JNK (c-Jun N-terminal kinase) and p38 activation in receptor-mediated and chemically-induced apoptosis of T-cells: differential requirements for caspase activation

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Activation of the stress-activated mitogen-activated protein kinases (MAP kinases), c-Jun N-terminal kinase (JNK) and p38, is necessary for the induction of apoptosis in neuronal cells; however, in other cell types their involvement may be stimulus-dependent. In the present study we investigate the activation of JNK and p38 in a single non-neuronal cell type, undergoing receptor-mediated (tumour necrosis factor-related apoptosis-inducing ligand and CD95) or chemically-induced (lactacystin) apoptosis. In Jurkat T-cells, receptor-mediated and chemically-induced apoptosis resulted in a time-dependent activation of the initiator caspases-8 and -9, respectively. Both types of stimuli resulted in a significant activation of JNK and p38, which closely paralleled the time-dependent induction of apoptosis. The caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.FMK) inhibited receptor-mediated apoptosis and suppressed JNK and p38 activation. In contrast, inhibition of lactacystin-induced apoptosis with z-VAD.FMK, as assessed by phosphatidylserine exposure and poly(ADP-ribose) polymerase cleavage, did not inhibit activation of JNK or p38, demonstrating that during chemically-induced apoptosis, activation of JNK and p38 is independent of effector caspases. The role of p38 in apoptosis was assessed using the specific p38 inhibitor, SB203580. No effect on the induction of apoptosis or caspase activation was observed, although activation of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2), an immediate downstream target of p38, was inhibited. Therefore neither p38 activation nor activation of MAPKAPK-2 is critical for induction of either receptor- or chemically-induced apoptosis. Thus, within a single cell type, (1) the mechanism of p38 and JNK activation during apoptosis is stimulus-dependent and (2) activation of the p38 pathway is not required for caspase activation or apoptosis, assessed by phosphatidylserine exposure, but may still be required to elicit other features of the apoptotic phenotype.

Key words: CD95, Jurkat cells, lactacystin, stress-activated protein kinases, TRAIL.

INTRODUCTION

Apoptosis is an evolutionary conserved mode of cell death essential for the normal development and homoeostasis of multicellular organisms [1]. It is characterized by distinct morphological changes and is regulated by a series of biochemical events that lead to cell death. The biochemical events have been divided into two distinct phases: an initial commitment phase where cells receive a signal that results in commitment to cell death, followed by an execution phase when all of the characteristic morphological and biochemical features of apoptosis occur [2]. Caspases, a family of aspartate-specific cysteine proteases, which exist as single-chain inactive zymogens, play a critical role in the execution phase of apoptosis. 'Initiator' caspases, with long prodomains such as caspases-8 and -9, either directly or indirectly activate 'effector' caspases, such as caspases-3, -6 and -7 [3,4]. These effector caspases then cleave intracellular substrates, including poly(ADP-ribose) polymerase (PARP) and lamins, resulting in the dramatic morphological changes of apoptosis [2–4].

Triggering of the CD95 (Fas/Apo1) receptor with its cognate ligand or agonistic antibody results in apoptosis in a variety of cells, via recruitment of the Fas-associated death domain (FADD, also known as MORT1). FADD in turn recruits and activates caspase-8 through its N-terminal death effector domain [5,6]. The ability of caspase-8 to activate all known caspases *in vitro* means that it is a prime candidate for an initiator caspase in many other forms of receptor-mediated apoptosis. The cytotoxic tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is the most recently identified apoptosis-inducing member of the TNF ligand family [7]. The effects of TRAIL are mediated by a family of five receptors, two of which resemble CD95 in that they contain an intracellular 'death domain', which is indispensable for the initiation of the intracellular signalling cascade leading to cell death. Thus the death receptors TRAIL-R1 (also known as death receptor 4) and TRAIL-R2 (also known as death receptor 5, 'TRICK2' or 'KILLER') engage and activate initiator caspases, such as caspase-8, resulting in direct activation of effector caspases. Interestingly, experiments using FADD $^{-/-}$ mice suggest that an adaptor molecule, distinct from FADD, is required for initiation of the apoptotic programme induced by TRAIL [8].

Procaspase-9 has also been proposed as an initiator caspase; in the presence of dATP and cytochrome *c*, released from mitochondria, the N-terminus of caspase-9 interacts with Apaf-1

Abbreviations used: ERK, extracellular-signal-regulated kinase; FADD, Fas-associated death domain; GST, glutathione S-transferase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MAPKAPK-2, MAP kinase-activated protein kinase-2; MEKK1, MAP or ERK kinase kinase 1; PARP, poly(ADP-ribose) polymerase; TNF, tumour necrosis factor; TRAF, TNF receptor-associated protein; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL–LZ, TRAIL–leucine-zipper fusion protein; z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone.

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resulting in proteolytic activation of caspase-9 [9]. This caspase cascade, with caspase-9 at the apex, is the major pathway initiated in many forms of chemically-induced apoptosis [10]. The induction of apoptosis by inhibition of the major cellular protease, the proteasome, using peptide aldehydes and lactacystin almost certainly results in activation of this pathway. Apoptosis induced by proteasome inhibitors has recently been demonstrated to occur mainly in proliferating cells, while in quiescent cells, similar inhibition of the proteasome blocked apoptosis induced by a variety of agents [11,12]. Since many important short-lived regulatory molecules, e.g. p53, are substrates of the proteasome, it has been proposed that inhibition of the degradation of these proteins results in their accumulation, thereby leading to apoptosis.

Mammalian cells respond to various stressful stimuli by the activation of the stress-activated mitogen-activated protein kinases (MAP kinases), c-Jun N-terminal kinase (JNK) and p38. An increasing number of studies have shown the activation of the JNK and p38 pathways during apoptosis. The JNK and p38 pathways are activated by stimulation of TNF receptor family members, such as TNFR1 [13], CD40 [14], Herpes virus entry mediator ('HVEM')/another TNF receptor-associated protein (TRAF)-associated receptor ('ATAR') [15], CD95/Fas/Apo1 [16–21], TRAIL/Apo2L receptors [22] and the TNF-related activation-induced cytokine ('TRANCE') receptor, receptor activator of NF-κB ('RANK') [23]. JNK activation appears to be a requirement for ceramide-induced apoptosis [24] and apoptosis induced by γ- and UV-irradiation [25]. In addition, activation of the JNK pathway, using activated MAP or extracellular-signal-regulated kinase (ERK) kinase kinase 1 (MEKK1), leads to an enhanced apoptotic response to UV irradiation [26]. Experiments in transgenic mice show that a JNK3 knockout leads to decreased neuronal cell death in the hippocampus in response to the excitotoxic glutamate-receptor agonist kainic acid [27]. JNK and p38 activation are also implicated in apoptosis induced by nerve growth factor withdrawal from PC12 cells [28]. JNK interacting protein-1 ('JIP-1'), a binding partner for, and specific inhibitor of, JNK, is able to block nerve growth factor withdrawal-induced apoptosis of PC12 cells [28,29]. In addition, experiments using dominant negative constructs show that the phosphorylation of the transcription factor c-Jun, a major target for JNK, is required for trophic factor withdrawal-induced cell death in both PC12 cells and in rat primary neuronal cultures [28,30,31]. Taken together, these data strongly suggest that, at least in neuronal cells, JNK and/or p38 activation is a requirement for apoptosis. However, in other non-neuronal cell types the role of JNK and p38 activation in apoptosis is more controversial. For example, induction of apoptosis in HeLa cells by TNF, or Jurkat T-cells by anti-CD95 treatment, leads to activation of JNK and p38 but this does not appear to be a requirement for apoptosis [17,32].

To clarify this issue we have investigated the activation of JNK and p38 during apoptosis in a single, non-neuronal, cell type challenged by diverse stimuli. We report that in the human T-cell line, Jurkat, TRAIL-, CD95- and lactacystin-mediated apoptosis result in activation of JNK and p38. In the case of receptor-mediated apoptosis, activation of JNK and p38 is dependent on caspase activation, whereas in lactacystin-induced apoptosis JNK and p38 activation do not require effector caspase activation. Thus, we propose that in a single cell type, the mechanism of JNK and p38 activation during apoptosis is stimulus-dependent. In addition, using the specific p38 inhibitor, SB203580, we demonstrate that neither p38 nor mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2) activation is required for receptor-mediated or chemically-induced apoptosis.

EXPERIMENTAL

Materials

Media and serum were purchased from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Anti-CD95 monoclonal antibody, anti-(MAPKAPK-2) sheep polyclonal antibody and MAPKAPK-2 substrate peptide (KKLNRTLSVA) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Rabbit polyclonal anti-JNK antiserum was raised to a full-length glutathione S-transferase (GST) fusion of human JNK1β. Rabbit polyclonal antibody to murine p38α was a gift from Dr J. Raingeaud (Institut Curie, Orsay, France). The caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.FMK) was purchased from Enzyme Systems (Dublin, CA, U.S.A.). The p38 inhibitor SB203580 and the proteasome inhibitor lactacystin were from Calbiochem (Nottingham, U.K.). Annexin V/fluorescein isothiocyanate was obtained from Bender Medsystems (Vienna, Austria). All other reagents were from Sigma-Aldrich (Poole, Dorset, U.K.).

Methods

Cell culture and quantification of apoptosis

Jurkat E6-1 cells, a human T-cell lymphoma cell line (European Collection of Animal Cell Cultures, Wiltshire, U.K.), were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 2 mM Glutamax™, in an atmosphere of air/CO_2 (19:1) at 37 °C. The cells were maintained in exponential growth phase by routine passage every 3–4 days. Jurkat T-cells were incubated either alone or in the presence of anti-CD95 (50 ng/ml), TRAIL–leucine-zipper fusion protein (TRAIL–LZ; 50 ng/ml) [33] (a gift from Dr T. Griffiths, Immunex Corp., Seattle, WA, U.S.A.) or lactacystin (5 μM) for up to 8 h. Apoptosis was quantified by phosphatidylserine exposure, determined by Annexin V/fluorescein isothiocyanate binding and FACS analysis coupled with propidium iodide staining to exclude necrotic cells [10]. Where indicated, cells were pretreated for 1 h with z-VAD.FMK (20–100 μM) or SB203580 (10 μM) before exposure to the apoptotic stimulus.

Measurement of caspase cleavage

After the required treatments, approximately 0.4×10^6 Jurkat cells were lysed in SDS/PAGE sample buffer and separated by SDS/PAGE (7 or 13% acrylamide) followed by electrophoretic transfer on to nitrocellulose membranes (Hybond-C extra, Amersham, Bucks, U.K.) as described previously [34]. Equivalent protein loading was confirmed by Ponceau-S staining of the nitrocellulose membranes. Primary antibodies to caspase-3 (kindly provided by Dr D. Nicholson, Merck Frosst Centre for Therapeutic Research, Quebec, Canada), caspase-8, caspase-9 (kindly provided by Dr D. Green, La Jolla Institute for Allergy and Immunology, San Diego, CA, U.S.A.) and PARP (kindly provided by Dr. G. Poirier, Laval University, Quebec, Canada) were used, for Western-blot analysis, as described previously [10]. Immunodetection was carried out using the enhanced chemiluminescence ('ECL®') detection system (Amersham).

Immune-complex kinase assays

After treatment approximately 5×10^6 Jurkat cells were harvested by brief centrifugation, at 300 ***g*** for 3 min, and lysed in 800 μl of ice-cold Triton lysis buffer [20 mM Hepes (pH 7.5)/137 mM NaCl/25 mM β-glycerol phosphate/2 mM sodium pyrophosphate/2 mM EDTA/10% (v/v) glycerol/1% (v/v) Triton

X-100/1 mM PMSF/2.5 μg/ml each of pepstatin, antipain and leupeptin/2 mM benzamidine/0.5 mM dithiothreitol/1 mM Na_3VO_4]. Lysates were clarified by centrifugation at 20000 ***g*** for 15 min at 4 °C. Clarified lysates were then incubated with 5 mg of Protein A–Sepharose and rabbit polyclonal antisera to JNK, p38 or MAPKAPK-2 for 3 h at 4 °C. After this period immunoprecipitates were washed three times with 1 ml of ice-cold Triton lysis buffer and once with 1 ml of kinase assay buffer [25 mM Hepes (pH 7.4)/25 mM β-glycerol phosphate/25 mM $MgCl_2$/0.5 mM Na_3VO_4/0.5 mM dithiothreitol]. Immunoprecipitates were resuspended in kinase assay buffer to a final volume of 50 μl containing 50 μM [γ-^{32}P]ATP (2000 c.p.m./pmol) and either 5 μg of GST–activating transcription factor-2 (1–109) for JNK and p38 assays or 50 μM MAPKAPK-2 substrate peptide for MAPKAPK-2 assays. JNK and p38 assays were terminated, after incubation for 30 min at 30 °C, by the addition of SDS/PAGE sample buffer. The samples were then subjected to SDS/PAGE (10% acrylamide). ^{32}P-incorporation into GST–activating transcription factor 2 was determined by PhosphorImager analysis of the dried gels (Molecular Dynamics, Sunnyvale, CA, U.S.A.). MAPKAPK-2 assays were terminated after 15 min at 30 °C by spotting 30 μl aliquots of the reaction mixture on to p81 phosphocellulose paper squares (Whatman International Ltd., Maidstone, Kent, U.K.) and immersion into 150 mM H_3PO_4. Papers were washed extensively in several changes of H_3PO_4, once in acetone, dried and ^{32}P incorporation into the substrate was measured by Cerenkov counting. Background incorporation was measured in reactions lacking substrate peptide.

RESULTS

TRAIL and anti-CD95 induce time-dependent apoptosis together with processing of the initiator caspase-8 and the effector caspase-3 in Jurkat T-cells

Both TRAIL and anti-CD95 caused a time-dependent induction of apoptosis in Jurkat T-cells as assessed by an increase in externalization of phosphatidylserine (Figure 1A). Following TRAIL exposure, apoptosis was first detected at 60 min and was maximal at 6 h, whereas anti-CD95-induced apoptosis was slower in onset; not detectable until 2 h after exposure, and still increasing at 8 h (Figure 1A). Cells not exposed to TRAIL or anti-CD95 did not undergo apoptosis (Figure 1A). To determine which caspases were activated and their order of activation, Western-blot analysis was performed using antibodies to the initiator caspase-8 and the effector caspase-3. In untreated Jurkat T-cells, caspase-8 was present primarily as two isoforms of approx. 55 kDa (Figure 1B, upper panel), corresponding to caspases-8a and -8b [35]. Induction of apoptosis by TRAIL resulted in a time-dependent processing of caspase-8 initially to two fragments of approx. 43 and 41 kDa, corresponding to cleavage of the small subunit from caspases-8a and -8b. This was followed by the appearance of an 18 kDa subunit as a result of cleavage of the death effector domains from the 43 and 41 kDa fragments (Figure 1B, upper panel). A substantial increase in the processing of caspase-8 (to yield fragments of 41 and 43 kDa) was first observed approx. 30–45 min after TRAIL treatment. The effector caspase-3 was present in control Jurkat T-cells primarily as its intact 32 kDa precursor form (Figure 1B, lower panel). Induction of apoptosis by TRAIL resulted in loss of the proform of caspase-3 and a time-dependent appearance of three fragments of approx. 20, 19 and 17 kDa following cleavage at Asp^{175}, Asp^{9} and Asp^{28}, respectively [36]. Processing of caspase-3 was first detected 30 min after exposure to TRAIL (Figure 1B, lower panel). Thus induction of apoptosis by TRAIL was accompanied by the processing/activation of the initiator caspase-8 and and the effector caspase-3. Activation of both initiator and effector caspases appeared to occur simultaneously, approx. 30–45 min after exposure to TRAIL, and essentially accompanied the detection of apoptosis. Apoptosis induced by anti-CD95 resulted in the same pattern of caspase-8 and -3 activation (Figure 1C, upper and lower panels, respectively) as that observed with TRAIL (Figure 1B, upper and lower panels, respectively) but paralleled the slower time course of anti-CD95-induced apoptosis shown in Figure 1(A).

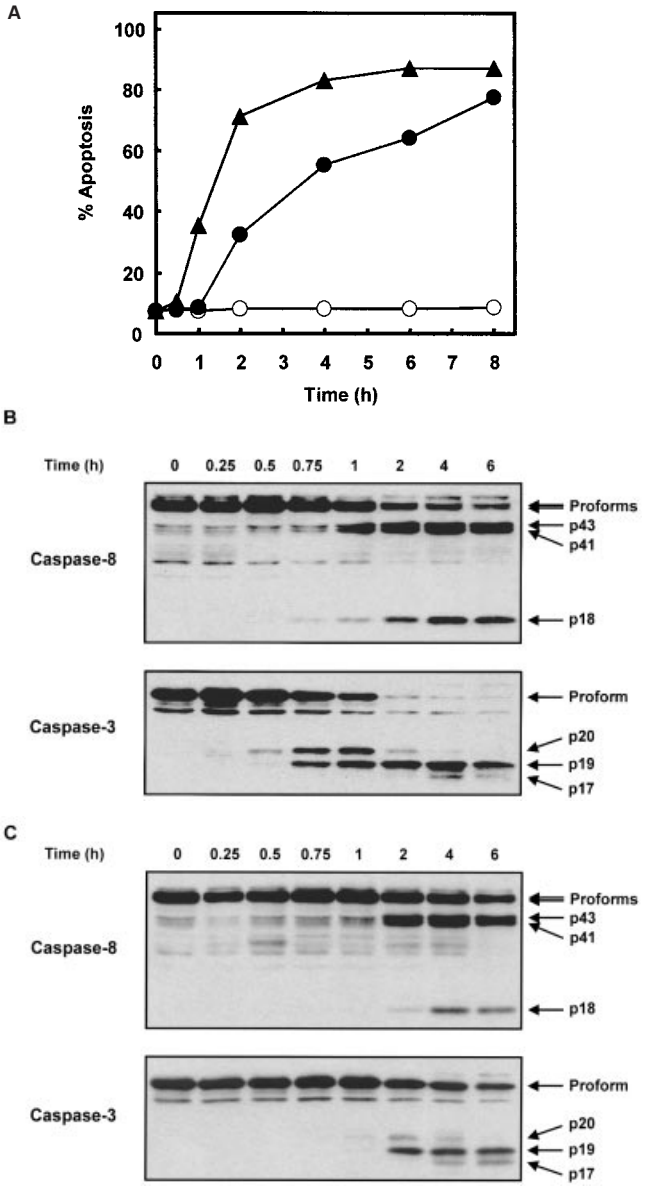

Figure 1 Time course of apoptosis and caspase activation induced by anti-CD95 and TRAIL in Jurkat T-cells

Jurkat T-cells were incubated in the absence (○-○) or presence of 50 ng/ml anti-CD95 (●-●) or 50 ng/ml TRAIL (▲-▲). The percentage of apoptotic cells was determined at the times indicated by FACS analysis as described in the Experimental section (**A**). Cleavage and activation of caspases-3 and -8 were determined after treatment with TRAIL (**B**) and anti-CD95 (**C**) by Western blotting, as described in the Experimental section. The data shown are representative of three independent experiments.

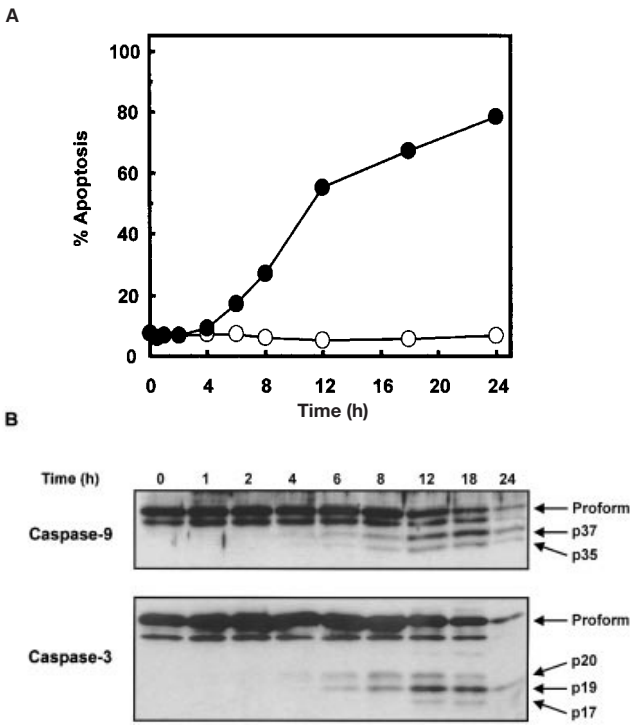

Figure 2 Time course of apoptosis and caspase activation induced by lactacystin in Jurkat T-cells

Jurkat T-cells were incubated in the absence (○-○) or presence of 5 μM lactacystin (●-●). The percentage of apoptotic cells was determined at the times indicated by FACS analysis as described in the Experimental section (**A**). Cleavage and activation of caspases-3 and -9 were determined by Western blotting as described in the Experimental section (**B**). The data shown are representative of three independent experiments.

Lactacystin induces time-dependent apoptosis together with processing of the initiator caspase-9 and the effector caspase-3 in Jurkat T-cells

Similar to TRAIL and anti-CD95, lactacystin induced a time-dependent induction of apoptosis (Figure 2A). The time course, however, was much slower than that induced by TRAIL and anti-CD95, with the effect of lactacystin only becoming apparent after 4–6 h and reaching a maximum at greater than 24 h. Cells not exposed to lactacystin did not undergo apoptosis (Figure 2A). The slower induction of apoptosis was paralleled by a slower activation of the effector caspase-3 and the major initiator caspase involved in chemically-induced apoptosis, caspase-9 (Figure 2B, upper panel). The proform of caspase-9 was processed to yield fragments of 37 and 35 kDa, first detectable 6 h after exposure to lactacystin, which result from cleavage at Asp^{315} and Asp^{330} [37]. Induction of apoptosis by lactacystin resulted in loss of the proform of caspase-3 and a time-dependent appearance of three fragments of approx. 20, 19 and 17 kDa (Figure 2B, lower panel). Processing of caspase-3 was first detected 6 h after exposure to lactacystin (Figure 2B, lower panel).

JNK and p38 are activated by TRAIL, anti-CD95 and lactacystin in Jurkat T-cells

In order to correlate the induction of apoptosis with the activation of the JNK and p38 pathways, we examined the time course of JNK and p38 activation in Jurkat T-cells treated with TRAIL, anti-CD95 and lactacystin. Treatment of Jurkat T-cells with TRAIL and anti-CD95 led to the activation of both JNK and p38, with no activation in unstimulated cells (Figures 3A and 3B). The time course of activation of both JNK and p38 closely correlated with that for the induction of apoptosis by both TRAIL and anti-CD95, as measured by phosphatidylserine exposure (Figure 1A) and caspase processing (Figures 1B and 1C). Activation of JNK by TRAIL was maximal at 2 h (7–8-fold stimulation) and declined thereafter to approx. 3–4-fold stimulation at 8 h. JNK activation by anti-CD95 was slower in comparison, peaking at 4 h (approx. 5-fold stimulation) and decreasing to approx. 4-fold at 8 h. The fold-activation of p38 by TRAIL and anti-CD95 was larger, but followed a similar time course. TRAIL-stimulated p38 activity was maximal at 2 h (12-fold) and declined to approx. 4-fold at 8 h. Anti-CD95 treatment led to p38 activation that peaked at 4 h (6-fold), declining marginally to approx. 4-fold at 8 h.

Treatment of Jurkat T-cells with the proteasome inhibitor, lactacystin, also activated JNK and p38, but with slower kinetics (Figures 3C and 3D). JNK activity was stimulated approx. 3.5-fold and was maximal at 8 h declining rapidly thereafter. p38 activity peaked at 8–12 h but was activated more strongly (approx. 7-fold), again declining to basal activity by 24 h. Negligible JNK or p38 activity was detected in unstimulated cells. Similarly to the activation of JNK and p38 by TRAIL and anti-CD95, the time course of activation of JNK and p38 by lactacystin closely correlated with that for the induction of apoptosis (Figures 2A and 2B), although the kinetics of kinase activation and apoptosis were slower.

The caspase inhibitor z-VAD.FMK but not the p38 inhibitor SB203580 blocks apoptosis in Jurkat T-cells

In order to clarify the role of p38 in the induction of apoptosis and caspase activation, we examined the effect of the caspase inhibitor z-VAD.FMK [3] and the p38 inhibitor SB203580 on the induction of apoptosis and activation of caspase-3 by TRAIL, anti-CD95 and lactacystin in Jurkat T-cells (Figures 4A and 4B).

In Jurkat T-cells exposed to TRAIL, anti-CD95 or lactacystin, there was a marked induction of apoptosis (Figure 4A). As expected, apoptotic induction by these agents was completely blocked by the caspase inhibitor z-VAD.FMK (20 μM). However, the specific p38 inhibitor, SB203580 (10 μM), had no effect on the induction of apoptosis by any of these agents (Figure 4A). Treatment of cells with either z-VAD.FMK or SB203580 alone for 8 h did not increase apoptosis above that seen in untreated cells (Figure 4A).

These observations were consistent with the effects of z-VAD.FMK and SB203580 on the activation/processing of pro-caspase-3 induced by TRAIL, anti-CD95 and lactacystin (Figure 4B). Caspase-3 processing in response to TRAIL and anti-CD95 was blocked completely by z-VAD.FMK but not by SB203580. Caspase-3 processing in response to lactacystin was partially blocked by z-VAD.FMK. This resulted in accumulation of a characteristic 20 kDa cleavage product, observed following z-VAD.FMK inhibition of chemically-induced caspase-3 activation. SB203580 had no effect on caspase-3 processing induced by lactacystin (Figure 4B).

Jurkat cells treated with either TRAIL, anti-CD95 or lactacystin showed a potent activation of MAPKAPK-2, an immediate downstream target of p38 (Figure 4C). This effect was completely abolished by SB203580, demonstrating the complete inhibition of p38 activity by this inhibitor (Figure 4C). Taken together these data demonstrate that in Jurkat T-cells, neither p38 nor MAPKAPK-2 activation by TRAIL, anti-CD95 or

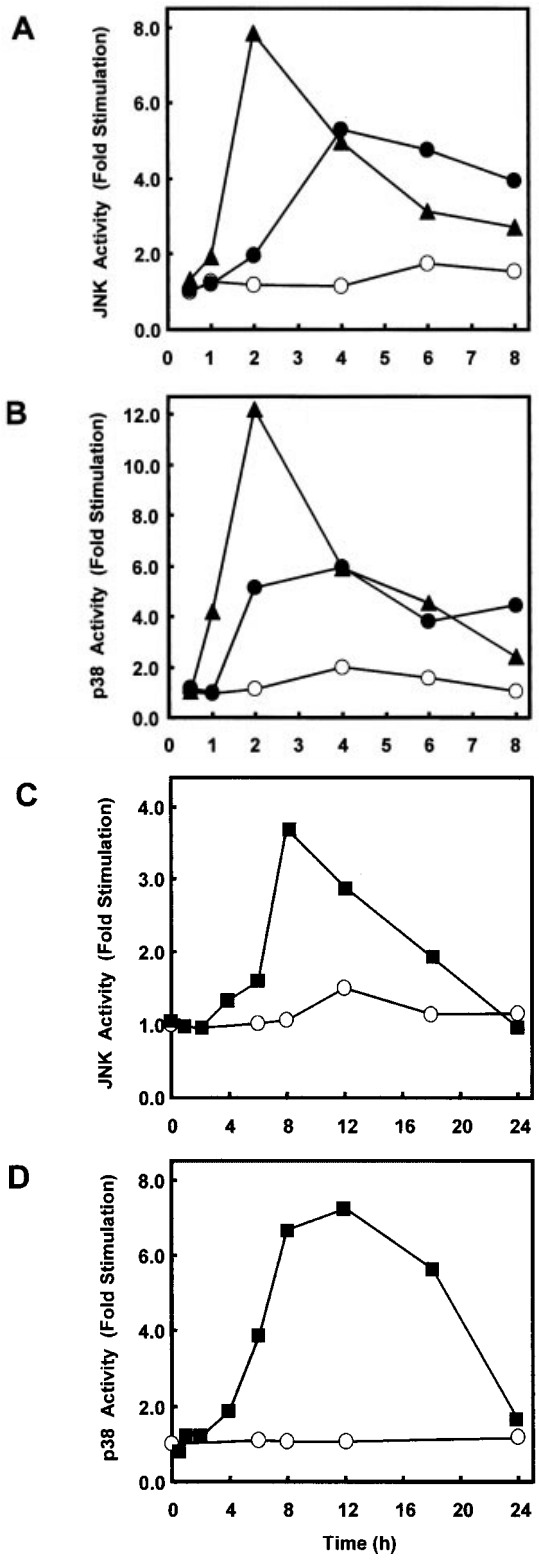

Figure 3 Activation of JNK and p38 by TRAIL, anti-CD95 and lactacystin in Jurkat T-cells

Jurkat T-cells were incubated in the absence (○-○) or presence of 50 ng/ml anti-CD95 (●-●), 50 ng/ml TRAIL (▲-▲) or 5 μM lactacystin (■-■). The activities of JNK (**A** and **C**) and p38 (**B** and **D**) were measured by an immune-complex kinase assay as described in the Experimental section. The data shown are representative of three independent experiments.

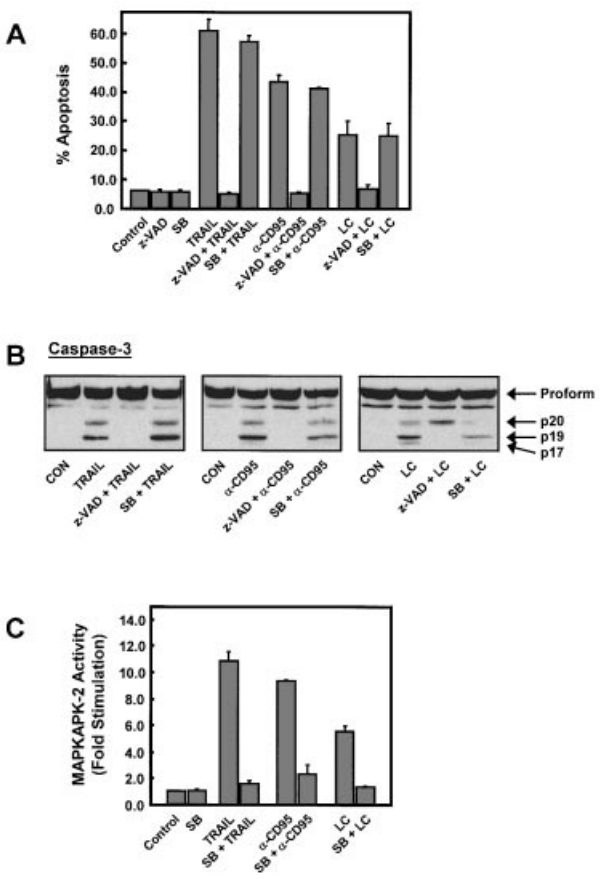

Figure 4 Effect of z-VAD.FMK and SB203580 on apoptosis and caspase activation in Jurkat T-cells

Jurkat T-cells were incubated with or without 50 ng/ml TRAIL for 2 h, 50 ng/ml anti-CD95 for 4 h, or 5 μM lactacystin (LC) for 8 h in the presence or absence of 20 μM z-VAD.FMK (z-VAD) or 10 μM SB203580 (SB) as indicated in the Figure. The percentage of apoptotic cells was determined by FACS analysis (**A**), activation of caspase-3 was determined by Western blotting (**B**) and MAPKAPK-2 activity was measured by an immune-complex kinase assay (**C**), as described in the Experimental section. The data shown in the bar charts represent the means ± S.E.M. ($n = 3$). CON, control; α-CD95, anti-CD95.

lactacystin is required for caspase-3 processing or induction of apoptosis.

z-VAD.FMK blocks JNK and p38 activation in response to TRAIL and anti-CD95 but not in response to lactacystin

To test if JNK and p38 activation by TRAIL, anti-CD95 or lactacystin was dependent on the activation of caspases, we examined the effect of z-VAD.FMK (20 μM) on JNK and p38 activation by these agents. Treatment of Jurkat T-cells with TRAIL, anti-CD95 or lactacystin led to the activation of both JNK and p38 as expected (Figures 5A and 5B, respectively). The activation of JNK and p38 by TRAIL and anti-CD95 was completely blocked by z-VAD.FMK. However, JNK and p38 activation by lactacystin was not blocked by z-VAD.FMK (Figures 5A and 5B). Interestingly, in lactacystin-treated cells z-VAD.FMK (20 μM) did not block the appearance of the 20 kDa fragment of caspase-3 but did block its further processing to its 19 and 17 kDa fragments (Figure 4B). These results implied that caspase-9 was activated and able to process caspase-3 to its 20 kDa fragment but that z-VAD.FMK had inhibited the

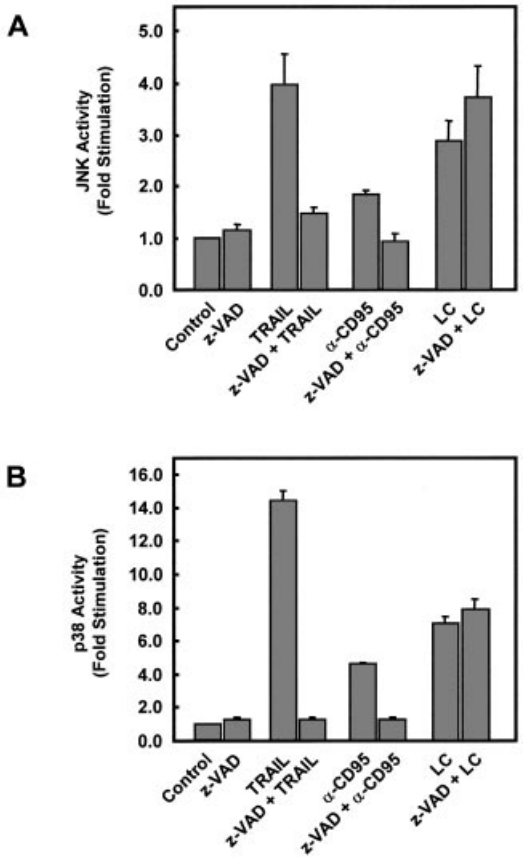

Figure 5 Effect of z-VAD.FMK on JNK and p38 activation in Jurkat T-cells

Jurkat T-cells were incubated with or without 50 ng/ml TRAIL for 2 h, 50 ng/ml anti-CD95 for 4 h, or 5 μM lactacystin (LC) for 8 h in the presence or absence of 20 μM z-VAD.FMK (z-VAD) as indicated in the Figure. The activities of JNK (**A**) and p38 (**B**) were measured by an immune-complex kinase assay as described in the Experimental section. The data shown represent the means $\pm$ S.E.M. ($n = 3$).

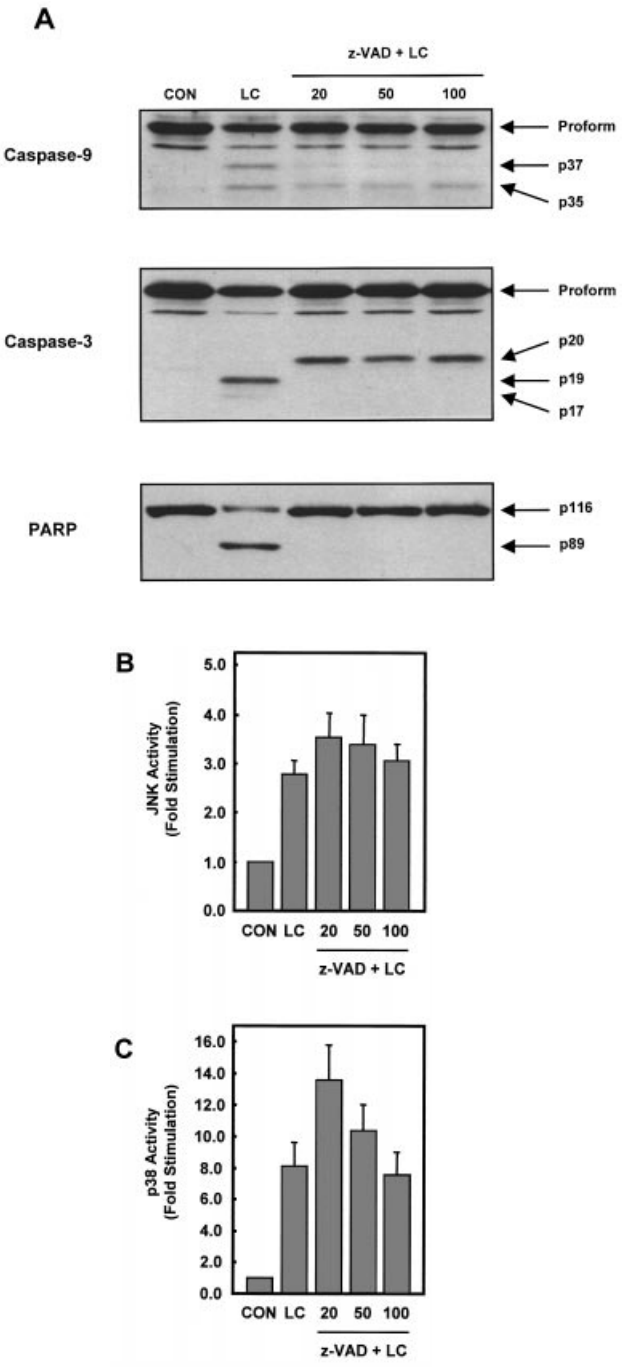

Figure 6 Effect of increasing concentrations of z-VAD.FMK on lactacystin-mediated caspase processing, PARP cleavage, and JNK and p38 activation

Jurkat T-cells were incubated with or without 5 μM lactacystin (LC) for 8 h in the presence of increasing concentrations of z-VAD.FMK (z-VAD, 20–100 μM) as indicated in the Figure. Activation of caspases-3 and -9 and cleavage of PARP were determined by Western blotting (**A**). The activities of JNK (**B**) and p38 (**C**) were measured by an immune-complex kinase assay as described in the Experimental section. The data shown in the bar charts represent the means $\pm$ S.E.M. ($n = 3$). CON, control.

autocatalytic activation of caspase-3. As both p38 and JNK were also activated at this concentration of z-VAD.FMK (Figure 5), it was possible that this activation was also mediated by caspase-9.

In order to test this hypothesis, we used a range of concentrations of z-VAD.FMK (20–100 μM) and examined both the processing of caspases-3 and -9 as well as the cleavage of PARP as a measure of caspase-3/-7 activity in the cells. Whereas almost all recombinant caspases can cleave PARP *in vitro*, in cells it appears that the effector caspases-3 and -7 are primarily responsible for cleavage of PARP at DEVD$\downarrow$G to yield its characteristic 24 kDa and 89 kDa fragments [38]. Treatment of Jurkat cells with lactacystin resulted in activation of effector caspases and cleavage of PARP to its characteristic 89 kDa fragment (Figure 6A). All concentrations of z-VAD.FMK (20–100 μM) completely blocked PARP cleavage, confirming that the activity of the effector caspases-3 and/or -7 was totally inhibited even at the lowest concentrations of z-VAD.FMK. In the lactacystin-treated cells, caspase-9 was again processed to its 37 and 35 kDa fragments (Figure 6A). Increasing concentrations of z-VAD.FMK only partially inhibited the formation of the 35 kDa fragment of caspase-9, while the appearance of the 37 kDa fragment of caspase-9 was reduced to basal levels. Interestingly, the processing of caspase-3 to its 20 kDa fragment was not inhibited at any concentration of z-VAD.FMK (20–100 μM), whereas further processing to its 19 and 17 kDa fragments was completely inhibited at all concentrations (Figure 6A). These results are compatible with increasing concentrations of z-

VAD.FMK being unable to inhibit the processing of caspase-9 at Asp^{315} resulting in formation of the 35 kDa fragment. However, these concentrations were able to inhibit the activity of caspase-3, so preventing its ability to feedback and further process caspase-9 at Asp^{330} resulting in formation of the 37 kDa fragment.

In order to test if activation of JNK and p38 by lactacystin was dependent on activation of effector caspases, we examined the effect of increasing concentrations of z-VAD.FMK on JNK and p38 activation by this agent. Treatment of Jurkat T-cells with lactacystin led to the activation of both JNK and p38 (Figures 6B and 6C). Treatment of cells with z-VAD.FMK had no significant inhibitory effect on JNK and p38 activation by lactacystin, even at concentrations up to 100 μM (Figures 6B and 6C).

These results, together with the observation that z-VAD.FMK is able to block PARP cleavage, autocatalytic activation of caspase-3 and caspase-9 processing to its 37 kDa fragment (Figure 6A), strongly suggest that effector caspases are not required for the activation of JNK and p38 in response to lactacystin. This is in marked contrast with the activation of JNK and p38 by TRAIL and anti-CD95, which were significantly inhibited by z-VAD.FMK, even at 20 μM (Figure 5). These data demonstrate that activation of both JNK and p38 during receptor-mediated and chemically-induced apoptosis of Jurkat T-cells exhibits a differential requirement for caspase activation.

DISCUSSION

Caspase activation by TRAIL, anti-CD95 and lactacystin

In order to characterize the differences between receptor-mediated and chemically-induced apoptosis in Jurkat T-cells, we examined the activation of caspases by TRAIL, anti-CD95 and lactacystin. Treatment of cells with TRAIL and anti-CD95 resulted in processing of the initiator caspase-8 and the effector caspase-3 with concomitant induction of apoptosis. Induction of apoptosis chemically, using the proteasome inhibitor lactacystin, resulted in the activation of the initiator caspase-9 and the effector caspase-3, although with slower kinetics than that observed with the receptor-mediated agents, TRAIL and anti-CD95. During both receptor-mediated and chemically-induced apoptosis, the activation of caspases exactly paralleled the appearance of Annexin V reactivity, a well-characterized marker of apoptosis. Thus activation of the initiator caspases-8 and -9 in TRAIL/anti-CD95- and lactacystin-mediated apoptosis, respectively, was entirely consistent with earlier observations in Jurkat and other cell lines, and suggested different mechanisms for activation of the core apoptotic pathway by receptor-dependent and -independent apoptotic agents [10,39].

JNK and p38 activation by TRAIL, anti-CD95 and lactacystin

To explore these differences further, and to determine the role of JNK and p38 in both receptor-mediated and chemically-induced apoptosis we examined the activation of JNK and p38 in Jurkat T-cells after induction of apoptosis by treatment with TRAIL, anti-CD95 and lactacystin. In Jurkat T-cells, our results showed that the JNK and p38 pathways were activated in parallel with caspases during both receptor-mediated and chemically-induced apoptosis. However, the kinetics of activation of both JNK and p38 were slower during lactacystin-induced apoptosis and this matched the profile of caspase activation by this apoptotic agent. Hence, for both receptor-mediated and chemically-induced apoptosis, JNK and p38 were activated in parallel with caspases even though the kinetics of apoptosis differed between the two types of stimuli. This suggested a causal link between caspase activation and JNK/p38 activation.

Role of caspases in JNK/p38 activation

The close match of the kinetics of activation of caspases and kinases did not allow us to assess whether kinase activation was dependent on caspase activation by simple examination of the time courses of activation. In order to address this we used z-VAD.FMK, a broad-spectrum caspase inhibitor.

z-VAD.FMK blocked activation of caspase-3 and apoptosis in response to TRAIL and anti-CD95 in Jurkat T-cells. In lactacystin-treated cells z-VAD.FMK inhibited phosphatidylserine exposure, PARP cleavage and processing of caspase-9 to its 37 kDa fragment. However, activation of JNK and p38 were blocked by z-VAD.FMK only if apoptosis was initiated by TRAIL or anti-CD95; their activation in response to lactacystin was not blocked by z-VAD.FMK, even at concentrations as high as 100 μM (Figures 5, 6B and 6C). Thus the mechanism of activation of JNK and p38 during TRAIL- and anti-CD95-induced apoptosis was caspase-dependent, whereas effector caspase activation was not required for JNK and p38 activation during apoptosis induced by lactacystin.

Our results with a range of concentrations of z-VAD.FMK demonstrated that the initial processing of caspase-9, presumably mediated by Apaf-1, and its subsequent processing of caspase-3 to yield the 20 kDa fragment were not markedly inhibited even at the highest concentration of inhibitor (100 μM; Figure 6A). In contrast, the activity of caspase-3 was completely inhibited by the lowest concentration of z-VAD.FMK as shown by inhibition of PARP cleavage, inhibition of the processing of caspase-9 to its 37 kDa form and inhibition of the autocatalytic activation of caspase-3 to its 19 and 17 kDa fragments (Figure 6A). As z-VAD.FMK is generally believed to be a broad-spectrum caspase inhibitor, these results were rather surprising and suggested either that it is a very poor inhibitor of both caspase-9 processing and caspase-9 activity or that caspase-9 is processed either by a caspase that is insensitive to z-VAD.FMK or by a distinct enzyme. Of interest in this regard, granzyme B can cleave caspase-3 directly and in the presence of z-VAD.FMK the generation of the 20 kDa fragment is largely unaffected [40]. However, as Jurkat T-cells do not possess significant endogenous granzyme B activity [41], it is unlikely that this enzyme is responsible for cleavage of caspase-3 in this system.

Our observations are consistent with the results of others. In U937 cells, apoptosis, as measured by PARP cleavage, and JNK activation are induced by the proteasome inhibitor MG132 [42]. In contrast with our study, these authors report JNK activation prior to apoptosis, as assessed by PARP cleavage. However, this may simply reflect the rather late cleavage of PARP compared with phosphatidylserine exposure or caspase activation, which were the markers used to measure apoptosis in our study.

z-VAD.FMK blocks CD95-mediated JNK activation in SKW6.4 cells, and in Jurkat T-cells inhibits all CD95-mediated apoptotic responses and the activation of JNK and p38 [16,17,21,43]. However, more specific caspase-3 inhibitors, such as acetyl-Asp-Glu-Val-Asp-aldehyde, block CD95-induced chromatin condensation and DNA fragmentation, but neither JNK and p38 activation nor CD95-induced morphological changes such as cell shrinkage and membrane blebbing or cell death [43]. Together these data suggest that CD95-induced activation of JNK and p38 does not require effector caspases, such as caspase-3, which are essential for nuclear apoptotic events, but may instead require activation of upstream initiator caspases such as caspases-8 and/or -9 [43,44]. However, the situation is more complex. CD95-induced JNK activation is not blocked by z-VAD.FMK in neuronal or 293 cells [18,45]. In addition, TRAIL activates JNK in HeLa cells and Kym-1 cells

and this is blocked by z-VAD.FMK in the former but not the latter [22]. Together these data suggest that there are both caspase-dependent and caspase-independent pathways for JNK activation by both anti-CD95 and TRAIL depending on the cell type.

Mechanism of JNK/p38 activation

Using z-VAD.FMK, a broad-spectrum caspase inhibitor, we were unable to determine which caspase is required for activation of JNK and p38 by TRAIL and anti-CD95 in Jurkat cells. However, recent evidence suggests that for CD95 and TNFα-mediated apoptosis, JNK and p38 activation may lie downstream of caspase-8.

CD95 and TNFα-dependent activation of JNK appears to be slower in caspase-8 $^{-/-}$ embryonic fibroblasts than in caspase-8 $^{+/+}$ cells, suggesting that caspase-8 contributes to JNK activation by these ligands [46,47]. Jurkat T-cells deficient in caspase-8 are completely refractory to CD95-induced caspase activation, cell death and induction of both JNK and p38 activity [44]. In addition, overexpression of caspase-8 results in JNK activation; the activation of JNK by caspase-8 overexpression is blocked by dominant negative TRAF and MEKK1 constructs, suggesting the involvement of both TRAF and MEKK1 in JNK activation by caspase-8 [47]. Indeed it is likely that caspase-mediated cleavage of MEKK1 leads to activation of the JNK pathway in response to both receptor-mediated and chemically-induced apoptosis [48,49]. Experiments in transfected cells and with transgenic mice suggest that TRAF is required for JNK activation by most of the TNF family of receptors [50,51]. Although CD95 ligation leads to activation of JNK, this receptor does not associate with TRAF. JNK activation by CD95 is therefore thought to require another adapter protein, Daxx [18,52]. Daxx is a death domain-containing adapter protein, which facilitates the recruitment of the apoptosis signal-regulated kinase to CD95 and its concomitant activation upon receptor ligation. Activation of the apoptosis signal-regulated kinase is then thought to lead to activation of the JNK pathway [18,52].

Role of p38 in apoptosis

The activation of JNK and/or p38 appears to be a requirement for induction of the apoptotic programme in neuronal cells. In order to determine if the activation of p38 was required for apoptosis in T-cells, we investigated the effect of the specific p38 inhibitor, SB203580. This inhibitor did not inhibit apoptosis or caspase-3 activation in Jurkat T-cells treated with TRAIL, anti-CD95 or lactacystin. SB203580 did however inhibit the activation of a direct downstream target of p38, MAPKAPK-2, in response to TRAIL, anti-CD95 and lactacystin, thus demonstrating the efficacy of the inhibitor. This result shows that the activation of p38 and MAPKAPK-2 is not required for the induction of apoptosis by these agents in Jurkat T-cells. However, their activation may still be required to elicit other features of the apoptotic phenotype.

In conclusion we have shown that in a single cell line, Jurkat T-cells, the induction of both receptor-mediated and chemically-induced apoptosis is accompanied by a parallel activation of the JNK and p38 MAP kinase pathways. However, the mechanism of p38 and JNK activation during apoptosis is stimulus-dependent; caspase cleavage being required for JNK and p38 activation in response to the receptor-mediated apoptotic agents TRAIL and anti-CD95, whereas their activation chemically, using lactacystin, is effector caspase-independent. In addition, activation of the p38 pathway does not appear to be required for apoptosis as assessed by caspase activation and phosphatidylserine exposure. Further studies will be required to determine if JNK and p38 are required to elicit other features of the apoptotic phenotype.

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