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Lack of a Role for Jun Kinase and AP-1 in Fas-Induced Apoptosis

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Cross-linking of Fas (CD95) induces apoptosis, a response that has been reported to depend upon the Ras activation pathway. Since many examples of apoptosis have been reported to involve AP-1 and/or the AP-1-activating enzyme Jun kinase (JNK), downstream effectors of Ras or Ras-like small GTP-binding proteins, we evaluated the role of these molecules in Fas-mediated apoptosis. Although cross-linking of Fas on Jurkat T cells did result in JNK activation, increased activity was observed relatively late, being detectable only after 60 min of stimulation. Expression of a dominant negative form of SEK1 that blocked Fas-mediated induction of JNK activity had no effect on Fas-mediated apoptosis. Furthermore, maximally effective concentrations of anti-Fas did not cause JNK activation if apoptosis was blocked by a cysteine protease inhibitor, suggesting that under these conditions, activation of JNK may be secondary to the stress of apoptosis rather than a direct result of Fas engagement. Despite the activation of JNK, there was no induction of AP-1 activity as determined by gel shift assay or induction of an AP-1-responsive reporter. The lack of a requirement for AP-1 induction in Fas-mediated death was further substantiated with Jurkat cells that were stably transfected with a dominant negative cJun, TAM-67. While TAM-67 effectively prevented AP-1-dependent transcription of both the interleukin-2 and cJun genes, it had no effect on Fas-induced cell death, even at limiting levels of Fas signaling. Thus, induction of JNK activity in Jurkat cells by ligation of Fas at levels sufficient to cause cell death is likely a result, rather than a cause, of the apoptotic response, and AP-1 function is not required for Fas-induced apoptosis.

Fas is a widely expressed transmembrane molecule whose cross-linking by a ligand or antibody results in apoptotic cell death (50). There is great interest in the mechanism by which Fas signals cells to die, and a growing number of Fas-associated molecules and signaling pathways are being described. Two molecules that bind to an essential intracellular domain of Fas, the so-called death domain, have been identified by the yeast two-hybrid technique: MORT1/FADD (10, 14) and RIP (66). Coprecipitation studies have revealed that serine-phosphorylated MORT1/FADD (CAP1 and CAP2) and two other molecules (CAP3 and CAP4) rapidly associate with antibody-cross-linked Fas, forming a death-inducing signaling complex (34). The latter two molecules have recently been shown to represent an interleukin-1β-converting enzyme (ICE)-like protease (FLICE or MACH) that binds to the death effector domain of MORT1/FADD (9, 49). Although these proteins can induce apoptosis when expressed in transfected cell lines, how they signal for apoptosis is unknown. In addition, two enzymes that associate with intracellular Fas have been reported. FAST is a serine/threonine kinase that is dephosphorylated, and thereby activated, when Fas is cross-linked (71). It, in turn, phosphorylates a nuclear transcription factor, TIA-1, a nuclear RNA-binding protein that has been suggested to participate in signaling for apoptosis (70). FAP-1 is a tyrosine phosphatase that binds to the C-terminal portion of Fas, distal to the death domain (59). Transfection studies have suggested that FAP-1 may inhibit rather than enhance Fas-induced killing (59). Finally, sphingomyelinase-produced ceramides have been shown to be generated by a number of stimuli that induce apoptosis, such as tumor necrosis factor (TNF) (33) and UV irradiation (25), and have recently been found to be elevated in cells induced to die by cross-linking Fas (23, 69). Given that cell-permeative ceramides can induce apoptosis of Jurkat cells, it has been hypothesized that ceramides may actually be essential effector molecules in signaling for Fas-induced death.

Recent reports have indicated that AP-1 may be required for some forms of apoptosis. For example, cJun has been implicated in nerve growth factor (NGF) withdrawal-induced apoptosis of PC-12 pheochromocytoma cells (78). AP-1, and in particular cJun, has also been reported to be required for ceramide-induced apoptosis of HL-60 human promyelocytic cells and U937 monoblastic leukemia cells (60, 75). AP-1 is a transcription factor that binds DNA in a sequence-specific manner (at a site called a TRE) and activates gene transcription (3, 17, 58). All of the identified Jun family proteins (cJun, JunB, and JunD) can homodimerize or form heterodimers with Fos family members. Fos family proteins (cFos, FosB, Fra-1, and Fra-2) cannot form intrafamily homodimers and thus cannot bind a TRE in the absence of Jun. Dimerization of Jun and Fos is mediated by leucine zippers, and binding of the dimer to DNA is via a highly basic region in the complex (3). Transactivation of gene transcription is dependent upon the presence of clusters of negatively charged amino acids found in the amino termini of Jun and Fos family members (1). As with any potent transcriptional regulator, the activity of AP-1 is tightly regulated, in this case at both the transcriptional and posttranslational levels. In the latter case, dephosphorylation of serine and threonine residues in the C terminus of Jun is necessary for binding to DNA, while phosphorylation of serine-63 and serine-73 promotes transactivation (8, 11, 48, 65). Phosphorylation of cJun is mediated by cJun NH_2-terminal protein kinases (JNKs), which are in turn induced as part of the Ras activation pathway (26). JNKs, also known as stress-

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activated protein kinases, are members of the mitogen-activated protein kinase (MAPK) group (18, 63). These kinases are activated in response to UV irradiation, NGF withdrawal, and Ras activation. In addition to cJun, the ATF2 and Elk-1 transcription factors are substrates for JNK (24, 77). Given that activation of Ras has been implicated as an essential step in Fas-mediated apoptosis (23) and that JNK and AP-1 activation is downstream of Ras or other Ras-like small GTP-binding proteins in the signaling cascade, we asked whether JNK is activated during Fas signaling and, if so, whether AP-1 activity is induced and/or essential for Fas-mediated killing. The results indicate that while cross-linking of Fas does indeed activate JNK, at low levels this is a consequence of a stress response induced in dying cells. At higher levels of cross-linking, JNK appears to be activated as a consequence of Fas signaling. However, AP-1 is neither activated by Fas nor required for Fas-induced apoptosis.

MATERIALS AND METHODS

Cell lines and reagents. Jurkat T leukemia cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 4 mM glutamine, 100 U of penicillin per ml, 150 μg of gentamicin per ml, and 5×10^{-5} M 2-mercaptoethanol (complete medium). The construction of mammalian expression vectors containing the human cJun transcriptional activation mutant (TAM-67) has been described previously (1, 12). Briefly, TAM-67 expression is driven by the mouse metallothionein I promoter in pMexMth, which also contains the bacterial neomycin resistance gene. Jurkat cells were electroporated with either a control vector (pMexMth without TAM-67) or the pMexMth-TAM-67 construct and were selected with G418. After subcloning, Jurkat cells expressing high levels of TAM-67 were chosen for further studies (J-TAM1 and J-TAM9). Phorbol myristate acetate (PMA) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ionomycin was purchases from Calbiochem (La Jolla, Calif.). AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] was purchased from ICN Biochemicals (Aurora, Ohio). The ICE inhibitor acetyl-Tyr-Val-Ala-Asp-CHO (YVAD) was purchased from Bachem (King of Prussia, Pa.), and the ICE-like protease inhibitor Z-Val-Ala-Asp(*O*-methyl)-fluoromethylketone (ZVAD) was purchased from Enzyme Systems Products (Dublin, Calif.). Fluorescein-conjugated annexin V was purchased from R&D Systems (Minneapolis, Minn.). cJun/AP-1 (Ab-1) antibody reactive against the DNA-binding domain for Western blotting was purchased from Oncogene Science (Uniondale, N.Y.). Supershifting pan-Jun antibodies or antibodies to JunB, JunD, and pan-Fos family members were kindly provided by Rodrigo Bravo (Bristol-Myers Squibb, Princeton, N.J.). Anti-human Fas immunoglobulin M (IgM) antibody (CH-11) was purchased from Kamiya Biomedical Company (Thousand Oaks, Calif.). Anti-JNK1 antibody (C-17) (Santa Cruz) was made against amino acids 368 to 384 of human JNK1 and is reactive to JNK1 p46 and, to a lesser degree, JNK2 p54α and p54β. The glutathione *S*-transferase (GST)–c-Jun(1-79) cDNA construct was kindly provided by Omar Coso and Silvio Gutkind (15) (National Institute of Dental Research, National Institutes of Health [NIH]). The luciferase reporter construct TRE-Luc contains four copies of the consensus TRE site from the collagenase gene, and the NFAT-Luc construct contains three copies of the NFAT-binding site from the murine interleukin-2 (IL-2) gene (52). A TK-βgal reporter was kindly provided by Juan Carlos Zuniga-Pflucker and Michael Lenardo (National Institute of Allergy and Infectious Diseases, NIH). Jurkat cells were cotransfected with the TRE-luciferase reporter and pSV-neo and selected in G418, to make the Jurkat-TRE-luciferase stable clones. The probes used for Northern blotting for c-*jun*, *junB*, and *junD* were kindly provided by Michael Birrer (National Cancer Institute, NIH). [^{3}H]thymidine (6.7 Ci/mmol), ^{125}I-protein A (30 μCi/μg), [^{32}P]dCTP (3,000 Ci/mmol), and [^{32}P]ATP (4,500 Ci/mmol) were purchased from ICN (Costa Mesa, Calif.). Oligonucleotides used in these studies were purchased from Stratagene (La Jolla, Calif.). The sequences of oligonucleotides used in these studies are as follows: AP-1, 5′ CTAGTGATGAGTCAGCCGGATC 3′ and 3′ GATCACTACTCAGTCGGCCTAG 5′; and SP-1, 5′ GATCGATCGG GGCGGGGCGATC 3′ and 3′ CTAGCTAGCCCCGCCCCGCTAG 5′. The pEBG expression vector containing a cDNA insert encoding an enzymatically inactive dominant negative form of SEK1 (pEBG-SEK1 K→R) (57) was the kind gift of Silvio Gutkind. The pcI expression vector (Promega Corporation, Madison, Wis.) containing a cDNA encoding the human IL-2Rα (Tac) (36) and the anti-Tac antibody (73) were obtained from Warren Leonard (National Heart, Lung, and Blood Institute, NIH).

Nuclear extraction protocol. Ten million J-neo, J-TAM1, or J-TAM9 cells were stimulated with 10 ng of PMA per ml plus 0.5 μg of ionomycin per ml or various concentrations of anti-human Fas antibody CH-11 for various times. Nuclear protein extracts were prepared by the hypotonic lysis method of Goldstone and Lavin (22). Briefly, cells were harvested and washed twice in ice-cold phosphate-buffered saline (PBS). The pellets were resuspended in 400 μl of ice-cold lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 1 mM PMSF, 1 mM AEBSF, 1 μg [each] of aprotinin and leupeptin per ml) and left to swell for 5 min at 4°C. The suspension was centrifuged for 10 s in a Microfuge. The nuclear pellet was resuspended in 50 μl of storage buffer (10 mM HEPES [pH 7.9], 50 mM KCL, 300 mM NaCl, 1 mM EDTA, 1 mM DTT, 20% glycerol, 1 mM PMSF, 1 mM AEBSF, 1 μg [each] of aprotinin and leupeptin per ml) and incubated on ice for 30 min with periodic mixing. The resulting solution was cleared by centrifugation for 5 min at 4°C in a microcentrifuge, and the supernatant was collected and stored at −80°C until required. Nuclear protein concentrations were estimated by the Bradford assay (Bio-Rad, Hercules, Calif.).

Immunoblotting. Nuclear extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10.5% polyacrylamide) under reducing conditions and transferred to Immobilon P (Millipore, Bedford, Mass.). After blocking overnight at 4°C with 5% bovine serum albumin (BSA)–Tris-saline (pH 7.4), the membrane was immunoblotted with an anti-cJun antibody from Oncogene Science diluted to 1.25 mg/ml in Tris-saline–0.5% BSA–0.05% Tween 20–0.02% azide, for 2 h. The membranes were washed three times in Tris-saline–0.5% BSA–0.5% Nonidet P-40, incubated for 1 h with ^{125}I-protein A diluted 1:2,000 in Tris-saline–0.5% BSA, washed several times, and exposed to Kodak X-Omat film overnight.

Electrophoretic mobility shift assays (EMSAs). Two micrograms of nuclear extracts from unstimulated or stimulated J-neo, J-TAM1, or J-TAM9 cells was incubated with a ^{32}P-labeled consensus TRE oligonucleotide for 30 min. Binding reaction mixtures consisted of 10% glycerol, 10 mM HEPES, 5 mM Tris (pH 8), 4 mM $MgCl_2$, 0.5 mM EDTA, 60 mM KCl, 0.5 μg of poly dI · dC, 100 mg of BSA per ml, 2 mM DTT, 10 μg (each) of aprotinin and leupeptin per ml 0.5 mM PMSF, and 45,000 cpm of the end-labeled oligomer probe. A 50-fold excess of unlabeled oligonucleotide was added in the cold competition assays. The nonspecific oligonucleotide used in these studies contained the Sp-1 consensus binding sequence. In supershift competition, the nuclear extracts were preincubated in the presence of 1 to 2 μl of antiserum for 15 min before addition of the labeled oligonucleotide. Following incubation, the complexes were separated by nondenaturing 4% PAGE in chilled buffer. Gels were dried and exposed to Kodak X-Omat film overnight.

Transient transfections and stimulation of luciferase reporter of endogenous JNK activity. Transfections were performed by electroporation. Cells were washed in RPMI 1640 and resuspended at 10^7 cells/ml in RPMI 1640 containing 20 mM HEPES and 2 mM glutamine. The cell suspension (0.2 ml) was mixed with 10 μg of TRE-Luc or NFAT-Luc and 9.4 μg of TK-β-gal in electroporation cuvettes (0.4-cm electrode gap) (Bio-Rad Laboratories, Richmond, Calif.). Electroporation was performed at 960 μF and 0.25 V with a Bio-Rad gene pulser. The cells were then transferred into complete medium and cultured in triplicate under various conditions. After 9 h, the cells were harvested, washed in PBS, pelleted, and lysed in 40 μl of lysis buffer containing 25 mM Tris-HCl (pH 7.8), 2 mM DTT, 10% glycerol, 1 mM EDTA, and 1% Triton X-100. Samples (20 μl) were analyzed for luciferase activity according to the manufacturer's instructions (Promega). β-Galactosidase (β-gal) activity was quantified as described previously (31). Reporter activity was measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.) and is expressed as relative light units. For induction of luciferase activity in J-TRE-luciferase cells, cells were incubated in duplicate or triplicate at a concentration of 10^6 cells/ml in complete medium with or without various stimuli. After 2 h, the cells were harvested and lysed, and protein was quantitated by the bicinchoninic acid method (Pierce, Rockford, Ill.). Equal amounts of protein from each sample were then assayed for luciferase activity. To determine the efficacy of transfected dominant negative SEK1 in inhibiting JNK activity, 50×10^6 to 75×10^6 cells/ml were electroporated as described above in the presence of 50 μg/ml of pcI Tac per ml plus 50 μg of pEBG or 50 μg of pEBG-SEK1 (K→R) per ml. The electroporated cells were transferred into complete medium with 20 mM HEPES and incubated at 37°C. After 14 h, the cells were harvested and washed with cold RPMI 1640 once, and dead cells were removed with lymphocyte separation medium (LSM Lymphocyte Separation Medium; Organon Teknika Corporation, Durham, N.C.). Under these conditions, 30 to 50% of the cells in each group were positive for surface IL-2Rα as measured by flow cytometry with the anti-Tac monoclonal antibody (73). The cells were counted, and Dynabeads (Dynabeads M-450 goat anti-mouse IgG; Dynal, Lake Success, N.Y.) coated with anti-Tac antibody were added to each sample at a ratio of four Dynabeads per Tac$^+$ cell. After 1 h of incubation at 4°C with gentle rotation, the positive cells were separated with a magnet and washed once with complete medium. Cells that adhered to the beads (positively selected; 0.5 to 1.5% recovery) and those that did not (negatively selected) were adjusted to 10^6 cells/ml and cultured in medium alone or with 1 μg of CH-11 per ml. After 60 min at 37°C, the tubes were placed on ice and the cells were washed once with cold PBS, lysed, and normalized for total protein by using the bicinchoninic acid protein assay. A JNK assay was performed with 20 μg of protein from each sample.

IL-2 production. One million J-neo, J-TAM1, or J-TAM9 cells were incubated in a volume of 1 ml of complete medium with or without various stimuli. After 20 h, supernatants were collected and assayed for IL-2 with the IL-2-dependent CTLL cell line as an indicator (45). One unit of IL-2 activity is defined as the

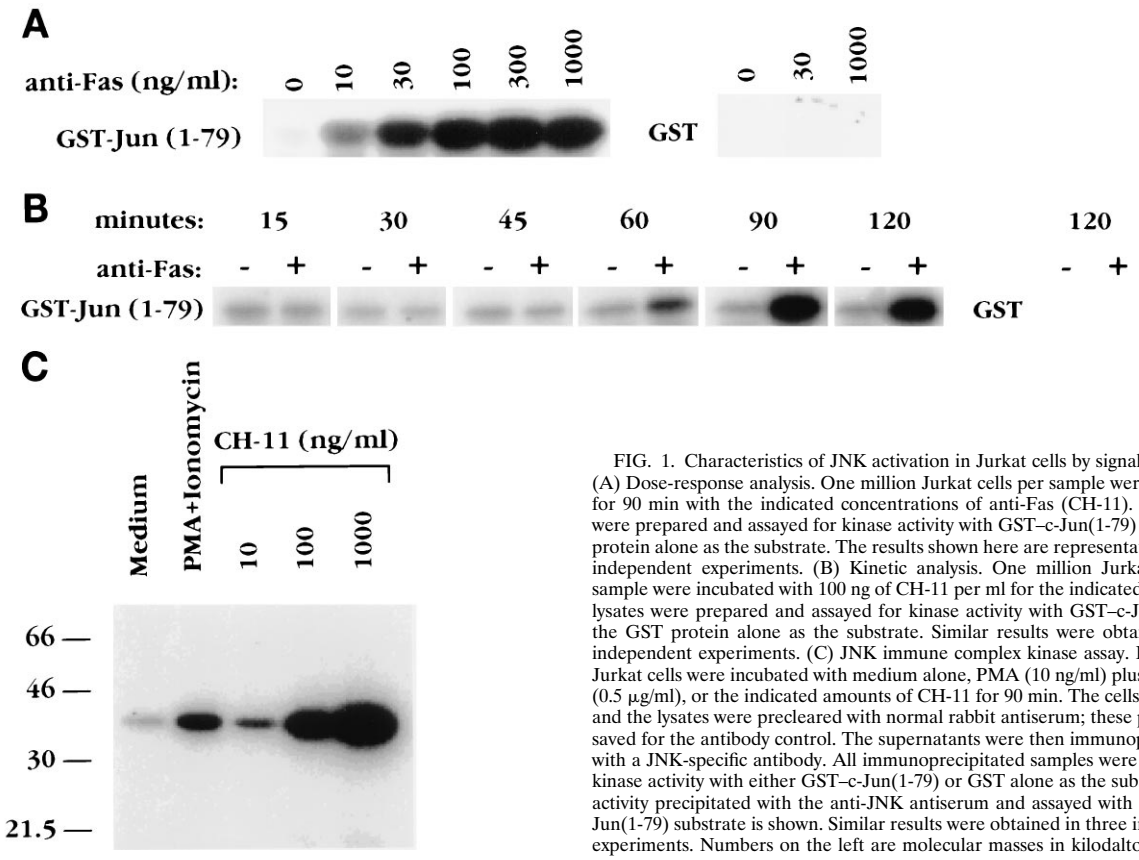

FIG. 1. Characteristics of JNK activation in Jurkat cells by signaling via Fas. (A) Dose-response analysis. One million Jurkat cells per sample were incubated for 90 min with the indicated concentrations of anti-Fas (CH-11). Cell lysates were prepared and assayed for kinase activity with GST–c-Jun(1-79) or the GST protein alone as the substrate. The results shown here are representative of eight independent experiments. (B) Kinetic analysis. One million Jurkat cells per sample were incubated with 100 ng of CH-11 per ml for the indicated times. Cell lysates were prepared and assayed for kinase activity with GST–c-Jun(1-79) or the GST protein alone as the substrate. Similar results were obtained in five independent experiments. (C) JNK immune complex kinase assay. Five million Jurkat cells were incubated with medium alone, PMA (10 ng/ml) plus ionomycin (0.5 μg/ml), or the indicated amounts of CH-11 for 90 min. The cells were lysed, and the lysates were precleared with normal rabbit antiserum; these pellets were saved for the antibody control. The supernatants were then immunoprecipitated with a JNK-specific antibody. All immunoprecipitated samples were assayed for kinase activity with either GST–c-Jun(1-79) or GST alone as the substrate. Only activity precipitated with the anti-JNK antiserum and assayed with the GST–c-Jun(1-79) substrate is shown. Similar results were obtained in three independent experiments. Numbers on the left are molecular masses in kilodaltons.

dilution of supernatant that caused CTLL cells to incorporate half-maximal amounts of [^{3}H]thymidine.

RNA preparation and Northern blotting. Total RNA was prepared from 5×10^6 to 10×10^6 cells by using RNA-STAT-60 (Tel-Test, Inc., Friendswood, Tex.) according to the manufacturer's instructions. Total RNA (10 μg) was separated on a 0.5% formaldehyde–1.0% agarose gel in 1× MOPS buffer (4.2 g of MOPS [morpholine propane sulfonic acid], 2.67 ml of 3 M sodium acetate, 2.0 ml of 0.5 M EDTA, 0.72 ml of 10 N NaOH) and transferred to Hybond membranes (Amersham, Arlington Heights, Ill.) by using 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Ethidium bromide staining of the gels was routinely performed to ensure the integrity of the RNA. After transfer, the membrane was cross-linked by using a UV Stratalinker (Promega). The filters were hybridized for 2 h in QuikHyb (Stratagene) at 68°C with ^{32}P-labeled c-*jun*, *junD*, or *GAPDH* probes (Prime-It II; Stratagene). The blots were washed twice in 2× SSC–0.1% SDS at 65°C for 30 min and once in 0.5× SSC–0.1% SDS at 65°C for 30 min, air dried, and autoradiographed at −80°C with Kodak X-Omat film.

Apoptosis induction. Cells (10^6/ml) were preincubated for 4 h in complete medium plus 10 to 20 μCi of [^{3}H]thymidine per ml. The cells were washed four times in PBS and resuspended in complete medium. Fifty thousand cells per well were plated in 96-well flat-bottomed plates in triplicate and incubated with or without CH-11 at various concentrations. After 5 h, the cells were harvested and incorporation of [^{3}H]thymidine was measured. The percent specific DNA fragmentation was calculated as described previously (80): $(M - E)/M \times 100$, where M is the retained label in cells cultured in medium and E is the retained label in cells cultured under experimental conditions. In experiments using ZVAD, the cells were preincubated for 30 min with ZVAD at various concentrations before addition of anti-Fas. In some experiments, anti-Fas-induced JNK activity (in vitro kinase assay) and apoptosis (DNA fragmentation, staining with fluorescein-ated annexin V plus propidium iodide, and trypan blue exclusion) were evaluated in parallel cultures. Apoptosis of transiently transfected Jurkat cells was determined as described previously (44). In this assay, cells are transfected with an expression vector for the gene of interest along with a β-gal reporter plasmid that identifies transfected cells and allows cell death to be quantitated. Briefly, Jurkat cells were electroporated, as described above, in the presence of 15 μg of CMV-β-gal (39) per ml plus 100 μg of either the empty pEBG vector or pEBG-SEK1 (K→R) per ml. The transfected cells were incubated for 12 h at 37°C and then treated in duplicate with various concentrations of CH-11 for 5 h at 37°C. The cells were harvested, centrifuged in microcentrifuge tubes, and lysed in 50 μl of buffer containing 25 mM Tris-HCl (pH 7.8), 2 mM DTT, 10% glycerol, 1 mM EDTA, and 1% Triton X-100. Samples (20 μl) were analyzed for β-gal activity as described previously (31). The percent specific cytotoxicity was calculated with the formula $(M - E)/M \times 100$, where M is the β-gal activity in cells cultured in medium alone and E is the β-gal activity in cells cultured under experimental conditions.

In vitro kinase assays. The solid-phase JNK assays were carried out by using a GST–c-Jun(1-79) fusion protein coupled to glutathione beads as a substrate. Briefly, to measure JNK activity in cell lysates, 10^6 cells were incubated for various periods of time with various stimuli and then lysed in lysis buffer (25 mM HEPES [pH 7.5], 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 μg of leupeptin per ml, 1 mM PMSF). Fifty micrograms of cellular extract was mixed with glutathione-agarose beads to which 10 μg GST–c-Jun(1-79) was bound. After incubation at 4°C for 3 h, the beads were washed three times with PBS containing 1% Nonidet P-40 and 2 mM vanadate, once with 100 mM Tris (pH 7.5) and 0.5 M LiCl, and once with final wash buffer (12.5 mM MOPS [pH 7.5], 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4). The beads were then incubated in kinase reaction buffer (12.5 mM MOPS [pH 7.5], 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4, 20 μM ATP, 3.3 mM DTT) containing 1 μCi of [γ-^{32}P]ATP at 30°C for 30 min. The reaction was terminated by addition of 10 μl of Laemmli buffer, and the phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography. For the immune complex JNK1 assay, 5×10^6 cells were incubated for 90 min with various stimuli. The cells were then collected and lysed in lysis buffer plus 0.1% SDS, 0.5% Na deoxycholate, and 1% Triton X-100. The supernatants were first immunoprecipitated with normal rabbit antiserum followed by protein A-Sepharose (Zymed, San Francisco, Calif.) and then immunoprecipitated with anti-JNK1 antibody (C-17; Santa Cruz Biotechnology, Inc.), followed by protein A-Sepharose (Zymed). Washed immunoprecipitates were resuspended in 30 μl of kinase buffer (12.5 mM MOPS [pH 7.5], 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4, 3.3 mM DTT, 20 μM cold ATP) containing 2 μg of GST–c-Jun(1-79) and 1 μCi of [γ-^{32}P]ATP. Samples were incubated at 30°C for 30 min, and the reaction was terminated by adding 10 μl of Laemmli buffer. The samples were separated by SDS–12% PAGE followed by autoradiography. For some experiments, phosphorimaging studies were carried out on the original gels with a Personal PhosphorImager SI from Molecular Dynamics.

RESULTS

Activation of JNK by Fas ligation. Given that JNK activity is increased in some forms of apoptosis (78) and that Fas has been reported to activate Ras (23), the question of whether signaling via Fas results in the activation of JNK was addressed. Jurkat cells were cultured in medium or with increasing concentrations of CH-11, an anti-Fas antibody that induces apoptosis, and after 90 min cell lysates were prepared. The lysates were assayed for NH_2-terminal JNK activity with either an NH_2-terminal (residues 1 to 79) cJun-GST fusion protein or GST alone as the substrate (Fig. 1A). There was a small amount of baseline activity in unstimulated cells that was detectably greater after stimulation with 10 ng of CH-11 per ml, a concentration that induces apoptosis (see Fig. 9), and reached a plateau at 100 to 300 ng/ml. The kinase activity was specific for the NH_2-terminal portion of cJun in that no phosphorylation of the GST protein alone could be detected, even at 1,000 ng of anti-Fas antibody per ml. In two independent experiments in which JNK activity was quantitated with a PhosphorImager, the maximal induction was found to be 15- and 24-fold over that with medium alone. Activation of JNK was a relatively late event after Fas ligation, not being detected until 60 min after stimulation and reaching a plateau at approximately 90 min (Fig. 1B).

To determine if the phosphorylation of the GST–c-Jun(1-79) substrate accurately reflected the activity of established JNKs, an immune complex kinase assay was performed. Jurkat T cells were cultured in medium alone, stimulated with PMA plus the Ca^{2+} ionophore ionomycin, or stimulated with various amounts of anti-Fas. After 90 min, the cells were lysed, the lysates were immunoprecipitated with a nonspecific rabbit antiserum followed by an anti-JNK antiserum, and the beads were incubated with either GST–c-Jun(1-79) or GST alone in the presence of [γ-^{32}P]ATP (Fig. 1C). As with cell lysates, immunoprecipitated JNK had a small amount of activity when isolated from cells cultured in medium alone, and this activity was enhanced by stimulation with PMA plus ionomycin. Anti-Fas readily induced JNK activity; a small increase was detected at 10 ng/ml, a concentration that causes substantial cell death, and very large increases were detected at 100 and 1,000 ng/ml. This assay was specific, since no activity was immunoprecipitated by an irrelevant rabbit antiserum, and no activity was detected in the anti-JNK immunoprecipitate assayed with GST protein as the substrate (data not shown). Thus, ligation of Fas results in the activation of JNK.

Fas ligation does not induce AP-1 activity. Several approaches were taken to determine if activation of JNK resulted in enhanced AP-1 activity. AP-1 was detected by its ability to shift a TRE-containing oligonucleotide in an EMSA. Nuclear extracts from Jurkat cell nuclei cultured in medium alone yielded a single band (Fig. 2). As shown below, this band was specifically competed by an unlabeled TRE-containing oligonucleotide. As expected, AP-1 binding was induced by PMA plus ionomycin. However, at 30 ng/ml, a concentration that is maximally effective at inducing apoptosis, anti-Fas had no effect on AP-1 activity in Jurkat cells. In fact, no induction of AP-1 could be detected even at a CH-11 concentration of 1,000 ng/ml (data not shown). To address the possibility that the gel shift analysis was not sensitive enough to detect induction of a small amount of AP-1, Jurkat cells that stably express a TRE-luciferase reporter were stimulated with PMA or various amounts of anti-Fas antibody for 2 h, and TRE-reporter activity was determined (Fig. 3). As expected, PMA caused a dose-dependent increase in TRE-regulated reporter activity. However, despite the greatly enhanced levels of JNK activity induced by anti-Fas, stimulation with this antibody caused no induction of luciferase activity even at very high concentrations. Anti-Fas did not have a negative effect on the TRE-Luc reporter itself, since luciferase induction by PMA was not inhibited by coculture with 100 ng of CH-11 per ml (data not shown). Therefore, despite activating JNK, anti-Fas does not cause a detectable increase in AP-1 activity.

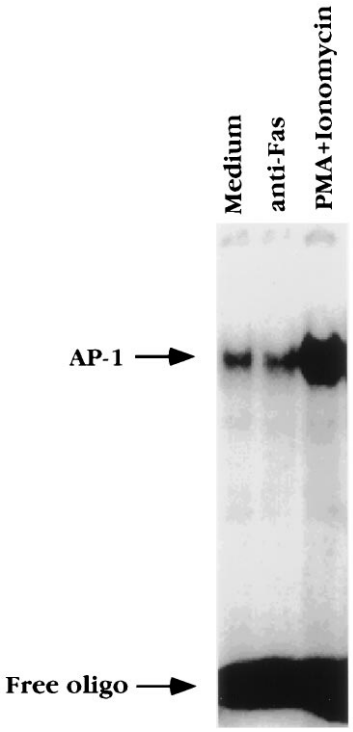

FIG. 2. Anti-Fas does not induce AP-1 in Jurkat cells. Cells were incubated with medium alone, 30 ng of anti-Fas per ml, or 10 ng of PMA per ml plus 0.5 μg of ionomycin per ml for 75 min prior to the preparation of nuclear protein extracts. EMSAs with labeled oligonucleotides containing the binding site for AP-1 were performed.

Expression of dominant negative cJun in Jurkat cells. Although it appeared that Fas engagement does not lead to appreciable AP-1 induction in Jurkat cells, it is still possible that low levels of AP-1 induction, or even basal activity, are required for Fas-mediated killing. To address this, we used a dominant negative cJun to inhibit formation of active AP-1 and to determine whether this inhibition would interfere with Fas-induced apoptosis. TAM-67 is a variant of cJun in which amino acids 3 to 122 have been deleted (1, 12). The resulting 28-kDa protein dimerizes and binds DNA but lacks the transactivation domain. Jurkat cells were transfected with a cDNA expression vector encoding the TAM-67 protein. Multiple independent G418-resistant clones were screened for expression of TAM-67 by immunoblotting nuclear extracts with an antiserum that recognizes the DNA-binding region of cJun, and which therefore binds both cJun and TAM-67. As shown in Fig. 4, a 28-kDa protein was detected in two independent transfectants, J-TAM1 and J-TAM9. Another transfectant, J-neo, was G418 resistant but did not express TAM-67 protein. In J-TAM1 and J-TAM9, TAM-67 was greatly overexpressed compared to endogenous cJun, to the extent that endogenous cJun, which migrates with a molecular mass of 39 kDa, cannot be seen in Fig. 4, although it could be detected in prolonged exposures of the film (data not shown). In multiple analyses, J-TAM9 cells

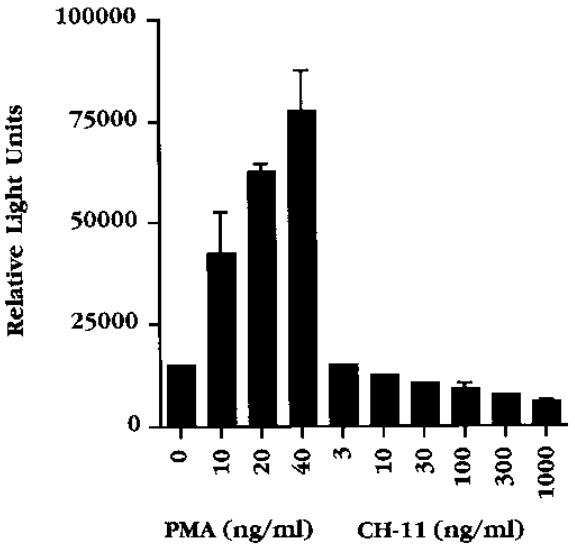

FIG. 3. Anti-Fas does not increase transcription of an AP-1-regulated reporter. J-TRE-luciferase cells were incubated at a concentration of 10^6 cells/ml in complete medium with or without the indicated stimuli. After 2 h, the cells were harvested and lysed, and equal amounts of protein from each sample were assayed for luciferase activity. The standard deviations for duplicate cultures are shown. Similar results were obtained in six independent experiments.

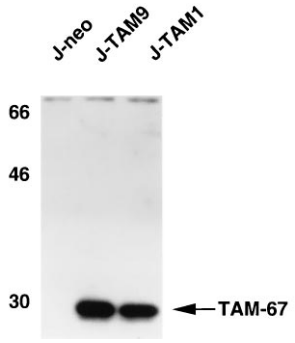

FIG. 4. Constitutive expression of TAM-67 in two stably transfected Jurkat cell lines, J-TAM9 and J-TAM1. Immunoblotting of nuclear proteins was performed with an anti-Jun antibody. Numbers on the left are molecular masses in kilodaltons.

were found to express TAM-67 protein at a higher level (ca. twofold) than J-TAM1 cells. Densitometric analysis revealed that the level of TAM-67 in these transfectants was 20-fold higher than that of endogenous cJun (data not shown).

AP-1 DNA-binding complexes in TAM-67$^+$ and TAM-67$^-$ Jurkat cells. TAM-67 protein produced by in vitro transcription and translation dimerizes with normal Jun and Fos and binds DNA (12). EMSAs with the consensus AP-1 site were performed to determine what kind of DNA-binding molecular complexes form in T cells that express TAM-67 (Fig. 5). Nuclear protein extracts from unstimulated J-neo cells were assayed in the cold to enhance Jun:Jun dimerization and yielded one major AP-1-binding complex (Fig. 5, lane 1). The mobility of this band was not altered by incubation with an excess of a nonspecific oligonucleotide (lane 3) but was eliminated by incubation with an excess of unlabeled AP-1 oligonucleotide (lane 5). Since unactivated Jurkat cells do not express detectable Fos mRNA or protein (reference 2 and our unpublished observation) and since we did not observe any alteration in the gel shift pattern after incubation with anti-Fos antibody (data not shown), this complex likely represents a Jun dimer. Gel shift analysis performed with nuclear lysates from J-TAM1 cells yielded a very different pattern, showing two major bands with mobilities different from that obtained with nuclear lysates from J-neo cells (Fig. 5, lane 2). The binding of these complexes to the AP-1 consensus site was specific, as shown by competition studies performed with unlabeled nonspecific (lane 4) and specific (land 6) oligonucleotides. Similar results were obtained with J-TAM9 (data not shown).

Antisera that recognize different components of the AP-1 complex were used to further characterize the nature of the DNA-binding proteins. All of the complexes contained a Jun family member (or TAM-67) as shown by incubation with a pan-anti-Jun antibody (Fig. 5, lanes 7 and 8). Additionally, the AP-1 complex from J-neo cells, as well as the slower-migrating complex from J-TAM1 cells, appeared to contain JunB, since a JunB-specific antiserum caused a diminution of intensity of the original bands and the appearance of supershifted bands (Fig. 5, lanes 9 and 10). Anti-Fos, anti-JunD, and anti-NFATp antibodies had no effect on the gel shift pattern (data not shown and Fig. 5, lanes 11 and 12), indicating that in unactivated Jurkat cells these proteins do not contribute to the AP-1-binding complex. Thus, the expression of TAM-67 resulted in the formation of altered AP-1 complexes, with the faster-migrating complex most likely representing a cJun:TAM-67 or a TAM-67:TAM-67 dimer. As all antibodies that recognize TAM-67 also recognize endogenous cJun, these possibilities cannot be distinguished by supershift analysis. However, if the complex were solely a TAM-67 homodimer, a more intense band might be expected, implying that this faster-migrating band probably consists of a TAM-67:cJun heterodimer. The composition of the slower-migrating complex is unknown, but based upon the effect of the protein-specific antisera, at least a portion of it appears to contain JunB.

TAM-67 inhibits induction of endogenous cJun. c-*jun*, which has nonconsensus TRE sites at positions −70 and −185 of its promoter, is positively regulated by AP-1 that is induced by T-cell activation or stimulation with phorbol esters (2, 28). Since an increase in AP-1 activity is essential for full induction of c-*jun* transcription following T-cell activation (3), we ana-

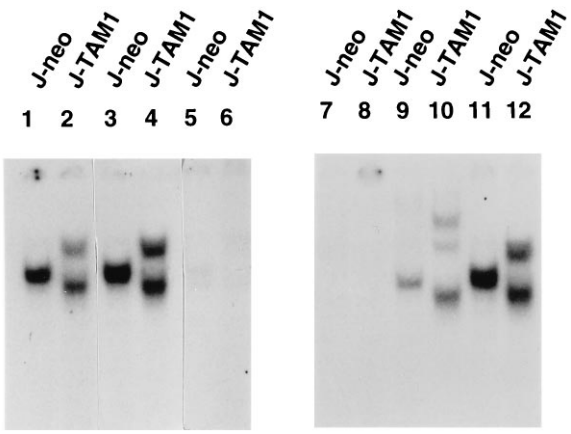

FIG. 5. EMSA of the consensus AP-1 site reveals an altered AP-1 binding pattern. EMSAs with nuclear extracts from unstimulated J-neo or J-TAM1 cell are shown. Unlabeled oligonucleotides bearing the Sp-1-binding site (lanes 3 and 4) or the AP-1-binding site (lanes 5 and 6) were added as nonspecific and specific competitors, respectively, at a 50-fold molar excess. For supershifting, binding reaction mixtures were preincubated with a pan-anti-Jun antibody (lanes 7 and 8), a specific anti-JunB antibody (lanes 9 and 10), or a specific anti-JunD antibody (lanes 11 and 12).

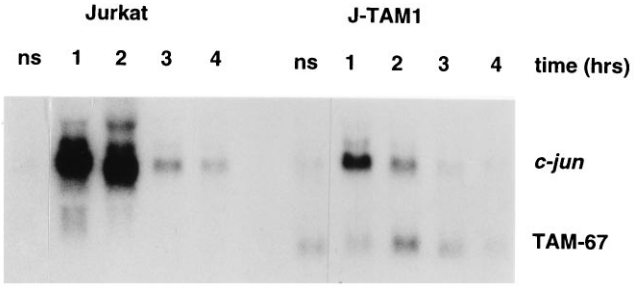

FIG. 6. Expression of TAM-67 hinders induction of c-*jun* RNA. RNA was isolated from J-neo and J-TAM1 cells stimulated with 10 ng of PMA per ml for the indicated periods of time, and blots were prepared and hybridized with a c-*jun*-specific probe. ns, nonstimulated cells.

lyzed the induction of c-*jun* mRNA levels in normal and TAM-67-expressing cells. Stimulation of J-neo cells with PMA caused an increase of c-*jun* mRNA within 30 min (data not shown) that peaked 2 h after activation (Fig. 6). In contrast, stimulation with PMA caused a much smaller increase in c-*jun* mRNA in J-TAM1 cells. The inhibition of c-*jun* mRNA upregulation was reflected in the induction of cJun protein (Fig. 7). Treatment of J-neo cells with PMA caused cJun levels to increase within 60 min, reaching a plateau at 2 to 4 h. Treatment of J-TAM1 cells (Fig. 7) and J-TAM9 cells (data not shown) with PMA also caused an increase in cJun expression with kinetics similar to that for J-neo cells, but the levels achieved were much lower. The expression of TAM-67 protein itself was unaffected by stimulation with phorbol ester. Thus, dominant negative cJun prevents the AP-1-dependent upregulation of c-*jun.*

Stable expression of dominant negative cJun blocks AP-1- and NFAT-dependent gene transactivation. The functional effects of TAM-67 on AP-1- and NFAT-dependent gene transactivation were determined. Jurkat cells were transiently transfected with reporter constructs consisting of the luciferase gene under the control of either AP-1 (Fig. 8A) or NFAT (Fig. 8B) responsive elements. Stimulation of J-neo cells with PMA resulted in up to a 160-fold increase in AP-1 reporter activity. Expression of TAM-67 resulted in a substantially decreased response in both J-TAM1 and J-TAM9 cells. Likewise, NFAT, whose induction requires a Ca^{2+} ionophore in addition to phorbol ester (30, 43, 51), was induced poorly in these cells. The relative levels of inhibition tended to correlate with TAM-67 expression, with slightly greater inhibition in J-TAM9 cells than in J-TAM1 cells. Since AP-1 and NFAT are both involved in activation-induced IL-2 production, Jurkat cells with or without TAM-67 were stimulated and IL-2 production was measured (Fig. 8C). Neither PMA nor ionomycin alone caused J-neo cells to produce IL-2, but cells stimulated with the combination secreted IL-2. Expression of TAM-67 markedly inhibited IL-2 production, as J-TAM1 and J-TAM9 secreted only 15 and 7% of the IL-2 made by J-neo cells, respectively. Thus, stable expression of TAM-67 in Jurkat T cells inhibits AP-1- and NFAT-dependent gene transactivation.

Effect of TAM-67 on Fas-mediated apoptosis. Since TAM-67 prevents AP-1 and NFAT induction by a strong stimulus (PMA plus a Ca^{2+} ionophore), we asked if it would have any effect on apoptosis of Jurkat cells. Cell surface staining revealed equivalent levels of Fas on J-neo, J-TAM1, and J-TAM9 cells (data not shown). J-neo cells incubated with anti-Fas underwent apoptosis in a dose-dependent fashion, with death of approximately 50% of the cells at 5 h with antibody concentrations of 1 to 50 ng/ml (Fig. 9). There was no difference in the dose-response curves of J-neo cells and TAM-67-expressing cells. Therefore, despite activating JNK, Fas stimulation neither activates nor requires AP-1 to induce apoptotic death in Jurkat cells.

Inhibition of JNK activity with dominant negative SEK1 does not inhibit Fas-mediated apoptosis. Although we were unable to detect any increase in AP-1 activity, or any dependence of apoptosis on AP-1 function, in Fas-activated Jurkat cells, it is possible that JNK might play a role in apoptosis via a nontranscriptional mechanism. To address this, we attempted to inhibit JNK activity in Jurkat cells by expression of a dominant negative form of SEK1, the kinase that activates JNK (57). The plasmid pEBG-SEK1 (K→R) encodes a variant of the wild-type gene in which a single point mutation results in a lysine-to-arginine substitution at position 129, abrogating enzymatic activity, and expression of this molecule prevents the activation of JNK in a dominant negative fashion (57). To determine if SEK1 (K→R) prevented Fas-dependent JNK activation, Jurkat cells were transfected with a plasmid encoding IL-2Rα chain with either the empty pEBG vector or pEBG-SEK1 (K→R). Successfully transfected cells were isolated with magnetic beads coated with anti-IL-2Rα antibodies. Cells that bound (expressing dominant negative SEK1) and that did not bind (not expressing dominant negative SEK1) to the beads were assayed for JNK activity after being cultured in medium alone or with CH-11 (1,000 ng/ml). As shown in Fig. 10A, Fas induced JNK activity in Jurkat cells that had been electroporated in the presence of pcI Tac and the control pEBG plasmid or the pEBG-SEK1 (K→R) plasmid but did not bind the anti-IL-2Rα beads (i.e., nontransfected cells). In Jurkat cells

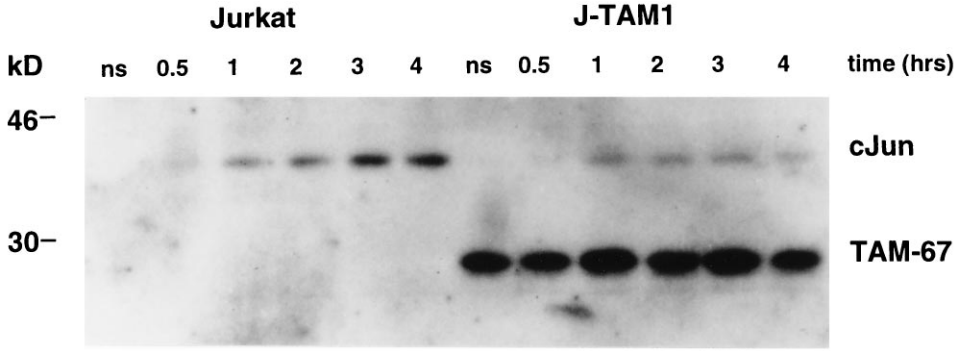

FIG. 7. TAM-67 expression inhibits induction of cJun protein. Cells were stimulated with PMA and ionomycin for the indicated times, after which nuclear extracts were prepared and subjected to immunoblot analysis with a cJun-specific antibody. Nonstimulated cells (ns) were collected at 4 h.

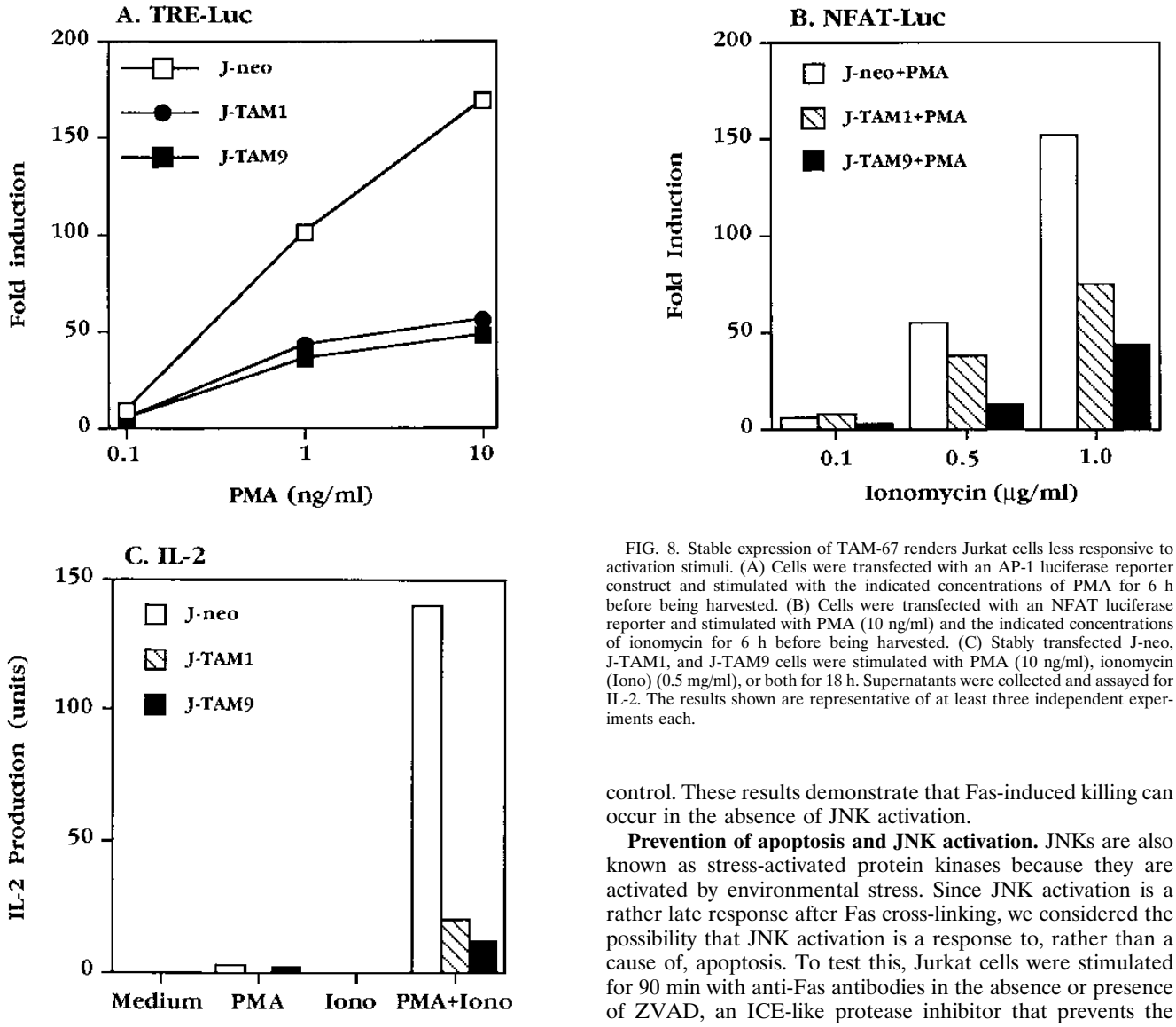

FIG. 8. Stable expression of TAM-67 renders Jurkat cells less responsive to activation stimuli. (A) Cells were transfected with an AP-1 luciferase reporter construct and stimulated with the indicated concentrations of PMA for 6 h before being harvested. (B) Cells were transfected with an NFAT luciferase reporter and stimulated with PMA (10 ng/ml) and the indicated concentrations of ionomycin for 6 h before being harvested. (C) Stably transfected J-neo, J-TAM1, and J-TAM9 cells were stimulated with PMA (10 ng/ml), ionomycin (Iono) (0.5 mg/ml), or both for 18 h. Supernatants were collected and assayed for IL-2. The results shown are representative of at least three independent experiments each.

from the same electroporations that did bind to anti-IL-2Rα beads, anti-Fas induced JNK activity in cells that received the empty vector. In contrast, anti-Fas failed to induce JNK activity in cells that had been transfected with dominant negative SEK1. Similar results were obtained in four independent experiments. Therefore, the dominant negative SEK1 was able to prevent induction of JNK activity by anti-Fas in Jurkat cells.

To determine what effect inhibition of Fas-induced JNK activity might have on apoptosis, the death of similarly transfected Jurkat cells stimulated with anti-Fas was assessed. This was done by transfecting Jurkat cells with a plasmid encoding β-gal plus the empty pEBG vector or the pEBG-SEK1 (K→R) vector. After 12 h the cells were cultured for an additional 5 h in the absence or presence of CH-11, and the percent specific cytotoxicity was determined as described previously (44). As shown for one representative experiment of seven (Fig. 10B), anti-Fas induced cell death in a dose-responsive manner. Importantly, the sensitivity of transfected Jurkat cells to Fas-mediated killing was the same in cells transfected with dominant negative SEK1 as in those that received the vector control. These results demonstrate that Fas-induced killing can occur in the absence of JNK activation.

Prevention of apoptosis and JNK activation. JNKs are also known as stress-activated protein kinases because they are activated by environmental stress. Since JNK activation is a rather late response after Fas cross-linking, we considered the possibility that JNK activation is a response to, rather than a cause of, apoptosis. To test this, Jurkat cells were stimulated for 90 min with anti-Fas antibodies in the absence or presence of ZVAD, an ICE-like protease inhibitor that prevents the activation of a Fas-induced protease as well as apoptosis and ceramide production caused by REAPER (54, 61). Apoptosis was measured with fluoresceinated annexin V, a protein that binds with affinity to phosphotidylserine moieties exposed on the surface of apoptotic cells, which has been found to be an early indicator of apoptosis (42). At CH-11 concentrations of 30 and 100 ng/ml, approximately 80% of the cells bound annexin V (Fig. 11). The large majority (85 to 90%) of these cells still excluded propidium iodide, indicating that they were viable at this time. ZVAD completely inhibited apoptosis as judged by binding of annexin V (Fig. 11A) and DNA fragmentation measured in parallel cultures (Fig. 11B). Consistent with recent reports (62), a related compound, the ICE inhibitor YVAD, had no effect on Fas-induced apoptosis. Both concentrations of CH-11 induced JNK activity (Fig. 11C). While YVAD had no effect, ZVAD completely prevented the JNK activity caused by 30 ng of CH-11 per ml. In contrast, although ZVAD prevented cell death at 100 ng of CH-11 per ml as well as it did at 30 ng/ml, it had little or no effect on JNK activity at 100 ng of CH-11 per ml. These data are representative of four independent experiments. We conclude that at relatively low levels of Fas cross-linking, which are nonetheless sufficient for

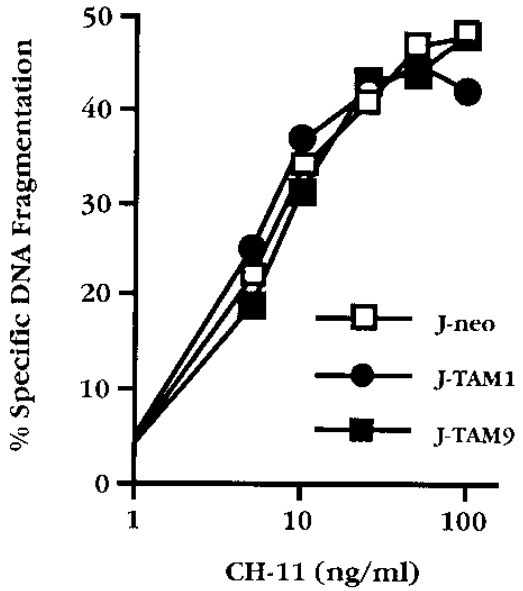

FIG. 9. TAM-67 expression does not alter Fas-mediated death in Jurkat cells. Cells labeled with [^{3}H]thymidine were incubated for 5 h with the indicated concentrations of anti-human Fas IgM. Samples were harvested, and the percent specific DNA fragmentation compared to that of untreated samples is shown. The data shown are representative of six independent experiments.

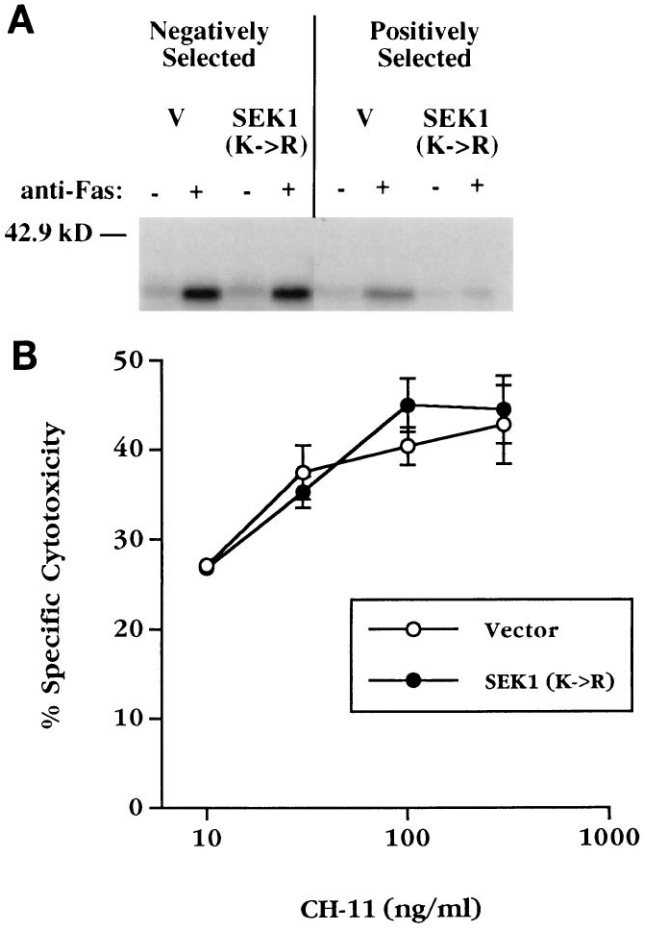

FIG. 10. Inhibition of JNK activity does not diminish Fas-mediated cytotoxicity. (A) Jurkat cells were electroporated in the presence of pcI Tac plus the empty pEBG vector (V) or pEBG-SEK1 (K→R). After 14 h, the cells were allowed to adhere to magnetic beads coated with anti-IL-2Rα antibodies for 60 min. After separation, equal numbers of adherent (positively selected) and nonadherent (negatively selected) cells were cultured for 60 min in medium alone or with CH-11 (1,000 ng/ml). Cell lysates were prepared, and JNK activity was measured. (B) Jurkat cells were electroporated in the presence of CMV-β-gal plus empty pEBG or pEBG-SEK1 (K→R). After 12 h, the cells were cultured in medium alone or with the indicated concentrations of CH-11. After 5 h, β-gal activity was measured and the percent specific cytotoxicity was determined as described in Materials and Methods. The error bars represent the standard deviations from duplicate samples.

induction of death, inhibition of apoptosis prevents JNK activation. At "supraphysiologic" levels of Fas cross-linking, however, JNK may be directly activated.

DISCUSSION

AP-1 is a ubiquitous transcription factor that binds specific DNA sequences found in the enhancer/promoter regions of many genes. AP-1 also contributes to transcriptional regulation by complexing with NFATp/c/x, Oct-1, NF-κB p65, and Ets family members (7, 67, 74). AP-1 and NFAT are distal components of the Ras signaling pathway (6, 27, 29, 64). Activation of Ras, a small GTP-binding protein, results in a cascade of events that begins with the activation of the Raf serine/threonine kinase (40). Raf phosphorylates and activates MAPK kinase (MEK), which in turn activates the ERK1 and ERK2 kinases. A parallel Ras-activated pathway induces MEK kinase (MEKK) activity, which causes the phosphorylation and activation of a family of kinases known as MEK/stress-induced ERK kinase (MEK/SEK) and results in the phosphorylation and activation of JNK (19, 38, 47, 63). In some cell lines JNK activity can be induced via a Ras-independent pathway, utilizing instead other small GTP-binding proteins, Rac1 and cdc42, to activate MEKK activity (16), while in others these Rho subfamily GTPases appear to serve as an intermediary between Ras and MEKK in the signaling cascade (46). JNK in turn activates cJun by phosphorylation of two serine residues in its amino terminus (48). Ras-dependent kinase activation also results in increased transcription of both c-*jun* and c-*fos* (64, 72, 79). Thus, activation of Ras results in increased transactivation activity of previously synthesized cJun as well as an increase in the levels of AP-1 components.

A number of studies have implicated Ras, JNK, and AP-1 in signaling for apoptosis. For example, withdrawal of NGF from PC-12 pheochromocytoma cells resulted in increased JNK activity followed by apoptotic death (78). The apoptosis was prevented by expression of TAM-67, suggesting a role for AP-1 in this process. Furthermore, expression of MEKK, which activates JNK, resulted in the apoptotic death of fibroblasts, suggesting that under some circumstances JNK signaling alone might be sufficient to cause cell death (32). In other studies it has been shown that ceramides, which are generated at the plasma membrane by the action of sphingomyelinases, activate MAPK and JNK and induce apoptosis in a variety of cells (55, 76). In one study using HL-60 cells, pharmacologic inhibition of AP-1 activity or treatment with c-*jun* antisense oligonucleotides prevented ceramide-induced apoptosis (60). It has recently been reported that stress-inducing stimuli (ceramides, X-rays, UV light, and TNF-α) also induce JNK activity in U937 monoblastic leukemia and bovine aortic endothelial cells and that dominant negative SEK1 (which inhibits JNK activity) and TAM-67 prevent apoptosis (75). How JNK and cjun participate in these forms of apoptosis is not clear, since the death of these cells, just as for Fas-induced apoptosis (20), is not prevented by inhibitors of RNA or protein synthesis (41). It was

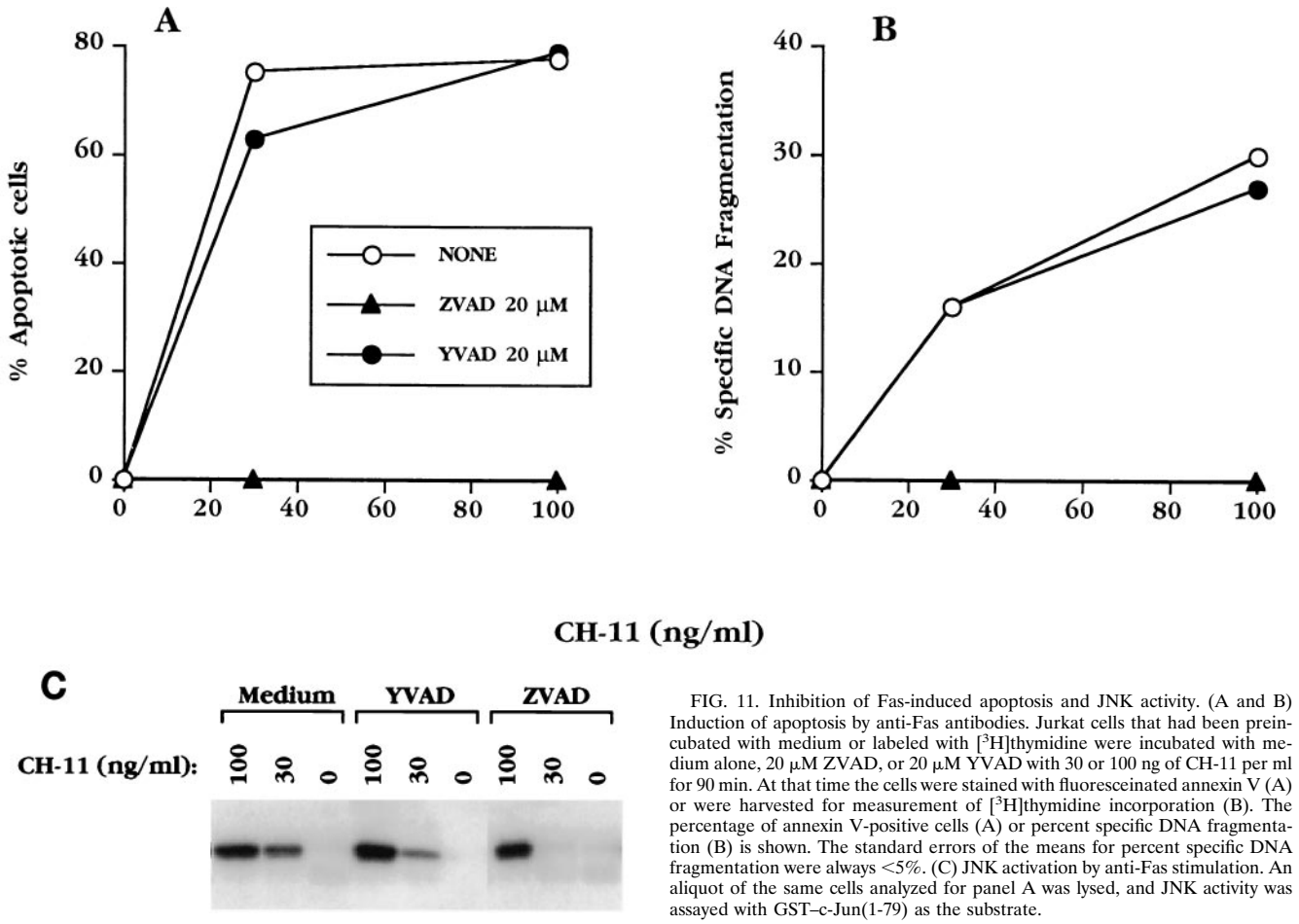

FIG. 11. Inhibition of Fas-induced apoptosis and JNK activity. (A and B) Induction of apoptosis by anti-Fas antibodies. Jurkat cells that had been preincubated with medium or labeled with [^{3}H]thymidine were incubated with medium alone, 20 μM ZVAD, or 20 μM YVAD with 30 or 100 ng of CH-11 per ml for 90 min. At that time the cells were stained with fluoresceinated annexin V (A) or were harvested for measurement of [^{3}H]thymidine incorporation (B). The percentage of annexin V-positive cells (A) or percent specific DNA fragmentation (B) is shown. The standard errors of the means for percent specific DNA fragmentation were always <5%. (C) JNK activation by anti-Fas stimulation. An aliquot of the same cells analyzed for panel A was lysed, and JNK activity was assayed with GST–c-Jun(1-79) as the substrate.

suggested that because of the well-described ability of AP-1 to bind to other regulatory molecules, cJun might participate in apoptosis by regulating the function of as-yet-unknown proteins involved in this response (75). Fas cross-linking also results in the production of ceramides, and it has been reported that signaling via Fas in Jurkat cells results in the activation of Ras and that this is a critical step in the process leading to cell death (23). In the present study, we investigated the consequences of Fas signaling for JNK and AP-1 activation. Fas ligation did cause a large increase in JNK activity. It is likely, however, that this is not a simple response to Ras activation. First, the kinetics of JNK activation are delayed, occurring at 60 to 90 min after cross-linking of Fas. This is in contrast to JNK induction in fibroblasts or epithelial cells by epidermal growth factor, UV irradiation, or TNF, which occurs within 15 min (48). We have also found that ceramide induction of JNK in Jurkat cells is already at plateau levels by 15 min (our unpublished observation). Another study recently reported that treatment of Jurkat cells with CH-11, at a concentration of 2,000 ng/ml, induced detectable JNK activity 10 min after stimulation (35). We have also been able to detect JNK activity 10 min after stimulation with very high concentrations of CH-11 (1,000 to 2,000 ng/ml) (data not shown), although as shown in Fig. 1B, 60 to 90 min is required with CH-11 concentrations of 30 to 100 ng/ml. Since in Jurkat cells the concentration of CH-11 required to cause early JNK activation is approximately 100-fold greater than that required to achieve apoptosis (Fig. 9), whether this finding has biological significance is unclear. Second, JNKs are also known as stress-activated protein kinases because they are induced under conditions of environmental stress or exposure to proinflammatory cytokines (18). Since Fas cross-linking causes cell death, we considered the possibility that JNK activation was not a direct response to Fas ligation but rather was a response to the stress of apoptosis. Consistent with this, expression of dominant negative SEK1 did not inhibit Fas-induced apoptosis, and a protease inhibitor that completely inhibited apoptosis caused by 30 ng of CH-11 per ml also prevented the upregulation of JNK activity. These results support the hypothesis that it is cell death, not direct signaling via Fas, that induces JNK activity under these conditions. Interestingly, despite inhibiting apoptosis, ZVAD did not prevent JNK activation when higher concentrations of CH-11 were used. It is possible, therefore, that extensive ligation of Fas may induce JNK activity, although with relatively delayed kinetics. Since 30 and 100 ng of CH-11 per ml induced apoptosis to an equivalent degree, the physiologic significance of inducing JNK activity at high levels of Fas cross-linking is unknown.

Despite elevation of JNK activity, at no concentration did anti-Fas induce a detectable increase in AP-1 activity. The failure to detect an increase in AP-1 binding by gel shift was confirmed with a sensitive assay using an AP-1-responsive reporter construct. The reason for the dichotomy between JNK and AP-1 activation is not clear, but there are several interesting possibilities. First, it is possible that c-*fos* is not induced by signaling via Fas. In T cells, JNK and ERK appear to have

different requirements for activation, since while phorbol ester is sufficient to activate the latter, activation of JNK requires calcium ionophore as an additional stimulus (68). We are currently exploring the regulation of c-*fos* in Fas-stimulated cells. Second, AP-1 activity is regulated both positively and negatively by serine/threonine phosphorylation (11). Thus, if Fas induces JNK to phosphorylate the activating cJun NH_2-terminal residues without dephosphorylation of the negative regulatory residues (37), there may be no increase in AP-1 activity. Third, the activity of AP-1 can be inhibited by its interactions with other proteins, such as the glucocorticoid receptor, the retinoic acid receptor, and the adenovirus E1-A protein (3, 53). In addition, AP-1 activity is downregulated by binding to its inhibitory protein, IP-1, a protein whose function is reversibly regulated by phosphorylation (4, 5). In unactivated cells, unphosphorylated IP-1 associates with Fos and Jun; after phosphorylation by activated protein kinase A, IP-1 releases Fos and Jun so they may bind DNA and function as transactivators. It is possible that while signaling via Fas induces JNK activity, it also promotes an interaction between AP-1 and one or more molecules that inhibit its biological activity, a possibility that is currently being explored.

A dominant negative approach was used to test the possibility that AP-1, perhaps at constitutive levels, was involved in Fas-mediated apoptosis. To disable all AP-1-containing transcription factors, a dominant negative cJun was stably introduced into Jurkat T cells. TAM-67 binds both Jun and Fos family members and functionally inactivates AP-1 and NFAT (12, 52). TAM-67 has been shown to block TPA/Ras-dependent transformation of embryonal cells (1, 12) and the tumorigenicity of an epidermal cell line (21), and it inhibited apoptosis of PC-12 cells deprived of NGF (78). Although cJun can bind to Oct-1 and NF-κB p65 in vitro (67, 74) and NF-κB activation of the human immunodeficiency virus long terminal repeat is inhibited by antisense *c-jun* (67), the activity of these transcription factors is not inhibited by TAM-67 in Jurkat cells (52). Since TAM-67 retains the cJun DNA-binding domain, it has the potential to cause molecular interference via two different mechanisms: (i) homodimerization and direct competition with endogenous AP-1 for DNA binding or (ii) heterodimerization with endogenous Fos and/or Jun family members and quenching of their transactivational activity. For TAM-67, the latter appears to be the predominant mechanism, since a TAM-67/GCN4 chimera, which can only homodimerize and thus can act only by blocking AP-1-binding sites, did not inhibit AP-1 gene transactivation, while a TAM-67/Fos chimera, which retains the ability to heterodimerize with cJun, did (13). As shown in this report, TAM-67 employs an additional mechanism to inhibit AP-1 activity, in this case by preventing the autoregulated upregulation of cJun, the most transcriptionally active of the Jun family members. Nuclear extracts from unstimulated TAM-67-expressing Jurkat cells yielded an AP-1-binding complex containing JunB and a faster-migrating complex that probably contains the smaller TAM-67 protein. The other component of the slower-migrating complex is unknown, and in fact it is possible that it either represents multiples of dimers due to the high levels of TAM-67 or is a complex of TAM-67 with an Ets family member, transcription factors recently shown to bind to Jun family members independent of DNA binding (7). Our results differ from those obtained with epidermal cells transfected with TAM-67, in which no alteration in the AP-1 gel shift pattern was observed (21). This may reflect an alteration in the regulation of AP-1 family members expressed. It has recently been reported that developmental and/or experimentally induced programmed cell death of neuronal cells and thymocytes occurs normally in mice that lack c-*jun*, c-*fos*, or both (56). In this case it is possible that other members of these complex families might have substituted for c-*jun* or c-*fos*. Nonetheless, the results are consistent the notion that there are pathways leading to apoptosis that do not involve AP-1.

The results of this study indicate that AP-1 is neither activated by stimulation via Fas nor, unlike for TNFR-mediated killing (75), required for Fas-mediated apoptosis in Jurkat cells. Furthermore, JNK activation is likely to be a result, rather than a cause, of Fas-mediated apoptosis, at least at levels of Fas ligation that are sufficient to cause maximal levels of apoptosis. Further studies with cells transfected with cDNAs encoding other enzymes in the Ras signaling pathway, or their dominant negative forms, may help in exploring what role, if any, JNK and MAPK family members might play in Fas signal transduction.

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ADDENDUM IN PROOF

Since the submission of this article, Wilson et al. have reported that signaling via Fas induces JNK activity in T cells (D. J. Wilson, K. A. Fortner, D. H. Lynch, R. R. Mattingly, I. G. Macara, J. A. Posada, and R. C. Budd, Eur. J. Immunol. **26:**989–994).

REFERENCES

1. **Alani, R., P. Brown, B. Binetruy, H. Dosaka, R. K. Posenberg, P. Angel, M. Karin, and M. J. Birrer.** 1991. The transactivation domain of the *c-jun* proto-oncogene is required for cotransformation of rat embryo cells. Mol. Cell. Biol. **11:**6286–6295.
2. **Angel, P., K. Hattori, T. Smeal, and M. Karin.** 1988. The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell **55:**875–885.
3. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta **1072:**129–157.
4. **Auwerx, J., and P. Sassone-Corsi.** 1991. IP-1: a dominant inhibitor of Fos/Jun whose activity is modulated by phosphorylation. Cell **64:**983–993.
5. **Auwerx, J., and P. Sassone-Corsi.** 1992. AP-1 (Fos-Jun) regulation by IP-1: effect of signal transduction pathways and cell growth. Oncogene **7:**2271–2280.
6. **Baldari, C. T., A. Heguy, and J. L. Telford.** 1993. Ras protein activity is essential for T-cell antigen receptor signal transduction. J. Biol. Chem. **268:**2693–2698.
7. **Bassuk, A. G., and J. M. Leiden.** 1995. A direct physical association between ETS and AP-1 transcription factors in normal human T cells. Immunity **3:**223–237.
8. **Binetruy, B., T. Smeal, and M. Karin.** 1991. Ha-ras augments c-jun activation and stimulates phosphorylation of its activation domains. Nature **351:**122–127.
9. **Boldin, M. P., T. M. Goncharov, Y. V. Goltsev, and D. Wallach.** 1996. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell **85:**803–815.
10. **Boldin, M. P., E. E. Varfolomeev, Z. Pancer, I. L. Mett, J. H. Camonis, and D. Wallach.** 1995. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J. Biol. Chem. **270:**7795–7798.
11. **Boyle, W. J., T. Smeal, L. H. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter.** 1991. Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell **64:**573–584.
12. **Brown, P. H., R. Alani, L. H. Preiss, E. Szabo, and M. J. Birrer.** 1993. Suppression of oncogene-induced transformation by a deletion mutant of c-jun. Oncogene **8:**877–886.
13. **Brown, P. H., T. K. Chen, and M. J. Birrer.** 1994. Mechanism of action of a dominant-negative mutant of c-Jun. Oncogene **9:**791–799.
14. **Chinnaiyan, A. M., K. O'Rourke, M. Tewari, and V. M. Dixit.** 1995. FADD,

a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell **81:**505–512.
15. **Coso, O. A., M. Chiariello, G. Kalinec, J. M. Kyriakis, J. Woodgett, and J. S. Gutkind.** 1995. Transforming G protein-coupled receptors potently activate JNK (SAPK). Evidence for a divergence from the tyrosine kinase signaling pathway. J. Biol. Chem. **270:**5620–5624.
16. **Coso, O. A., M. Chiariello, J. C. Yu, H. Teramoto, P. Crespo, N. Xu, T. Miki, and J. S. Gutkind.** 1995. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell **81:**1137–1146.
17. **Curran, T., and B. R. Franza, Jr.** 1988. Fos and Jun: the AP-1 connection. Cell **55:**395–397.
18. **Davis, R. J.** 1994. MAPKs: new JNK expands the group. Trends Biochem. Sci. **19:**470–473.
19. **Derijard, B., M. Hibi, I. H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R. J. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
20. **Dhein, J., H. Walczak, C. Bäumler, K.-M. Debatin, and P. H. Krammer.** 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature **373:** 438–441.
21. **Domann, F. E., J. P. Levy, M. J. Birrer, and G. T. Bowden.** 1994. Stable expression of a c-JUN deletion mutant in two malignant mouse epidermal cell lines blocks tumor formation in nude mice. Cell Growth Diff. **5:**9–16.
22. **Goldstone, S. D., and M. F. Lavin.** 1994. Prolonged expression of c-jun and associated activity of the transcription factor AP-1, during apoptosis in a human leukaemic cell line. Oncogene **9:**2305–2311.
23. **Gulbins, E., R. Bissonnette, A. Mahboubi, S. Martin, W. Nishioka, T. Brunner, G. Baier, G. Baier-Bitterlich, C. Byrd, F. Lang, R. Kolesnick, A. Altman, and D. Green.** 1995. FAS-induced apoptosis is mediated via a ceramide-initiated RAS signaling pathway. Immunity **2:**341–351.
24. **Gupta, S., D. Campbell, B. Derijard, and R. J. Davis.** 1995. Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science **267:**389–393.
25. **Haimovitz-Friedman, A., C. C. Kan, D. Ehleiter, R. S. Persaud, M. McLoughlin, Z. Fuks, and R. N. Kolesnick.** 1994. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J. Exp. Med. **180:**525–535.
26. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:**2135–2148.
27. **Hirai, S., Y. Izumi, K. Higa, K. Kaibuchi, K. Mizuno, S. Osada, K. Suzuki, and S. Ohno.** 1994. Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKC δ. EMBO J. **13:**2331–2340.
28. **Hirai, S. I., R. P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv.** 1989. Characterization of junD: a new member of the jun proto-oncogene family. EMBO J. **8:**1433–1439.
29. **Imler, J. L., C. Schatz, C. Wasylyk, B. Chatton, and B. Wasylyk.** 1988. A Harvey-ras responsive transcription element is also responsive to a tumour-promoter and to serum. Nature **332:**275–278.
30. **Jain, J., P. G. McCaffrey, Z. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran, and A. Rao.** 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature **365:**352–355.
31. **Jain, V. K., and I. T. Magrath.** 1991. A chemiluminescent assay for quantitation of β-galactosidase in fentogram range: application to quantitation of β-galactosidase in lacZ-transfected cells. Anal. Biochem. **199:**119–124.
32. **Johnson, N. L., A. M. Gardner, K. M. Diener, C. A. Lange-Carter, J. Gleavy, M. B. Jarpe, A. Minden, M. Karin, L. I. Zon, and G. L. Johnson.** 1996. Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. J. Biol. Chem. **271:**3229–3237.
33. **Kim, M. Y., C. Linardic, L. Obeid, and Y. Hannun.** 1991. Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation. J. Biol. Chem. **266:**484–489.
34. **Kischkel, F. C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P. H. Krammer, and M. E. Peter.** 1995. Cytotoxicity-dependent AP-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. **14:**5579–5588.
35. **Latinis, K. M., and G. A. Koretzky.** 1996. Fas ligation induces apoptosis and Jun kinase activation independently of CD45 and Lck in human T cells. Blood **87:**871–875.
36. **Leonard, W. J., J. M. Depper, G. R. Crabtree, S. Rudikoff, J. Pumphery, R. J. Robb, M. Kronke, P. B. Svetlik, N. J. Peffer, T. A. Waldmann, and W. C. Greene.** 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor. Nature **311:**626–631.
37. **Lin, A., J. Frost, T. Deng, T. Smeal, N. al-Alawi, U. Kikkawa, T. Hunter, D. Brenner, and M. Karin.** 1992. Casein kinase II is a negative regulator of c-Jun DNA binding and AP-1 activity. Cell **70:**777–789.
38. **Lin, A., A. Minden, H. Martinetto, F. X. Claret, C. Lange-Carter, F. Mercurio, G. L. Johnson, and M. Karin.** 1995. Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science **268:**286–290.
39. **MacGregor, G. R., and C. T. Caskey.** 1989. Construction of plasmids that express E. coli β-galactosidase in mammalian cells. Nucleic Acids Res. **17:** 2365.
40. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.
41. **Martin, S. J.** 1993. Apoptosis: suicide, execution or murder? Trends Cell Biol. **3:**141–144.
42. **Martin, S. J., C. P. Reutelingsperger, A. J. McGahon, J. A. Rader, R. C. van Schie, D. M. LaFace, and D. R. Green.** 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J. Exp. Med. **182:**1545–1556.
43. **McCaffrey, P. G., C. Luo, T. K. Kerppola, J. Jain, T. M. Badalian, A. M. Ho, E. Burgeon, W. S. Lane, J. N. Lambert, T. Curran, G. L. Verdine, A. Rao, and P. G. Hogan.** 1993. Isolation of the cyclosporin-sensitive T cell transcription factor, NFATp. Science **262:**750–754.
44. **Memon, S. A., D. Petrak, M. B. Moreno, and C. M. Zacharchuk.** 1995. A simple assay for examining the effect of transiently expressed genes on programmed cell death. J. Immunol. Methods **180:**15–24.
45. **Mercep, M., J. A. Bluestone, P. D. Noguchi, and J. D. Ashwell.** 1988. Inhibition of transformed T cell growth in vitro by monoclonal antibodies directed against distinct activating molecules. J. Immunol. **140:**324–335.
46. **Minden, A., A. Lin, F. X. Claret, A. Abo, and M. Karin.** 1995. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell **81:**1147–1157.
47. **Minden, A., A. Lin, M. McMahon, C. Lange-Carter, B. Derijard, R. J. Davis, G. L. Johnson, and M. Karin.** 1994. Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science **266:**1719–1723.
48. **Minden, A., A. Lin, T. Smeal, B. Derijard, M. Cobb, R. Davis, and M. Karin.** 1994. cJun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol. Cell. Biol. **14:**6683–6688.
49. **Muzio, M., A. M. Chinnaiyan, F. C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J. D. Bretz, M. Zhang, R. Rentz, M. Mann, P. H. Krammer, M. E. Peter, and V. M. Dixit.** 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell **85:**817–827.
50. **Nagata, S., and P. Golstein.** 1995. The Fas death factor. Science **267:**1449–1456.
51. **Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree.** 1994. NF-AT components define a family of transcription factors targeted in T cell activation. Nature **369:**497–502.
52. **Petrak, D., S. Memon, M. J. Birrer, J. D. Ashwell, and C. M. Zacharchuk.** 1994. Dominant negative mutant of c-Jun inhibits NF-AT transcriptional activity and prevents IL-2 gene transcription. J. Immunol. **153:**2046–2051.
53. **Ponta, H., A. C. B. Cato, and P. Herrlich.** 1992. Interference of pathway specific transcription factors. Biochim. Biophys. Acta **1129:**255–261.
54. **Pronk, G. J., K. Ramer, P. Amiri, and L. T. Williams.** 1996. Requirement of an ICE-like protease for induction of apoptosis and ceramide generation by REAPER. Science **271:**808–809.
55. **Raines, M. A., R. N. Kolesnick, and D. W. Golde.** 1993. Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. J. Biol. Chem. **268:**14572–14575.
56. **Roffler-Tarlov, S., J. J. Brown, E. Tarlov, J. Stolarov, D. L. Chapman, M. Alexiou, and V. E. Papaioannou.** 1996. Programmed cell death in the absence of c-Fos and c-Jun. Development **122:**1–9.
57. **Sánchez, I., R. T. Hughes, B. J. Mayer, K. Yee, J. R. Woodgett, J. Avruch, J. M. Kyriakis, and L. I. Zon.** 1994. Roles of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature **372:** 794–798.
58. **Sassone-Corsi, P., W. W. Lamph, M. Kamps, and I. M. Verma.** 1988. fos-associated cellular p39 is related to nuclear transcription factor AP-1. Cell **54:**553–560.
59. **Sato, T., S. Irie, S. Kitada, and J. C. Reed.** 1995. FAP-1: a protein tyrosine phosphatase that associates with Fas. Science **268:**411–415.
60. **Sawai, H., T. Okazaki, H. Yamamoto, H. Okano, Y. Takeda, M. Tashima, H. Sawada, M. Okuma, H. Ishikura, H. Umehara, and N. Domae.** 1995. Requirement of AP-1 for ceramide-induced apoptosis in human leukemia HL-60 cells. J. Biol. Chem. **270:**27326–27331.
61. **Schlegel, J., I. Peters, and S. Orrenius.** 1995. Isolation and partial characterization of a protease involved in Fas-induced apoptosis. FEBS Lett. **364:** 139–142.
62. **Schlegel, J., I. Peters, S. Orrenius, D. K. Miller, N. A. Thornberry, T.-T. Yamin, and D. W. Nicholson.** 1996. CPP32/apopain is a key interleukin 1β converting enzyme-like protease involved in Fas-mediated apoptosis. J. Biol. Chem. **271:**1841–1844.
63. **Seger, R., and E. G. Krebs.** 1995. The MAPK signaling cascade. FASEB J. **9:**726–735.
64. **Sistonen, L., E. Holtta, T. P. Makela, J. Keski-Oja, and K. Alitalo.** 1989. The

cellular response to induction of the p21 c-Ha-ras oncoprotein includes stimulation of jun gene expression. EMBO J. **8:**815–822.
65. **Smeal, T., B. Binetruy, D. A. Mercola, M. Birrer, and M. Karin.** 1991. Oncogenic and transcriptional cooperation with Ha-ras requires phosphorylation of *c-jun* on serines 63 and 73. Nature **354:**494–496.
66. **Stanger, B. Z., P. Leder, T.-H. Lee, E. Kim, and B. Seed.** 1995. RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell **81:**513–523.
67. **Stein, B., A. S. Baldwin, D. W. Ballard, W. C. Greene, P. Angel, and P. Herrlich.** 1993. Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J. **12:**3879–3891.
68. **Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah.** 1994. JNK is involved in signal integration during costimulation of T lymphocytes. Cell **77:**727–736.
69. **Tepper, C. G., S. Jayadev, B. Liu, A. Bielawska, R. Wolff, S. Yonehara, Y. A. Hannun, and M. F. Seldin.** 1995. Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity. Proc. Natl. Acad. Sci. USA **92:**8443–8447.
70. **Tian, Q., M. Streuli, H. Saito, S. F. Schlossman, and P. Anderson.** 1991. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. Cell **67:**629–639.
71. **Tian, Q., J. Taupin, S. Elledge, M. Robertson, and P. Anderson.** 1995. Fas-activated serine/threonine kinase (FAST) phosphorylates TIA-1 during Fas-mediated apoptosis. J. Exp. Med. **182:**865–874.
72. **Uberall, F., S. Kampfer, W. Doppler, and H. H. Grunicke.** 1994. Activation of c-fos expression by transforming Ha-ras in HC11 mouse mammary epithelial cells is PKC-dependent and mediated by the serum response element. Cell Signal. **6:**285–297.
73. **Uchiyama, T., S. Broder, and T. A. Waldmann.** 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. J. Immunol. **126:**1393–1397.
74. **Ullman, K. S., J. P. Northrop, A. Admon, and G. R. Crabtree.** 1993. Jun family members are controlled by a calcium-regulated, cyclosporin A-sensitive signaling pathway in activated T lymphocytes. Genes Dev. **7:**188–196.
75. **Verheij, M., R. Bose, X. H. Lin, B. Yao, W. D. Jarvis, S. K. Grant, K, M. J. Birrer, E. Szabo, L. I. Zon, J. M. Kyriakis, A. Haimovitz-Friedman, Z. Fuks, and R. N. Kolesnick.** 1996. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature **380:**75–79.
76. **Westwick, J. K., A. E. Bielawska, G. Dbaibo, Y. A. Hannun, and D. A. Brenner.** 1995. Ceramide activates the stress-activated protein kinases. J. Biol. Chem. **270:**22689–22692.
77. **Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis.** 1995. Integration of MAP kinase signal transduction pathways at the serum response element. Science **269:**403–407.
78. **Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg.** 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270:**1326–1331.
79. **Yamauchi, K., K. Holt, and J. E. Pessin.** 1993. Phosphatidylinositol 3-kinase functions upstream of Ras and Raf in mediating insulin stimulation of c-fos transcription. J. Biol. Chem. **268:**14597–14600.
80. **Yang, Y., M. Mercep, C. F. Ware, and J. D. Ashwell.** 1995. Fas and activation-induced Fas ligand mediate apoptosis of T cell hybridomas: inhibition of Fas ligand expression by retinoic acid and glucocorticoids. J. Exp. Med. **181:** 1673–1682.