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Suppression of Fas/APO-1-Mediated Apoptosis by Mitogen-Activated Kinase Signaling[1]

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Jurkat T cells undergo rapid apoptosis upon stimulation of the Fas/APO-1 (CD95) receptor. We examined the role of the mitogen-activated protein kinase (MAPK) cascade as a negative regulator of Fas-mediated apoptosis. To this end, we used both physiologic and artificial activators of MAPK, all of which activate MAPK by distinct routes. MAPK activity could be efficiently elevated by two T cell mitogens, the lectin PHA and an agonistic Ab to the T cell receptor complex as well as by the type 1 and 2A phosphatase inhibitor, calyculin A, and the protein kinase C-activating phorbol ester, tetradecanoyl phorbol acetate. All these treatments were effective in preventing the characteristic early and late features of Fas-mediated apoptosis, including activation of caspases. Our results indicate that the elevated MAPK activities intervene upstream of caspase activation. The degree of MAPK activation by the different stimuli used in our study corresponds well to their potency to inhibit apoptosis, indicating that MAPK activation serves as an efficient modulator of Fas-mediated apoptosis. The role of MAPK in modulation of Fas-mediated apoptosis was further corroborated by transient transfection with constitutively active MAPK kinase, resulting in complete inhibition of the Fas response, whereas transfection with a dominant negative form of MAPK kinase had no effect. Furthermore, the apoptosis inhibitory effect of the MAPK activators could be abolished by the specific MAPK kinase inhibitor PD 098059. Modulation of Fas responses by MAPK signaling may determine the persistence of an immune response and may explain the insensitivity of recently activated T cells to Fas receptor stimulation. ***The Journal of Immunology*****, 1998, 160: 2626–2636.**

Apoptosis or programmed cell death is an essential mechanism for cell removal during embryonic development and in the mature organism as well as under various pathologic conditions. A number of physiologic mediators of apoptosis have been identified. One of them is the Fas/APO-1 receptor (CD95), a cell surface receptor belonging to the TNF receptor family. The Fas receptor is a 48-kDa transmembrane glycoprotein (1), the cytoplasmic domain of which contains a region with significant sequence homology to the p55 TNF receptor (1, 2). Ligation of the Fas receptor with its specific ligand (3) or with anti-Fas Abs (4) induces apoptosis in many cell types of both hemopoietic (1, 2, 5) and nonhemopoietic (6, 7) origin.

A number of recent studies have shown that caspases, i.e., cysteine proteases with homology to the IL-1β-converting enzyme and CED-3, act as both proximal and distal effectors in Fas-mediated apoptosis (8–10). However, the mechanisms of Fas receptor activation are still insufficiently characterized. Several studies have indicated that phosphorylation-based signaling could be involved in both inhibition and activation of Fas-mediated apoptosis (11–16). It is not known how the receptor activity is modulated. There are clear indications that the receptor, although expressed, may not always generate apoptosis, but may be inactive at times or even generate accelerated cell growth (17, 18). This kind of modulation could occur through phosphorylation-based signaling.

A possible signaling candidate for regulation of Fas receptor activity is the mitogen-activated protein kinase (MAPK)[3] cascade. This major signal transduction pathway is involved in regulation of cell proliferation and differentiation as well as in various metabolic functions (19, 20). Activation of the Raf-MAPK kinase-MAPK pathway is often associated with stimulation of cell division rates, and consequently, these kinases could promote cell survival by inhibition of apoptosis. Evidence in support of this hypothesis has been obtained from a study of PC-12 cells showing that inhibition of MAPK and activation of the stress-activated protein kinase, or Jun N-terminal kinase, and p38 kinase are critical for apoptosis induced by nerve growth factor deprivation (21) and from a study indicating that suppression of ceramide-mediated apoptosis by sphingosine 1-phosphate is associated with activation of the MAPK pathway (22). Activation of MAPKs requires their phosphorylation by dual specificity MAPK kinases (MKK) (23), which, in turn, can be activated by stimulation of cells with mitogens or phorbol esters (24). As the activating MKK1 (25) and also MAPK are under negative control of type 2A (PP2A) serine/threonine protein phosphatases, it has been proposed that MAPK can also be activated through PP2A inhibition (26–28).

In the present study we examined whether activation of MAPK can act as a negative regulator in Fas-mediated apoptosis in T cells. To this end, we used different activators of this pathway, all of

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[3] Abbreviations used in this paper: MAPK, mitogen-activated protein kinase; MKK1, mitogen-activated protein kinase kinase 1; PP2A, type-2A serine/threonine protein phosphatase; HMW, high molecular weight; PKC, protein kinase C; cl-A, calyculin A; TPA, tetradecanoyl phorbol acetate; PI, propidium iodide; ANPA, apoptotic nuclei promoting activity; MBP, myelin basic protein; HA, hemagglutinin.

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which act through distinct initial signaling routes, including activation through protein kinase C (PKC), receptor-mediated activation, activation through inhibition of regulatory protein phosphatases, as well as activation by transfection with constitutively active MKK1. To investigate the temporal aspects of possible mitogen-induced effects on Fas signaling, we made use of the characteristic cellular events of the apoptotic machinery. The earliest signs of Fas-mediated apoptosis in these cells are cytoskeletal alterations and appearance of high m.w. DNA fragments (HMW), occurring by 30 min after Fas stimulation (5). The activation of caspases can be followed by using cell-free assay systems and by immunoblotting analysis of specific components of the caspase cascade (29, 30). These initial phases are followed by the terminal apoptotic phases, distinguished by the characteristic cleavage of DNA into oligonucleosomal size fragments, occurring 1 to 2 h after Fas stimulation (5). By using the above-mentioned cellular and biochemical markers as a guideline, we were able to position the inhibition generated by MAPK signaling in the sequence of processes initiated by activation of the Fas receptor.

Our study reveals efficient inhibition of Fas-induced apoptosis by all included activators of MAPK. Previous studies have shown that the activation of MAPKs can be achieved through the interaction of various T cell surface receptors, such as CD2 (31), the TCR complex (32, 33), CD28 (34), and TGF-β (35) with their ligands or agonists. Our findings suggest that MAPKs may provide an integrating circuit for the signals arising from these receptors, which, in turn, would direct the sensitivity of cells to Fas-mediated apoptosis. This form of modulation is likely to constitute an important regulatory signal determining the persistence or termination of an immune response and may also explain the failure to delete autoreactive and persistently activated T cells in the periphery.

Materials and Methods

Cell culture

The human leukemic T cell line, Jurkat (clone EG-1), was received from American Type Cell Collection (Rockville, MD). The cells were cultured in RPMI 1640 medium supplemented with 10% inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator with 5% CO_2 in air at 37°C. The cells were kept at a density of 0.5 to 1.0 $\times$ 10^6/ml.

Jurkat T cells were incubated at a density of 2 $\times$ 10^6 with an agonistic anti-human Fas IgM Ab (250 ng/ml; Kamiya Biomedical Co., Thousand Oaks, CA) for the indicated periods in the absence or the presence of calyculin A (cl-A; 10 nM; LC Laboratories, Beverly, MA), tetradecanoylphorbol acetate (TPA; 20 nM; LC Laboratories), OKT3 (1 μg/ml), or PHA (20 μg/ml; Sigma Chemical Co., St. Louis, MO). When cl-A or TPA were used, the cells were pretreated with the respective drug for 5 min before adding the Fas Ab. With OKT3 or PHA, the cells were pretreated 30 min before adding the Fas Ab.

Analysis of cytoskeletal and nuclear morphology

For confocal microscopy, an aliquot of cells was removed and centrifuged, and the cell pellet was resuspended in PBS and fixed with 3% formaldehyde (in PBS) for 1 h on ice. The fixed cells were again washed with PBS and then left for 2 h in PBS containing 0.1% saponin and 200 mU Bodipy 558/68-phalloidin (Molecular Probes, Eugene, OR) at room temperature. After 1 h, 1 μM Syto-13 (Molecular Probes) was added to the samples. The cells were washed once with PBS before mounting with Mowiol on coverslips and viewed using a Leica TCS40 confocal laser microscope. To stain filamentous actin or F-actin for flow cytometry, cells were fixed with 3% formaldehyde (in PBS) for 1 h on ice and then left for 2 h in 100 μl of PBS containing 0.1% saponin and 200 mU Bodipy 558/68-phalloidin at room temperature. The cells were then resuspended in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) as previously described (36).

Analysis of DNA fragmentation

To detect DNA fragmentation on flow cytometry, purified nuclei were stained with propidium iodide (PI) and analyzed on a FACScan flow cytometer as previously described (37, 38). Detection of DNA fragmentation into oligonucleosomal DNA fragments by agarose gel electrophoresis was performed as previously described (39). Detection of HMW DNA fragments was performed using field inversion gel electrophoresis (40).

Assays to determine initiation of apoptotic signaling

The activation of caspases can be followed using cell-free assay systems (29, 30). In such an assay, cytoplasmic extracts from Fas-stimulated Jurkat T cells are able to induce characteristic apoptotic DNA fragmentation in isolated nuclei. Hence, activation of caspases is indicated by the presence of apoptotic nuclei promoting activity (ANPA) (29). This assay was performed essentially as previously reported (29). In brief, Jurkat T cells (30×10^6 cells/ml) were stimulated with 20 ng/ml anti-human Fas or were left untreated, with or without 15-min preincubation with TPA or cl-A, for 60 min at 37°C in RPMI 1640 (10% FCS). The cells were washed twice with ice-cold RPMI 1640 (without FCS) and resuspended ($\sim 6 \times 10^6$ cells/10 μl) in buffer A (40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES, pH 7.0). After three cycles of freezing and thawing, the samples were centrifuged for 30 min at 20,000 $\times$ *g*, and the pellets were discarded. The cell lysates were centrifuged for another 30 min at 100,000 $\times$ *g*, and the supernatants were retained. Rat thymocyte nuclei, prepared as previously described (41), were suspended in buffer B (5 mM $MgCl_2$, 2.1 M sucrose, and 50 mM Tris, pH 7.5), with a final concentration of 4 to 5 $\times$ 10^8 nuclei/ml before use. The reaction mixture containing 10 μg of the supernatant proteins and 5 $\times$ 10^6 nuclei was diluted to a final volume of 30 μl with buffer A and supplemented with an ATP generation system (42). Samples were incubated at 37°C for 60 min before analysis by conventional agarose gel electrophoresis.

Cleavage or processing (and activation) of a specific caspase, caspase-3 (CPP32), was assessed using an Ab specific for the p20 subunit of CPP32 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). This was performed by lysing treated cells in 3$\times$ Laemmli sample buffer and resolving the extracted proteins on 12.5% SDS-PAGE, followed by transfer to a nitrocellulose membrane that was blotted with the caspase-3 p20 Ab. After coupling to a secondary Ab (Zymed, San Francisco, CA), the proteins were visualized with the ECL system (Amersham, Arlington Heights, IL).

MAPK activity assays

Cells (2×10^6/sample) were lysed with 400 μl of lysis buffer (PBS (pH 7.4), 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM Na_3VO_4, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). To immunoprecipitate MAPKs, cell lysates were centrifuged (3000 $\times$ *g* for 15 min), and the supernatant was incubated with an Ab generated against p42 MAPK or ERK2 (Transduction Laboratories, Lexington, KY) coupled to protein A-Sepharose (Sigma). Immunoprecipitates were then washed three times in lysis buffer and three times in kinase assay buffer (10 mM Tris (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$, and 0.5 mM DTT). The kinase reaction was conducted by adding to the immunoprecipitate 20 μl of kinase assay buffer, including 25 μM ATP, 2.5 μCi of [γ-^{32}P]ATP (Amersham, Aylesbury, U.K.), and 1 mg/ml myelin basic protein (MBP; Sigma Chemical Co.) as substrate. The reaction was conducted for 15 min at 37°C and was stopped by addition of 3$\times$ Laemmli sample buffer. The samples were resolved on 12.5% SDS-PAGE, and MBP phosphorylation was quantified with a Bio-Rad phosphorimager (Richmond, CA). In parallel with the immunocomplex kinase assays, we determined MAPK activation using an Ab specific for the phosphorylated forms of MAPK (New England Biolabs, Beverley, MA). This was performed by lysing treated cells in 3$\times$ Laemmli sample buffer and resolving the extracted proteins on SDS-PAGE, followed by transfer to a polyvinylidene difluoride membrane that was blotted with the phospho-MAPK Ab. After coupling to a secondary Ab, the proteins were visualized with the CDP-Star chemiluminescence reaction (New England Biolabs).

The role of MAPK in the observed protection against apoptosis was confirmed using the synthetic MKK1 inhibitor PD 098059 (Calbiochem, La Jolla, CA) (43). In these experiments, cells were incubated for 30 min in the presence of 20 μM PD 098059 before addition of the MAPK-stimulating agonists.

Transfection studies

Cells were transiently transfected by using DEAE-dextran (Pharmacia LKB, Stockholm, Sweden). Briefly, 10^6 cells were resuspended in 0.5 ml of Tris-buffered saline (25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5 mM

KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.6 mM Na_2HPO_4) with 1 mg of DEAE-dextran and 50 μg of plasmid DNA and then incubated for 30 min at room temperature, after which 10 ml of RPMI 1640 containing 0.5 mg of chloroquine diphosphate (Sigma) was added, and the cells were further incubated for 1 h. Cells were then washed twice with RPMI 1640 and allowed to rest for 48 h before treatment. The DNA constructs used were pMCL-HA-MKK1-S218E/S222 and pMCL-HA-MKK1-K97 M, encoding for hemagglutinin (HA)-tagged constitutively active and dominant negative forms of the MKK1 (44, 45), respectively. Both plasmids were gifts from Natalie Ahn (University of Colorado, Boulder, CO). The pCDNA3-CD20 was used for transfection controls (a kind gift from Tomi Mäkelä, University of Helsinki, Helsinki, Finland). For detection of transfected cells, the cells were collected by centrifugation, resuspended in PBS, and fixed on ice for 1 h with 3% formaldehyde in PBS. The cells were then washed once with PBS and permeabilized with 0.2% Nonidet P-40 (Sigma) for 10 min at room temperature. After washing, cells were incubated for 2 h at room temperature with 10 μg/ml of a monoclonal HA-specific Ab (12CA5, Boehringer Mannheim, Mannheim, Germany) in PBS with 1% BSA. Cells were then washed once with PBS and incubated further for 1 h at room temperature with fluorescein-conjugated anti-mouse secondary Ab and 10 μg/ml Hoechst (Molecular Probes) in PBS with 1% BSA. Cells were mounted in 50% glycerol and viewed under a Leica RMB epifluorescence microscope. CD20 was detected by incubating live cells (100 μl; 10^7 cells/ml) with 5 μl of a fluorescein-conjugated CD20 mouse mAb (Becton Dickinson) and 10 μg/ml Hoechst in PBS with 1% BSA for 30 min at 4°C in dark.

Results

Mitogenic stimuli inhibit Fas-mediated apoptosis in Jurkat T cells

To study whether mitogenic and/or activating stimuli could affect the rate of apoptosis in Fas-stimulated cells, we used four different approaches to stimulate Jurkat T cells. The lectin, PHA, and the agonistic Ab, OKT3, were used to induce T cell activation. PHA operates through polyclonal activation by ligating various cell surface receptors (46), and OKT3 activates CD3 of the TCR complex (32). Furthermore, we used two modifiers of mitogenic signaling pathways, the phorbol ester TPA, which activates PKC (24), and cl-A, a type 1 and type 2A protein phosphatase inhibitor. Flow cytometric analysis of nuclei from Fas-stimulated Jurkat T cells incubated in the presence or the absence of the activating substances was used to assess the degree of apoptosis. DNA fragmentation can be measured by flow cytometric analysis of isolated nuclei stained with a suitable DNA-specific fluorochrome, since apoptotic nuclei will show a lower DNA content due to leakage of nucleosome-sized DNA fragments from the nuclei (31). Before addition of the Fas Ab (250 ng/ml), we incubated Jurkat T cells for 30 min with 20 μg/ml PHA or 1 μg/ml of OKT3 or for 5 min with either 10 nM cl-A or 20 nM TPA. After 2 h, the number of apoptotic cells was determined by PI staining and flow cytometric analysis. Ligation of the Fas receptor with the agonistic Ab promoted rapid apoptosis in the cells, as indicated by a large subdiploid peak on the flow cytometric histogram, the events of which represent apoptotic nuclei and nuclear fragments (Fig. 1*A*). Two hours after addition of the Ab, approximately 50 to 65% of the cells were apoptotic, and most of the apoptotic cells appeared to be recruited from the G1 phase of the cell cycle (Fig. 1*A*). In contrast, when the cells were preincubated in the presence of the activating substances, Fas-mediated apoptosis could be inhibited to a variable degree by all these treatments (Fig. 1*A*).

The organization of microfilaments is affected sensitively in cells undergoing apoptosis (36). To confirm the results obtained on DNA fragmentation, we used the disruption of F-actin organization, measured as F-actin-specific fluorescence from Bodipy 558/568-conjugated phalloidin, as a marker of apoptosis (Fig. 1*B*). While flow cytometric measurements of F-actin-specific fluorescence showed a clear decrease in F-actin staining after treatment with Fas Ab (Fig. 1*B*), pretreatment with all activating substances prevented the decrease in F-actin staining. The percentage of cells showing reduced F-actin-specific fluorescence was somewhat lower than the percentage of events in the subdiploid peak observed in the flow cytometric analysis of apoptotic nuclei. This is consistent with a slight overestimation when using the chromatin-based measurement, as both single nuclei and nuclear fragments are included in the subdiploid peak of nuclear flow cytometry. However, both methods show comparable degrees of induction or inhibition, also showing variation in the degree of protection against Fas-mediated apoptosis by different agonists (Fig. 1, *A* and *B*). The observed increase in F-actin-specific fluorescence in a fraction of PHA-treated cells is due to marked clumping of cells in the presence of this lectin.

To confirm that the observed inhibitory effects were not specific for Jurkat T cells, we also tested another lymphocyte (SKW 6.4) and a macrophage (U937) cell line. Pretreatment of SKW 6.4 and U937 cells with either TPA or cl-A before addition of Fas Ab was equally efficient in inhibiting Fas-mediated apoptosis as in Jurkat T cells (data not shown).

Fas-mediated apoptosis can be inhibited during the initial phases of the signaling machinery

We used various biochemical markers to assess whether inhibition of Fas-mediated apoptosis occurred at the initial or late phases of Fas signaling. As cl-A and TPA were the most efficient in inhibiting the Fas-generated signal, most assays are exemplified with data from experiments with these two compounds. To determine whether all DNA fragmentation could be inhibited, cells were incubated for 2 h, and the induction of apoptotic chromatin cleavage into HMW and oligonucleosome-sized DNA fragments was measured by conventional and field inversion agarose gel electrophoresis. Stimulation of the Fas receptor promoted rapid DNA fragmentation in the Jurkat T cells, as indicated by agarose gel electrophoresis that could be repressed by preincubation with cl-A or TPA (Fig. 2*A*), which is preceded by the formation of HMW DNA fragments (5, 40). Our data clearly show that pretreatment with TPA or cl-A inhibits Fas-induced formation of HMW DNA fragments (Fig. 2*B*). These biochemical analyses of DNA fragmentation confirm both qualitatively and quantitatively the results obtained with the flow cytometric analysis of apoptotic nuclei (Fig. 1*A*).

One of the characteristic early signs of Fas-mediated apoptosis is membrane blebbing together with simultaneous cytoskeletal alterations, both of which occur simultaneously with the formation of HMW fragments (5). To analyze in detail whether the chromatin and cytoskeletal organization remained intact in Fas-stimulated cells incubated in the presence of TPA or cl-A, we used confocal microscopy of cells double labeled with the DNA-specific fluorochrome Syto-13 and the F-actin-specific fluorochrome Bodipy 558/568-conjugated phalloidin. Confocal microscopy showed control cells with normal morphology and characteristic chromatin- and F-actin-specific fluorescence patterns (Fig. 3), whereas cells treated with the Fas Ab showed markedly condensed and disintegrated nuclei and abolished staining of F-actin (Fig. 3), indicating disruption of cytoskeletal structure and appearance of apoptotic nuclear bodies. Cells pretreated with TPA or cl-A showed no alterations in the nuclear morphology or F-actin staining patterns (Fig. 3) compared with control cells. These results further verify the flow cytometric analysis of F-actin-specific fluorescence (Fig. 1*B*).

The formation of ANPA in Fas-stimulated cells has been shown to involve activation of both serine and caspase activities, which are manifested as proteolysis-mediated nuclear chromatin cleavage

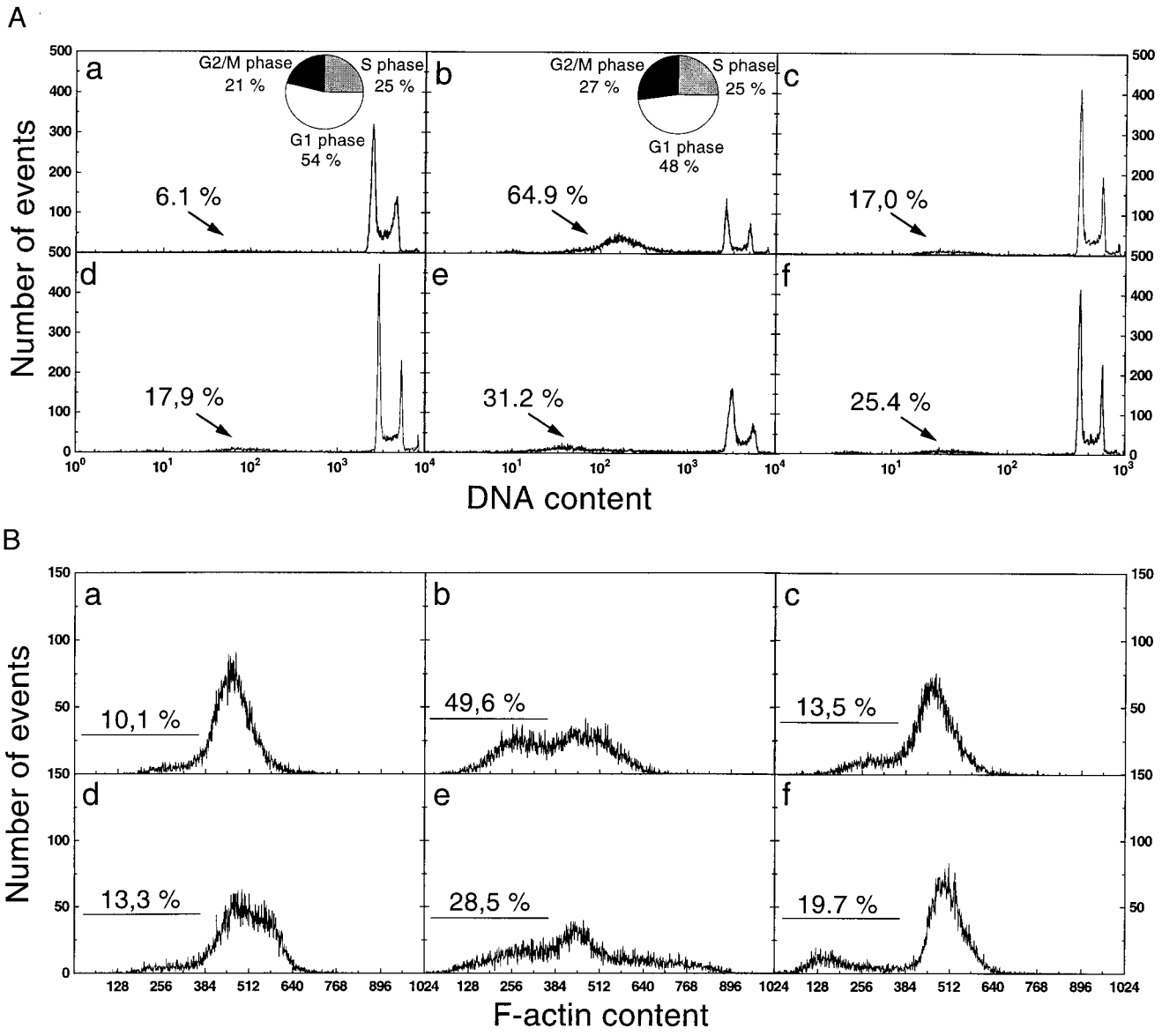

FIGURE 1. Effects of mitogenic agents on Fas-mediated apoptosis in Jurkat T cells. Fragmentation of DNA (*A*) and microfilament disassembly (*B*) were used as indicators for the presence of apoptotic cells following stimulation of the Fas receptor with an agonistic Ab. Cells were cultured for 2 h with medium alone (*a*) and in the presence of Fas Ab (250 ng/ml; *b*), TPA (20 nM) plus Fas Ab (*c*), cl-A (10 nM) plus Fas Ab (*d*), PHA (20 μg/ml) plus Fas Ab (*e*), or OKT3 (1 μg/ml) plus Fas Ab (*f*). After 2 h, aliquots of cells were stained with a hypotonic PI solution, and the proportion of apoptotic nuclei was determined using a FACScan flow cytometer (*A*). Arrows indicate the percentage of cells in the subdiploid peak, which is characteristic of apoptotic cells. Parallel aliquots of cells were stained with Bodipy 558/568-conjugated phalloidin, and the degree of Fas-induced microfilament disassembly was quantified by flow cytometry (*B*). The increased amount of cells with high Bodipy 558/568 fluorescence in *e* of panel *B* is due to clumping of cells caused by the PHA treatment.

in the reconstituted in vitro apoptosis assay system (29). Pretreatment of Jurkat T cells with TPA or cl-A inhibited the formation of ANPA and subsequently the activation of DNA cleavage into oligonucleosomal length fragments in isolated nuclei (Fig. 4, *lanes 3* and *6*). To rule out the nonspecific effect of TPA or cl-A on ANPA per se, both compounds were added to cells after 1-h treatment with the Fas Ab, when the onset of apoptosis was clearly detectable. The cell lysates derived from these cells remained active in causing DNA fragmentation in isolated rat thymocyte nuclei (Fig. 4, *lanes 4* and *7*), thus ruling out any inhibitory effects of TPA and cl-A on ANPA itself. Taken together, our results indicate that these compounds inhibit activation of proteases that have been established as crucial mediators of Fas-mediated apoptosis (8–10, 29, 30).

One of the caspases activated during apoptosis is caspase-3/CPP32 (10). The 32-kDa precursor protein is, via an intermediate step, cleaved into two subunits of 12 and 17 kDa, respectively (10), and it has been shown that caspase-3 is rapidly cleaved and activated in Fas-treated cells (10). This activation occurs within 15 min after cross-linking of the Fas receptor and has been considered to constitute one of the earliest phases in apoptotic induction (10). The effect of mitogenic stimuli on the Fas-induced cleavage of caspase-3 was assayed by immunoblotting with a caspase-3-specific Ab, using cell samples treated in the presence and the absence of mitogenic agents. As shown in Figure 5, the Ab recognized the caspase-3 proform in the control samples, which disappeared in Fas-treated samples but was retained in samples pretreated with the mitogenic agents (Fig. 5), indicating mitogen-induced arrest of

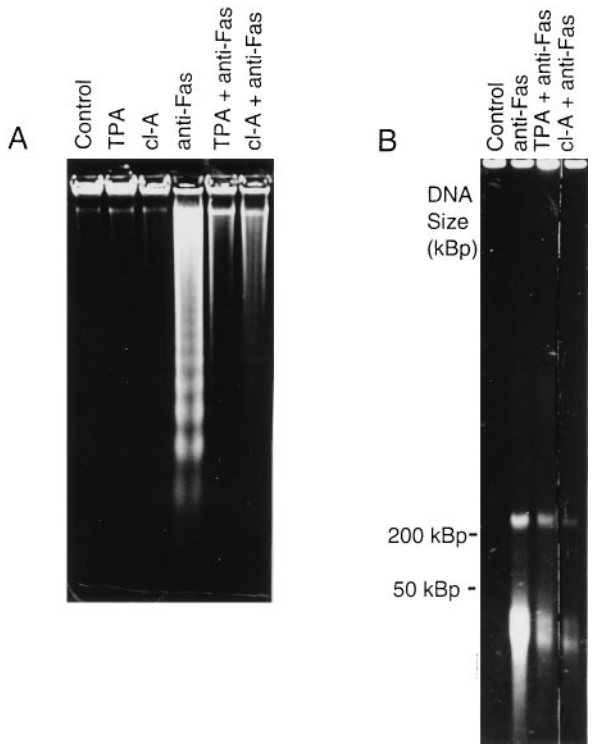

FIGURE 2. Effect of mitogenic stimuli on Fas-mediated chromatin fragmentation in Jurkat T cells. The formation of oligonucleosomal (*A*) and HMW (*B*) DNA fragments in Fas-stimulated cells was studied by agarose gel electrophoresis. Cells were incubated as indicated in Figure 1. After 2 h, aliquots of cells were taken and processed for one-stage (*A*) and field-inversion (*B*) agarose gel electrophoresis, respectively.

the caspase-3 activation. The Ab used, which is specific for the 20-kDa intermediate form of p17 (10), could not detect the p12 and p17 subunits of activated caspase-3.

MAPK activation in Jurkat T cells correlates with the relative inhibition of Fas-mediated apoptosis

As the MAPK pathway mediates stimulation of cell proliferation and has been suggested to be a negative regulator of apoptosis (21, 22), we examined whether the observed inhibition of a normal Fas response in Jurkat T cells could be a consequence of MAPK activation. MAPK activation can be attained both by physiologic stimuli and by using modifiers of kinase/phosphatase activities. Receptor-stimulating agents, such as OKT3 and PHA (32, 33); PKC-activating compounds, for example TPA (24); and protein phosphatase inhibitors, such as cl-A (26), have all been reported to induce MAPK activation (26, 32, 33). To determine whether these agents were able to activate MAPK in Jurkat T cells, we assayed the cells for MAPK activity using an immunocomplex assay (Fig. 6). The immunocomplex assay showed a clear increase in MAPK activation after treatment with the respective stimuli, as determined by MBP phosphorylation (Fig. 6).

To correlate the different degree of MAPK induction with the inhibition of Fas-mediated apoptosis in Jurkat T cells, we used concurrent treatments with PHA, the OKT3 Ab, cl-A, and TPA, all added before addition of the Fas Ab, followed by collection of parallel samples for flow cytometric analysis of apoptotic cells and MAPK activity measurements. While both receptor agonists, in accordance with previous studies (32, 33), were able to stimulate MAPK activities, OKT3 was clearly the more efficient of the two (Figs. 6 and 7). Cl-A and TPA were more efficient than the receptor agonists in activating MAPK (Figs. 6 and 7). The inhibition of

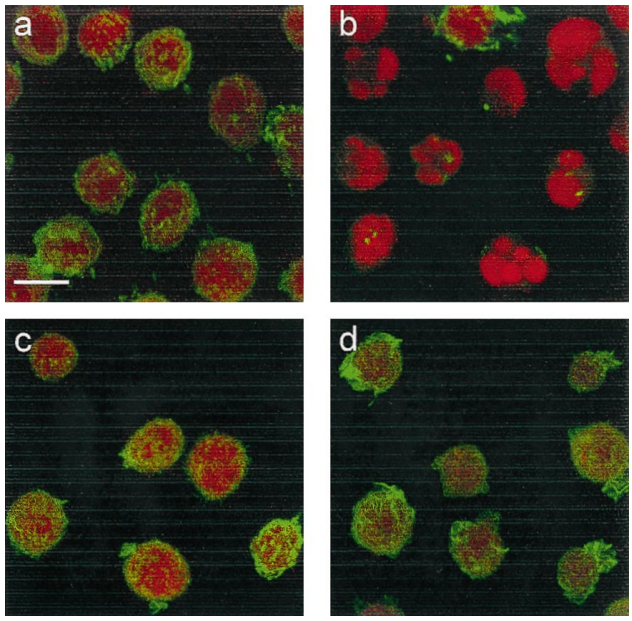

FIGURE 3. Inhibition of Fas-induced alterations of cytoskeletal morphology and nuclear organization. Confocal laser scanning micrographs of Jurkat T cells incubated for 2 h as indicated in Figure 1 with medium alone (*a*), Fas Ab (*b*), TPA plus Fas Ab (*c*), and cl-A plus Fas Ab (*d*). Cells were stained for F-actin with Bodipy-conjugated phalloidin (signal shown in green), and DNA was stained with Syto-13 (signal shown in red). Bar = 5 μm.

apoptosis by these different agents corresponded well with the level of MAPK activation induced by the respective activator (Fig. 7, *A* and *C*).

To further corroborate the factual involvement of MAPK signaling in the observed protective effects, we used a specific inhibitor of the MAPK pathway, PD 098059 (43). The protective effect on Fas-mediated apoptosis of all included MAPK activators, except for cl-A, could be abolished by preincubation of the cells with PD 098059 (Fig. 7*B*). Correspondingly, PD 098059 was effective in inhibiting MAPK activation by all agonists used, except that induced by cl-A (Fig. 7*D*). However, the inhibitor did not reduce the basal level of MAPK activity in control cells, as the action of this synthetic inhibitor of the MAPK pathway is based upon inhibition of the Raf-mediated activation of MKK1 rather than direct inhibition of MAPK (43). The inhibitor was not effective against cl-A as the cl-A-induced MAPK activity presumably arises from direct activation of MAPK through inhibition of MAPK- and MKK1-specific PP2A activities.

To investigate whether the MAPK-generated inhibitory effect on Fas-mediated apoptosis requires production of newly synthesized proteins, we examined whether cycloheximide would abolish or restrict this effect. No effect could be observed by cycloheximide as cells pretreated with any of the included activators were equally insensitive to Fas in both the presence and the absence of cycloheximide (Fig. 7*E*). Cycloheximide by itself had no effect on the viability of the cells.

The dose response of PD 098059 was also assessed on cells incubated with TPA followed by subsequent stimulation with the Fas-specific Ab. Cells were incubated for 30 min in the presence of increasing concentrations of PD 098059 before TPA was added. The half-maximal inhibition of TPA-generated protection occurred at approximately 2 μM (Fig. 8*A*), which corresponded well with the half-maximal inhibition of MAPK activity generated by PD 098059 (Fig. 8*B*). When all measured MAPK activities, analyzed in the presence or the absence of the various activating agents,

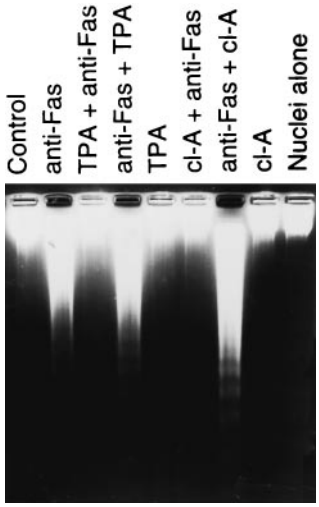

FIGURE 4. Inhibition of the formation of ANPA in Fas-stimulated Jurkat T cells. The figure shows DNA from isolated rat thymocyte nuclei following separation by agarose gel electrophoresis and staining with ethidium bromide. The thymocyte nuclei were incubated for 60 min at 37°C with cytoplasmic extracts from cells treated according to the following scheme: control cells (*lane 1*), cells treated with the Fas Ab for 60 min (*lane 2*), cells preincubated with TPA for 5 min before treatment with the Fas Ab for 60 min (*lane 3*), cells treated with the Fas Ab for 60 min before incubation with TPA for 5 min (*lane 4*), cells treated with TPA for 60 min (*lane 5*), cells preincubated with cl-A for 5 min before treatment with the Fas Ab for 60 min (*lane 6*), cells treated with the Fas Ab for 60 min before incubation with cl-A for 5 min (*lane 7*), cells treated with cl-A for 60 min (*lane 8*), and nuclei alone (*lane 9*). Similar inhibition of the formation of ANPA was obtained with PHA and OKT-3 (results not shown; see Fig. 5).

were plotted against the degree of apoptosis, a close negative correlation was obtained between the degree of apoptosis and the level of MAPK activity (Fig. 8*C*).

Possible interactions between Fas-mediated signaling and the MAPK cascade

To further analyze the MAPK-generated effects, we determined which form of MAPK was predominant in the observed MAPK activities and also assayed the activation kinetics in the presence and the absence of agonistic Fas Abs. The level of activating phosphorylation on MAPK was assessed by means of a phospho-MAPK-specific Ab. Using the two agents that were most efficient in MAPK activation, we observed that cl-A induced a marked phosphorylation of p42 MAPK (also referred to as ERK2),

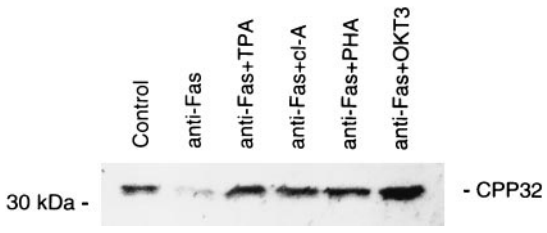

FIGURE 5. Inhibition of Fas-mediated CPP32 cleavage by MAPK-inducing agents. The cleavage of CPP32 was followed by Western blotting with a CPP32-specific Ab. The Jurkat T cells were treated as indicated in Figure 1. The signal obtained with the CPP32-specific Ab represents the inactive proform of CPP32. The dissipation of the proform indicates activation of the protease. Equal loading was confirmed by densitometric measurement of the Coomassie blue-stained protein bands remaining on the gel after electrotransfer. A representative immunoblot from three experiments is shown.

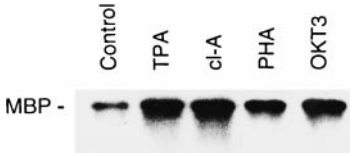

FIGURE 6. Activation of MAPK in Jurkat T cells. MAPK activation in cell extracts from Jurkat T cells was determined by an immunocomplex kinase assay. Cells were treated as described in Figure 1. A representative autoradiograph of the immunocomplex kinase assay is shown.

whereas TPA-induced phosphorylation could be seen on both p44 (ERK1) and p42 MAPK, although in this case also, p42 MAPK was the predominantly phosphorylated form (Fig. 9*A*). Upon long exposure of the Western blots, a faint basal p42 MAPK phosphorylation could be observed in samples from control cells (data not shown). As the cl-A-induced MAPK activation was at least as efficient as that induced by TPA, p42 MAPK kinase appears to be the predominant kinase in the observed activity increases. The activation kinetics of MAPK (Fig. 9*C*) by cl-A and TPA, respectively, was distinct as determined by the immunocomplex assay (Fig. 9*C*). Cl-A-induced activities peaked at 15 min after addition of the drug, while TPA-induced activation had still not leveled at 30 min. As mentioned previously, the Fas Ab alone showed, interestingly, a small increase in MAPK activities, which could also be detected as a minor increase in the phosphorylation signal obtained with the phospho-MAPK Ab. The kinetics of MAPK activation induced by the Fas Ab together with TPA or cl-A was different from the kinetics observed with cl-A or TPA alone. There appeared to be a synergistic effect on MAPK activation when cells were incubated in the presence of both cl-A and Fas.

Constitutively active MKK1 rescues Jurkat T cells from Fas-mediated apoptosis

To get further evidence that the above-described effects all relate to signaling by the MAPK cascade, we performed transient transfection experiments with the constitutively active and dominant negative forms of MKK1. While expression of the dominant negative form of MKK1 (K97 M) did not interfere with Fas-mediated apoptosis in Jurkat T cells, cells expressing the constitutively active form of MKK1 (S18E-S222D) were effectively protected (Fig. 10). These results give convincing evidence in support of the assumption that activation of MAPK in Jurkat T cells inhibits Fas-induced apoptosis and show, furthermore, that the MAPK pathway is not required for induction of Fas-mediated apoptosis.

Discussion

The evolutionary conserved cell death machinery appears to be composed of several distinct parts, including activators, inhibitors, and effectors (47). Recent advances concerning the Fas receptor indicate that a cascade of cysteine proteases, all of which display homology to the prototype proteases ICE, CPP32, and Ich-1, are responsible for a number of important activator and effector functions (for review, see Refs. 8 and 9). Specific adapter proteins associated with the death-inducing signaling complex (48) are responsible for conveying the Fas receptor signal to the effector machinery (49, 50). Although this signaling and effector cascade has been well established, very little is known about how this machinery can be modulated or suppressed during different stages of development and differentiation. The results in this study present a plausible instrument that could be responsible for determining the outcome of a Fas-generated signal. The involvement of one of the major signaling cascades, the MAPK cascade, is indicated in the

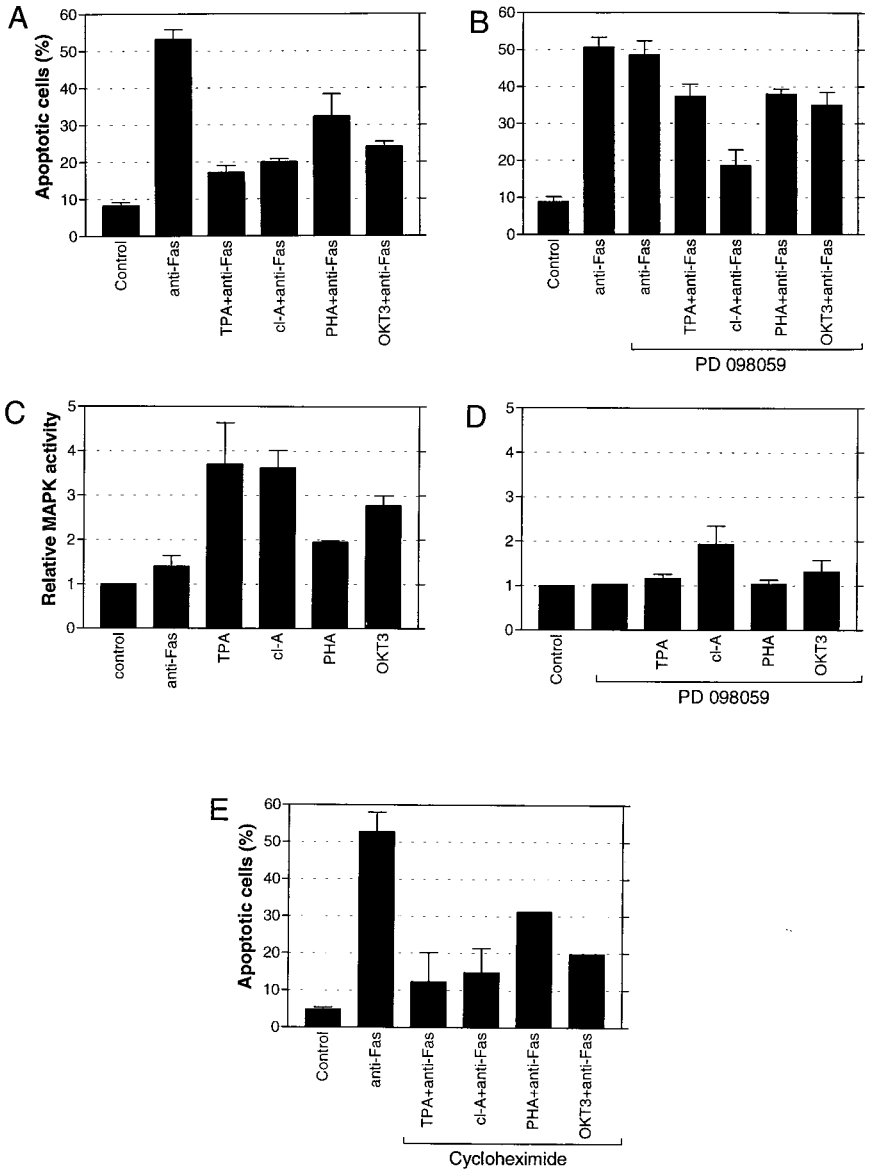

FIGURE 7. The inhibition of Fas-mediated apoptosis corresponds to the degree of MAPK activation and is independent of protein synthesis. The MAPK-stimulating effect was related to the degree of apoptosis inhibition. Cells were preincubated for 5 min with TPA or cl-A and for 30 min with PHA or OKT3 before incubation with Fas Ab. The relative amount of apoptotic cells after 2 h was measured by flow cytometry (*A*) and compared with the degree of MAPK activation after a 30-min treatment with the indicated compounds (*C*). The inhibition of Fas-mediated apoptosis by MAPK-activating agonists could be abolished by the MKK1-specific inhibitor PD 098059. A comparison of the inhibition of Fas-mediated apoptosis by different MAPK-activating drugs and agonists when cells were preincubated for 30 min with 20 μM PD 098059 is shown in graph *B*. Otherwise, cells were treated as described above. The induction of apoptosis in the presence of PD 098059 was compared with the degree of MAPK activation in these samples (*D*). To assess whether the observed inhibition of Fas-mediated apoptosis is dependent on protein synthesis, cycloheximide was added to a final concentration of 100 μM 5 min before addition of the MAPK-activating drugs and agonists (*E*). Otherwise, cells were treated as described above. The data represent mean values (mean $\pm$ SEM) from a minimum of three separate experiments.

suppression of Fas-mediated signals. It has been suggested that MAPKs are key factors in promoting cell survival and inhibiting apoptosis. According to this scheme, lack of growth stimulation through this signaling cascade results in apoptosis (21). Our study suggests that MAPKs play a central role in regulating the onset of apoptosis by intervening with a death signal from a specific apoptosis-promoting receptor.

Fas-mediated apoptosis can be inhibited by MAPK activation through different induction routes

Although the activating agents used operate through completely different initial signaling entities, they were able to render a protection against Fas-mediated apoptosis, which was proportional to their induction of MAPK activity. These results imply that MAPK activation was efficient in inhibiting Fas-mediated apoptosis regardless of the signaling route used to activate this pathway. Both PKC activation and phosphatase inhibitor-induced MAPK activation reduced the number of apoptotic cells after Fas stimulation almost to control levels. A number of studies have argued for a modulating function for PKC, since TPA has conferred Fas resistance to T cells (9, 51), and PKC inhibitors have sensitized certain cells to Fas-mediated apoptosis (6). The cl-A-induced activation of MAPK acts downstream of PD 098059-sensitive PKC-generated signals, presumably via inhibition of MKK1/MAPK-directed PP2A activities (26–28). As cl-A is at least as efficient as TPA in activating MAPK and inhibiting the Fas response, PKC-induced MAPK activation appears to be the significant part of the TPA-evoked inhibition, not the PKC activation per se. The physiologic T cell activators were not as efficient in activating MAPK as TPA and cl-A; consequently, they were less efficient in inhibiting apoptosis. This is plausible, as PHA and the OKT3 Ab are not likely to induce full activation of TCR complexes and may also generate signaling resulting in negative feedback on MAPK. In our assay system, a twofold MAPK activation seemed to be an approximate threshold level to significantly protect against Fas-mediated apoptosis. A previous study indicated that Ras activation would be required for Fas-induced apoptosis (52). This result is seemingly contradictory, as activation of Ras is generally associated with MAPK activation. While the role of Ras in Fas-mediated signaling is insufficiently characterized, Fas-induced Ras activation is presumably different from that induced by receptor tyrosine kinase activation. The specificity for either

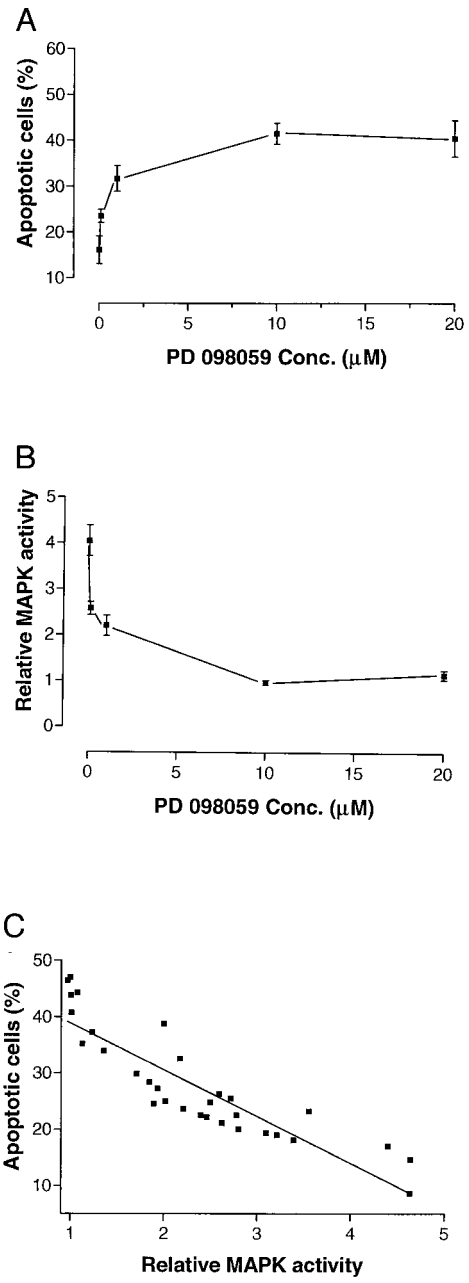

FIGURE 8. Dose-response of the PD 098059-induced reversal of TPA-generated protection against Fas-mediated apoptosis. There is a close correlation between the reduction in the number of apoptotic cells and MAPK activity when the effects on the amount of apoptotic cells (*A*) and MAPK activity (*B*) were studied. Cells were pretreated with 0.1, 1, 10, or 20 μM PD 098059 before addition of TPA and anti-Fas. The relative amount of apoptotic cells, as measured by flow cytometry (*A*), after the above-mentioned treatments was compared with the degree of MAPK activation after a 30-min treatment with the indicated compounds (*B*). An equally good correlation between MAPK activity and inhibition of apoptosis was obtained when the combined data from all MAPK assays was plotted against the percentage of apoptotic cells among correspondingly treated cells (*C*).

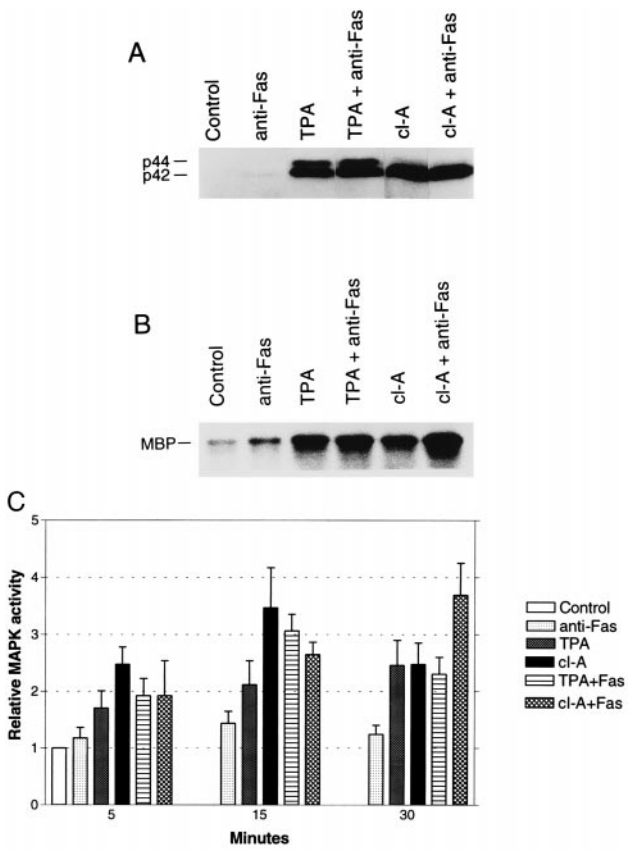

FIGURE 9. The kinetics of MAPK activation in Jurkat T cells. MAPK activation was followed in cell extracts incubated with medium alone; in the presence of anti-Fas, TPA, and cl-A; as well as with anti-Fas in combination with TPA or cl-A (5-min preincubation with both compounds followed by 30 min in the presence of anti-Fas). The activation was measured by immunoblotting using a phospho-MAPK-specific Ab (*A*) or was determined using an immunocomplex kinase assay (*B*). A representative autoradiograph of the immunocomplex kinase assay is shown (*B*), and multiple samples at different time points were quantified by phosphorimager analysis (*C*). The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

inducing apoptosis or cell growth is likely to be generated by accompanying signals.

The specific involvement of MKK1-MAPK in the observed inhibition of apoptosis was confirmed using the MKK1 inhibitor PD 098059 (43), which abolished both MAPK activation and the apoptosis-inhibiting effects of TPA, PHA, and OKT-3, respectively. This compound was not effective in inhibiting cl-A-induced MAPK activation. Consequently, cl-A protected against Fas-mediated apoptosis even in the presence of the inhibitor. The failure of PD 098059 to inhibit cl-A-induced MAPK activation is likely to be due to the fact that it inhibits MAPK activation by interfering with the Raf-generated activation of MKK1 (43). Hence, it does not abolish the cl-A-induced MAPK activation attributable to inhibition of PP2A activities directly regulating either MKK1 or MAPK. In accordance with previous studies (43), PD 098059 inhibits MKK1-generated activation of MAPK but does not affect the basal level of MAPK activity. Accordingly, it did not sensitize the cells to Fas-mediated apoptosis. The final evidence establishing involvement of the MAPK cascade in the observed inhibitory effects was obtained by transient transfections with constitutively active forms of MKK1, showing almost complete inhibition of Fas receptor signaling. Interestingly, transfections with the dominant negative form of MKK1 had no effect on Fas-induced apoptosis, which implies that the small increase in MAPK activity upon Fas receptor stimulation observed in our and in a previous study (52) is without significance in activation of the apoptotic effector machinery.

In the MAPK cascade, MAPKs are usually the effector kinases, regulating a vast number of signaling, regulatory, and transcription factor proteins (28). However, although MAPKs on the basis of

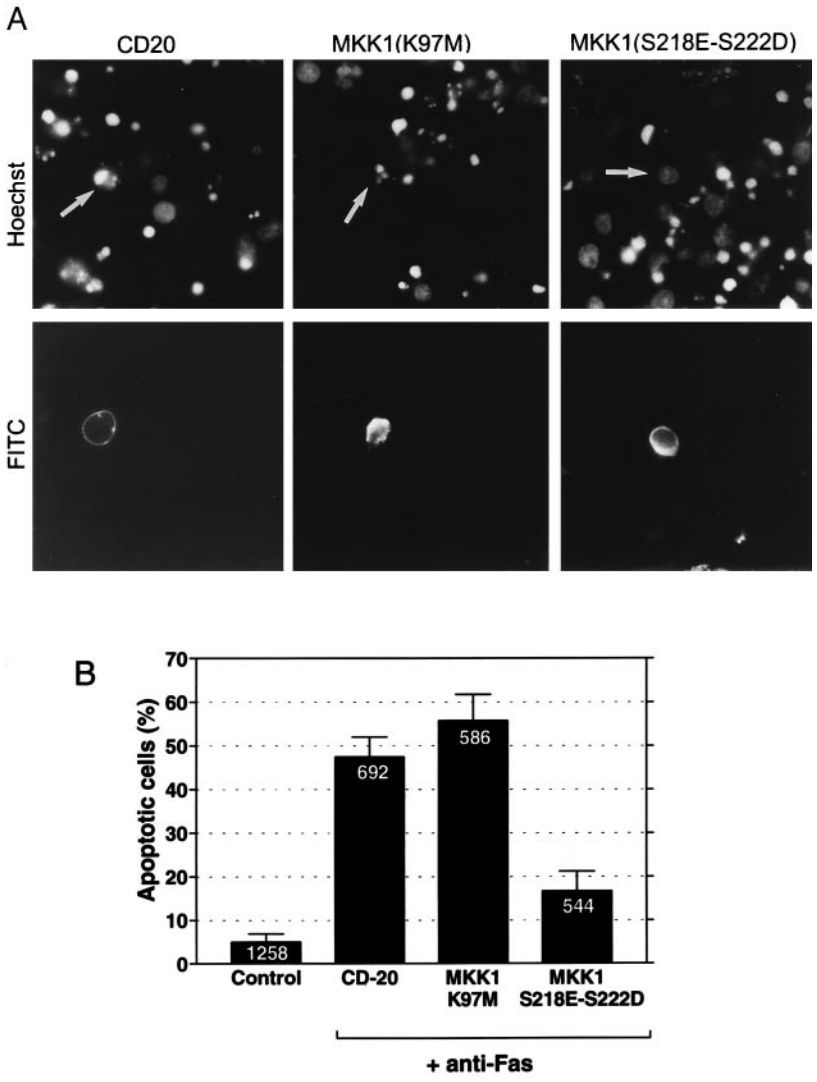

FIGURE 10. Inhibition of Fas-mediated apoptosis by constitutively active, but not dominant negative, MKK1. Representative immunofluorescence micrographs (*A*) of cells transfected with dominant negative (K97 M) and constitutively active (S218E-S222D) constructs of MKK1, and incubated in the presence of anti-Fas for 2 h. The pCDNA3-CD20 construct was used for transfection control. Nuclear alterations were visualized by Hoechst staining (*upper panel*), and transfected cells were detected by FITC immunofluorescence of HA or the CD20 receptor (*lower panel*). The nuclei of apoptotic cells are fragmented into apoptotic bodies with intense Hoechst staining, whereas live cells show a uniform and more dim staining. The arrows point at transfected cells, as detected by the CD20 and the HA-specific Abs, respectively. The percentage of apoptosis in transfected cells after Fas treatment was counted (*B*). The data are the mean value (± range of values) from at least five independent experiments. The total number of transfected cells counted is indicated within each bar.

previous experience may be more likely as effector signaling proteins, our assays do not allow us to distinguish whether the Fas inhibitory signal arises from MAPKs directly or possibly from MKK1. The latter kinase has, independently of MAPKs, been shown to phosphorylate SOS (53); therefore, this route possibly has to be taken into account.

MAPK signals generate inhibition at the initial phases of Fas-mediated apoptosis

In MAPK-activated cells, the inhibiting effect was not only seen at the terminal stages but also in the early features of apoptosis, including the appearance of HMW fragments, and cytoskeletal lesions were inhibited. Furthermore, the formation of ANPA was completely suppressed in MAPK-activated cells as well as the processing of caspase-3. As these events can be detected 15 to 30 min after Fas stimulation, the elevated MAPK activity must intervene at the initial phases of the Fas-mediated signal. As the processing of caspase-3 and elevated ANPA essentially represent initiation of the major effector machinery in Fas-mediated cell death, the MAPK-induced intervention is likely to occur upstream from these proteases. However, this experimental setup does not allow us to conclude whether the signal obstruction is proximal or distal to the receptor-associated protease caspase-8, as this protease is activated almost instantly upon Fas ligation (49, 50).

The mitogen-induced effect is almost immediate, and it interrupts the first steps of the effector cascade. In light of these results, it is unlikely that the effect could be generated through synthesis of an apoptosis-inhibiting protein(s). This assumption was confirmed by the results showing that cycloheximide did not abrogate the MAPK-generated protection. Furthermore, the transcription of immediate early genes in Jurkat T cells commences only 30 to 60 min following activation of MAPK-stimulating pathways, as indicated by elevated DNA-binding activity of activator protein-1 (data not shown).

Possible interactions between MAPK and Fas-generated signaling

Interestingly, the MAPK activation kinetics in the presence of the Fas Ab together with either compound seemed in both cases to be different from the activation kinetics observed when cells were incubated with the respective compounds alone. Furthermore, there seemed to be some degree of synergism when cells were stimulated with the agonistic Fas Ab in combination with a mitogenic stimulus. Hence, not only does the MAPK pathway affect Fas-mediated signaling, but there also seems to be cross-talk between the two signaling pathways. We observed a small, but significant, MAPK activity increase in Fas-stimulated cells, which gives further evidence of an interaction between the two signaling

pathways. Our data indicate a preferential involvement of the p42 MAPK in the observed MAPK activities. This is in agreement with a number of studies showing that p42 MAPK would be the predominantly active MAPK form in cell lines with B or T cell characteristics (26).

Possible roles of the MAPK pathway in the regulation of Fas signaling

In view of the remarkably wide role the MAPKs play in cell regulation, specifying not only cell fate, but also many cellular functions in fully differentiated cells, it is not surprising that MAPKs have been shown to be of crucial importance also in T cell-specific functions. Several regulatory factors involved in T cell activation and growth regulation are able to activate the MAPK cascade. Evidence is accumulating that the TCR complex with its associated coreceptors activate this signaling pathway. CD2 (31), CD3 (33), and CD28 (34) have been shown to activate MAPK. Interestingly, a recent study shows that preincubation with PHA and TGF-β inhibits reactivation-induced and Fas-mediated apoptosis in T cells (54). This study is in line with the data of the present study, since PHA (33) and TGF-β (35) are also known to induce MAPK activation. Consequently, the TGF-β-induced protection against Fas-mediated apoptosis observed by Cerwenka and coworkers (54) may have been a result of MAPK activation. The results in our study are also supported by the previous observation that TNF- and Fas-mediated apoptosis could be suppressed by sphingosine 1-phosphate-induced MAPK activation (20).

The Fas receptor has been established as especially important in peripheral T cell selection (55, 56). It has been indicated that peripheral T cells undergo apoptosis by two distinct mechanisms, one that is Fas independent and sensitive to the expression of bcl-x protein and bcl-2 protein, and the other that is Fas dependent and insensitive to bcl-x protein and bcl-2 protein (57, 58). As the MAPK pathway is obviously potent in inhibiting Fas-mediated signaling, a possible role for the MAPK pathway in this scheme would be to function as an integrating circuit for various incoming signals to determine whether the cell should undergo apoptosis or generate a different type of response. A sufficient activation by combined MAPK-targeted stimuli, induced either by the TCR and its associated components or by various cytokines, would result in rescue of the Fas-sensitive cells. The duration and efficacy of the protection against Fas-mediated apoptosis would be determined by the intensity and/or combination of stimuli. An important role for this type of protection would be during T cell activation. It has been established that activated T cells are terminated by Fas-mediated apoptosis that is triggered by autocrine production of the Fas ligand (59–61). However, this activation-induced apoptosis occurs several days after the activation, although the expression of both the receptor and the ligand is up-regulated within 24 h (56, 62). Hence, activated T cells appear to be unresponsive to Fas receptor stimulation over a prolonged period in the presence of both the receptor and the ligand. In fact, Fas stimulation during this period may be even advantageous, as indicated in studies showing, on the one hand, that agonistic Fas Abs can cooperate with suboptimal concentrations of TCR/CD3-specific Abs to induce mitogenic stimulation (63, 64) and, on the other hand, that T cells from mutant *lpr* mice are less responsive to antigenic stimuli than are normal T cells (65). The results presented in our study are likely to explain the unresponsiveness of activated T cells to Fas ligand before they enter the terminal phase and could also explain the possible synergistic effects of the Fas receptor and the TCR. The latter aspect is supported by the observation that there seemed to be cross-talk between the two signaling systems and even some degree of synergistic effect when MAPK-inducing stimuli were used in combination with the agonistic Fas antibody. Fas responsiveness could also be modulated through the actions of various MAPK-inducing cytokines. This type of modulation could explain the protective effects of TGF-β against Fas-mediated apoptosis, which has been suggested to be important for the generation of effector and/or long-lived memory T cells (52).

Apart from T cells, the MAPK-induced suppression of the Fas response could be important in other cells expressing the Fas receptor. Furthermore, it has even been shown that some tumor cell lines respond to Fas receptor stimulation by accelerated cell growth (17, 18). The MAPK pathway is a possible candidate to generate these kinds of variable responses to Fas receptor stimulation.

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