55, 59, 62, 86, 88, 98). In the present study, Fas activation of p38 required the action of ICE/CED-3 family proteases, based on the ability of both stably and transiently transfected *crmA* to partially antagonize (>50%) Fas-induced p38 activity (Fig. 6A and data not shown). Even more strikingly, cell treatment with YVAD-CMK, an irreversible tetrapeptide inhibitor of ICE (88), or Z-VAD-FMK, an irreversible tripeptide pan-ICE inhibitor (23), resulted in complete (>95%) and dose-dependent blockade of Fas stimulation of p38 (Fig. 7A and 7C). The stronger inhibition observed with YVAD-CMK and Z-VAD-FMK than with CrmA is consistent with the irreversible nature of the peptide inhibitors (88). These results demonstrate that Fas activation of p38 requires the action of ICE/CED-3 family proteases, therefore placing the MKK3/p38 activation pathway downstream of ICE/CED-3 family proteases.

Some controversy exists in the literature regarding the exact identity of the relevant protease(s) involved in Fas-mediated apoptosis (i.e., ICE or CPP32) (27, 28, 59, 77, 86). An excellent candidate for one of the proteases is the recently described FLICE/Mch5/MACH protein, which contains both FADD-like and protease modules and is an integral member of the Fas receptor complex (3, 64). Potentially downstream from FLICE in a protease cascade are ICE and CPP32; recent evidence indicates that ICE may reside upstream of CPP32 during Fas-mediated apoptosis (28). Although the use of CrmA, YVAD-CMK, and Z-VAD-FMK does not allow unambiguous identification of the target protease(s), the current work suggests

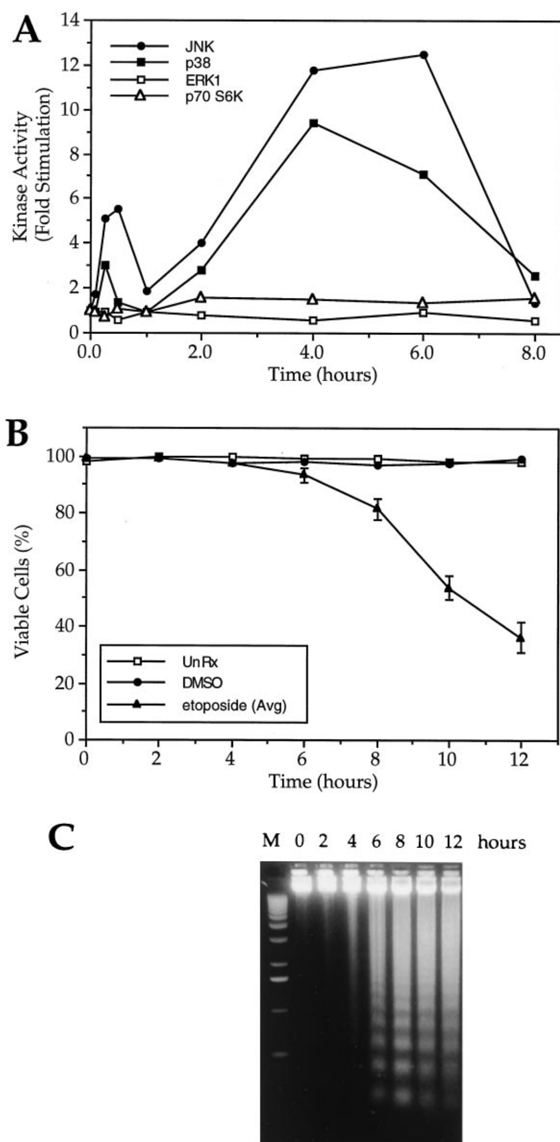



FIG. 8. Etoposide activation of p38 correlates with cell death. (A) Jurkat cells ($2 \times 10^6$/ml) were treated with etoposide (10 μg/ml) for various times. p38, JNK, $pp70^{S6k}$, and ERK1 kinase activities were measured in immune-complex kinase assays as described above. Equal numbers of cells were treated with etoposide for each time point. Kinase activities were not normalized for loss of cells due to apoptosis. Data are presented as fold stimulation over untreated cells. Similar results were obtained in three independent experiments. (B) Jurkat cells ($2 \times 10^6$/ml) were treated with etoposide (10 μg/ml) or dimethyl sulfoxide (DMSO) or left untreated (UnRx). At various times, an aliquot was removed and stained with trypan blue dye to distinguish dead cells. Cells were quantitated with a hemocytometer. At least 150 to 200 cells were counted per time point. Data are presented as the mean ± standard deviation ($n = 3$). (C) Aliquots of Jurkat cells ($10^6$) were lysed at various times after treatment with etoposide (10 μg/ml) and analyzed for DNA ladder formation. Similar results were observed in two independent experiments.

that Fas absolutely requires the action of an ICE/CED-3 protease family member(s) to activate p38.

While proteases of the ICE/CED-3 superfamily are unquestionably essential to the apoptotic process, their relevant downstream targets have not been clearly defined. One of the mechanisms by which these proteases have been postulated to act is by destructive cleavage of numerous molecules such as poly(ADP ribose) polymerase, lamin B1, β-actin, and topoisomerase I (reviewed in references 40 and 60). Significantly, the ICE/CED-3 family–p38 regulatory relationship defined in the present work expands the known spectrum of action of the cysteine proteases to also include the regulation of stress kinase pathways.

What mechanism might the ICE/CED-3 family proteases use to regulate stress kinases? The ability of CrmA and synthetic tri- and tetrapeptide ICE/CED-3 family inhibitors to block p38 induction, as in this study, certainly indicates that cysteine proteases, via direct or indirect mechanisms, are capable of initiating the kinase cascade(s) leading to p38 activation. In this regard, it is particularly relevant that Emoto et al. have recently described proteolytic cleavage and activation of protein kinase C-δ by a YVAD-inhibitable protease (26). The p38 and JNK kinases have been previously described to be activated by protein kinase cascades involving MKK3 or MKK6 and MEKK-1/MKK4, respectively (21, 42, 70), analogous to the HOG1 osmotic shock response pathway in yeast cells (6). It will therefore clearly be of interest to assay for proteolytic activation of regulatory kinases within the p38 activation pathway, such as MKK3, MKK4, and MKK6, as phosphorylation has been presumed to be their primary mode of regulation.

The specific p38 inhibitor SB 203580 (56) produced complete blockade of p38 activation by Fas (Fig. 5A) without inhibiting Fas-induced death (Fig. 5B), suggesting that p38 itself is not required for Fas-induced death. This result is consistent with other studies which have shown that SB 203580 can potently antagonize TNF-dependent gene expression without affecting TNF-dependent apoptosis (2). On the other hand, multiple studies overexpressing either wild-type or dominant-negative alleles of the upstream kinases MKK3 and MKK4 have implicated these enzymes in the regulation of apoptosis in multiple systems such as NGF withdrawal and ceramide (95, 90). In the present study as well, the constitutively activated upstream kinase MKK3(glu) enhanced Fas-mediated killing (Fig. 4). How might the ability of kinases such as MKK3 and MKK4 to promote death be reconciled with the ability to completely block the downstream kinase p38 without affecting apoptosis? The current results indicate that Fas activation of p38 and MAPKAP kinase 2 is not uniquely required for Fas-induced death but may function in a redundant pathway possibly involving JNK; using the p38-specific inhibitor SB 203580, we are unable to rule out this model. Alternatively, MKK3-augmented Fas killing may employ additional, non-p38 mechanisms, with p38 activation merely serving as a bystander marker for an apoptotic MKK3 activation cascade. However, we also cannot formally rule out the possibility that MKK3(glu) enhances Fas-induced death through nonapoptotic mechanisms. Since many of the known substrates of p38, such as ATF2 and ELK1 (69, 70), are transcription factors and since transcription and translation are not required for Fas-mediated apoptosis (47, 97), it should be interesting to identify other downstream targets of either MKK3 or p38 which may be more relevant to the apoptotic process.

Like Fas, the chemotherapeutic agent etoposide preferentially activated p38 and JNK over mitogen-stimulated kinases such as ERK1 and p70 S6 kinase with delayed (2 to 4 h) time kinetics paralleling the onset of apoptosis (Fig. 8A and B). Interestingly, etoposide stimulation of p38 activity was completely insensitive to the concentration of YVAD-CMK (120 μM) which completely inhibited Fas activation of p38 (compare Fig. 7 and 9). Moreover, etoposide activation of p38 was insensitive to concentrations of the pan-ICE inhibitor Z-VAD-FMK, which completely inhibited etoposide-induced cell death

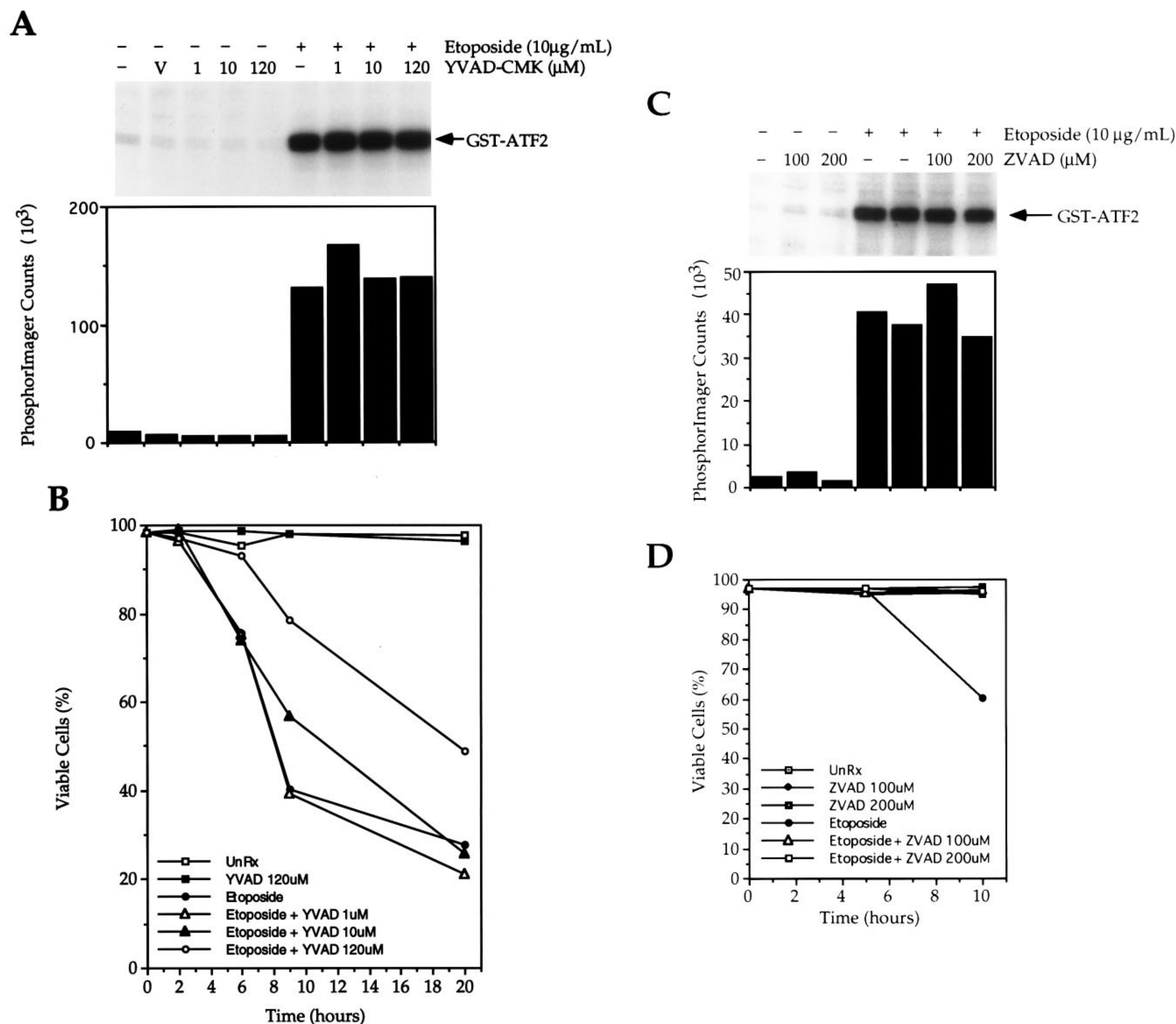



FIG. 9. Etoposide activation of p38 is not mediated by ICE/CED-3 family proteases. (A) Jurkat cells were left untreated or treated with vehicle alone, etoposide alone, or various concentrations of YVAD-CMK either alone or in combination with etoposide for 4 h. Cells were preincubated with YVAD-CMK for 1 h prior to etoposide treatment. p38 kinase activity was measured as described above (Fig. 7, legend). Results are representative of three experiments. (B) Aliquots of the cells from the experiment in panel A were monitored for cell death, using trypan blue dye exclusion. Cells treated with vehicle alone or YVAD-CMK alone (1 and 10 µM) were completely viable throughout the time course (data not shown). Data are presented as the average of two experiments. Similar results were observed with FACS analysis. UnRx, untreated. (C) Jurkat cells were left untreated (lane 1), treated with etoposide alone for 4 h (lanes 4 and 5), or treated with two concentrations of Z-VAD-FMK either alone (lanes 2 and 3) or in combination with etoposide for 4 h (lanes 6 and 7). p38 kinase activity was measured as described above. (D) Aliquots of the cells from the experiment in panel C were monitored for cell death, using trypan blue dye exclusion. The etoposide-alone data points are the average of a duplicate experiment. Results are representative of two independent experiments. Similar results were observed with FACS analysis.

(Fig. 9C and D). Sorbitol stimulation of p38 activity was similarly YVAD-CMK and Z-VAD-FMK resistant (Fig. 7A, right panel, and data not shown). These results thus demonstrate the existence of at least one additional mechanism of p38 regulation which involves either a distinct subset of proteases that are not inhibitable by the range of concentrations of YVAD-CMK or Z-VAD-FMK used or perhaps a novel cysteine protease-independent mechanism. A clear distinction therefore exists between Fas, which exhibits complete dependence on YVAD-CMK- and Z-VAD-FMK-sensitive protease(s) to activate p38, and etoposide and sorbitol, whose YVAD-CMK- and Z-VAD-FMK-insensitive p38 activation must utilize additional mechanism(s) (Fig. 10). These results suggest that p38 activation is not merely a secondary response to cell death, as etoposide-induced cell death can be completely inhibited by Z-VAD-FMK (Fig. 9D) without affecting etoposide activation of p38 (Fig. 9C). We are currently extending our investigation of the involvement of ICE/CED-3 family members in p38 activation by other apoptotic stimuli, including other chemotherapeutic agents, ionizing radiation, and ceramide. Investigations are also under way to elaborate the role of MKK3, MKK4, and MKK6 in Fas-induced death to determine which are the relevant regulators of p38 in vivo. The additional definition of the precise mechanism by which ICE/CED-3 family proteases activate MKK3/p38, as well as of the relevant downstream targets of MKK3 and/or p38, should shed considerable insight into signalling events downstream of the apoptotic receptor superfamily exemplified by Fas.

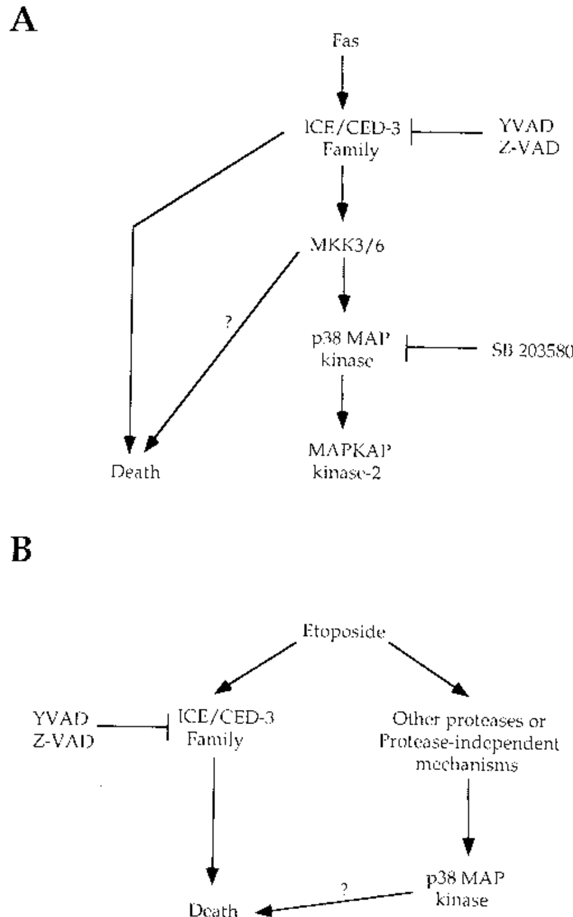



FIG. 10. Fas activation of p38 MAPK is dependent on ICE/CED-3 family proteases (A), whereas etoposide activation of p38 MAPK must utilize as yet unidentified additional mechanisms (B). Since Fas activation of p38 can be blocked by SB 203580 with no effect on Fas-induced death, the p38/MAPKAP kinase 2 pathway may branch from the MKK3-cell death pathway or function in a parallel, redundant cell death pathway (arrow not shown). (B) Etoposide-induced death can be inhibited by YVAD and Z-VAD with no effect on p38 MAPK activation, suggesting that etoposide activation of p38 involves proteases uninhibitable by YVAD and Z-VAD or some other protease-independent pathway. Although MKK3 has not formally been shown to be activated by Fas, MKK3 and MKK6 have been included since they are the only known upstream regulators of p38.

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