Molecular Biology of the Cell
Vol. 12, 3139–3151, October 2001

# p38-mediated Regulation of an Fas-associated Death Domain Protein-independent Pathway Leading to Caspase-8 Activation during TGFβ-induced Apoptosis in Human Burkitt Lymphoma B Cells BL41

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Submitted November 16, 2000; Revised June 22, 2001; Accepted July 19, 2001
Monitoring Editor: Tony Hunter

On binding to its receptor, transforming growth factor β (TGFβ) induces apoptosis in a variety of cells, including human B lymphocytes. We have previously reported that TGFβ-mediated apoptosis is caspase-dependent and associated with activation of caspase-3. We show here that caspase-8 inhibitors strongly decrease TGFβ-mediated apoptosis in BL41 Burkitt's lymphoma cells. These inhibitors act upstream of the mitochondria because they inhibited the loss of mitochondrial membrane potential observed in TGFβ-treated cells. TGFβ induced caspase-8 activation in these cells as shown by the cleavage of specific substrates, including Bid, and the appearance of cleaved fragments of caspase-8. Our data show that TGFβ induces an apoptotic pathway involving sequential caspase-8 activation, loss of mitochondrial membrane potential, and caspase-9 and -3 activation. Caspase-8 activation was Fas-associated death domain protein (FADD)-independent because cells expressing a dominant negative mutant of FADD were still sensitive to TGFβ-induced caspase-8 activation and apoptosis. This FADD-independent pathway of caspase-8 activation is regulated by p38. Indeed, TGFβ-induced activation of p38 and two different inhibitors specific for this mitogen-activated protein kinase pathway (SB203580 and PD169316) prevented TGFβ-mediated caspase-8 activation as well as the loss of mitochondrial membrane potential and apoptosis. Overall, our data show that p38 activation by TGFβ induced an apoptotic pathway via FADD-independent activation of caspase-8.

## INTRODUCTION

Apoptosis is a highly regulated process involving various intracellular signaling pathways and a large number of molecules. Among these molecules, the proteases of the caspase family play a crucial role in triggering and controlling the execution of apoptosis (Cohen, 1997). These caspases are cysteine-related proteases that are synthesized as inactive proenzymes and are activated by most apoptotic stimuli. The proenzymes are activated by proteolysis at specific aspartate sites. The cleavage products form dimers, which are the active enzymes (Alnemri, 1997). There are 14 known caspases, of which caspase-8 and caspase-3 play key roles in control of the various steps of apoptosis. In recent years, an increasing number of investigations has contributed to elucidate the mechanisms underlying the activation of these two caspases (Kumar, 1999). Thus, caspase-3 may be activated via mitochondria-dependent or -independent pathways (Porter and Janicke, 1999). One of these pathways is dependent on the release by mitochondria of cytochrome *c*, which, in the presence of ATP, associates with the cytoplasmic Apaf1 and inactive proforms of caspase-9 to form a complex called apoptosome (Li *et al.*, 1997; Qin *et al.*, 1999). Autocleavage and activation of caspase-9 occur in this complex. In turn, caspase-9 then directly cleaves and activates caspase-3 proforms.

An alternative pathway, observed in type I Jurkat T cells in response to Fas ligation, is independent of mitochondrial activation and requires the direct cleavage of caspase-3 proforms by activated caspase-8 (Stennicke *et al.*, 1998; Scaffidi *et al.*, 1999). Caspase-8 activation has been extensively studied

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in apoptosis mediated by members of the tumor necrosis factor-receptor (TNF-R) family such as Fas (CD95) and TNF-R itself (Ashkenazi and Dixit, 1998). Indeed, the activation of CD95 by its natural ligand or agonist antibody ligands results in Fas-associated death domain protein (FADD) recruitment through their respective death domain (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Interactions between Fas and FADD via their COOH-terminal death domains expose the $NH_2$-terminal death effector domain (DED) of FADD, which can interact with DED domains in the caspase-8 proform, resulting in the oligomerization of this protease and its subsequent autocleavage and activation (Kischkel *et al.*, 1995; Medema *et al.*, 1997). Caspase-8 by cleaving the proapoptotic member of the Bcl-2 family protein, Bid, is then responsible for changes in the mitochondria, including opening of the permeability transition pore, a decrease in mitochondrial membrane potential, and the release of cytochrome *c* into the cytoplasm (Li *et al.*, 1998; Luo *et al.*, 1998; Schendel *et al.*, 1999). Whereas Fas binds directly to FADD, it is generally believed that other members of the TNF-R family bind to FADD via the adaptor molecule TNF receptor-associated death domain (Hsu *et al.*, 1996). Thus, FADD is the final common link between the death domain-containing receptors and caspase-8. Activation of caspase-8 by caspase-3 has also been reported, and Wesselborg and colleagues recently reported that anticancer drug-mediated caspase-8 activation is FADD-independent (Slee *et al.*, 1999; Wesselborg *et al.*, 1999). However, the nature and regulation of these FADD-independent pathways of caspase-8 activation remain unknown.

The serine/threonine kinases of the mitogen-activated protein kinase (MAPK) family are also key modulators of cell activation, including apoptosis. To date, three major MAPKs have been identified: the extracellular signal-regulated kinases (ERK1/2), the c-Jun $NH_2$-terminal protein kinase (JNK), and the p38 mitogen-activated protein kinase (p38). These kinases differ in their involvement in the control of apoptosis (Ip and Davis, 1998; Tibbles and Woodgett, 1999). ERK1/2 are mainly activated by growth factors and are involved in the regulation of cell proliferation (Hartsough and Mulder, 1995; Seger and Krebs, 1995; Taieb *et al.*, 1995; Blanchard *et al.*, 2000). On the other hand, JNK and p38 are stress-associated protein kinases that may regulate apoptosis positively or negatively depending on the cell type and stimulus (Raingeaud *et al.*, 1995; Xia *et al.*, 1995; Yamaguchi *et al.*, 1995; Graves *et al.*, 1996; Ichijo *et al.*, 1997; Juo *et al.*, 1997; Kummer *et al.*, 1997; Seimiya *et al.*, 1997; Wang *et al.*, 1998; Franklin *et al.*, 1998; Callsen and Brune, 1999; Kimura *et al.*, 2000). Although the involvement of p38 in apoptosis has been reported in various systems, the mechanism by which p38 regulates apoptosis is still unclear. Hsu *et al.* (1999) reported that p38 activates the expression of Fas-L, thereby mediating apoptosis by regulating Fas signaling. More recently, Zhuang *et al.* (2000) reported that, during singlet oxygen-induced apoptosis, p38 may regulate the cleavage of Bid in a caspase-8-independent manner. To date, the role of p38 in caspase activation has not been clearly assessed.

We previously reported that TGF$\beta$ mediates the apoptosis of human B lymphocytes (Chaouchi *et al.*, 1995). This apoptosis is caspase-dependent and associated with caspase-3 activation (Schrantz *et al.*, 1999). However, the pathways responsible for caspase-3 activation are still poorly defined and, in particular, the role of caspase-8 in this response has not been clearly defined during TGF$\beta$-mediated B cell apoptosis. On the other hand, in addition to ERK activation, TGF$\beta$ can stimulate both MKK4-JNK and MKK3-p38 pathways by activating TGF$\beta$-activated kinase 1 (Hartsough and Mulder, 1995; Yamaguchi *et al.*, 1995; Atfi *et al.*, 1997; Frey and Mulder, 1997; Hanafusa *et al.*, 1999). This led us to investigate the roles of caspase-8 and p38 in the activation of caspase-3 observed during the TGF$\beta$-mediated apoptosis of B lymphocytes. We report here that TGF$\beta$ induces caspase-8 activation, which in turn regulates both the loss of mitochondrial membrane potential and caspase-3 activation. We also found that TGF$\beta$-mediated p38 phosphorylation controlled this caspase-8 activation in an FADD-independent manner.

## Materials and Methods

### Reagents

zAEVD-fmk, zIETD-fmk, zAEVD-pNA, and zIETD-pNA were purchased from R & D Systems (Wiesbaden, Germany), and zVAD-fmk was obtained from Bachem Biochimie SARL (Voisin le Bretonneux, France). Stock solutions of zAEVD-fmk, zIETD-fmk, and zVAD-fmk were prepared in dimethyl sulfoxide and stored at −20°C. The working dilutions were prepared immediately before use. 3,3′dihexylocarbocyanine iodide [$DiOC_6(3)$] was purchased from Molecular Probes (Leiden, The Netherlands), purified porcine TGF$\beta$ was obtained from R&D Systems, the cell-permeable fluorigenic substrate PhiPhilux $G_2D_2$ from OncoImmunin (Kensington, MD), and ionomycin from Sigma (St. Louis, MO). The CH11 and the ZB4 monoclonal antibodies (mAbs) were from Immunotech (Marseille, France). SB203580, PD169316, PD98059, and U0126 were from Calbiochem (San Diego, CA).

### Cell Lines

The Burkitt's lymphoma cell line BL41, kindly provided by Drs. Alan Calender and Gilbert Lenoir (Edward Herriot Hospital, Lyon, France), does not contain the EBV genome. The Jurkat cell line was obtained from American Type Culture Collection (Rockville, MD). Both cell lines were cultured in RPMI 1640 Glutamax culture medium (Seromed; Biochrom, Berlin, Germany) supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA).

### Cell Transfection

The pcDNA-3.0/FADD-DN vector (kindly provided by Dr. V. Dixit) carries a truncated FADD cDNA (aa 80/208) lacking the DED region (Chinnaiyan *et al.*, 1995, 1996). The dominant active human pcDNA-MKK3(b)E and pcDNA-MKK6(b)E vectors were kindly provided by Dr. J. Han and have been previously described (Han *et al.*, 1996). pIRES.hrGFP was from Stratagene (San Diego, CA). BL41 cells were transfected by electroporation (960 $\mu$F, 240 V in a Bio-Rad apparatus). Stable transfectants, expressing FADD-DN were selected by incubating the cells with 1 mg/ml G418 for ~3 wk. Stable clonal transfectants were isolated from resistant G418 cells with the use of the limiting dilution technique, and the expression of FADD-DN protein in the various clones was analyzed by Western blotting with the use of a rabbit anti-FADD antibody (StressGen Biotechnologies, British Columbia, Victoria, Canada). Cells were transiently transfected with green fluorescent protein (GFP) and either MKK3 or MKK6 vectors. Eighteen hours after transfection, dead cells, due to the electroporation shock, were removed from the cultures by centrifugation through Ficoll gradient. Viable cells were then cultured at 37°C for 24 h.

### *Detection of Apoptotic Cells*

Cells were washed in phosphate-buffered saline, pelleted, and resuspended in phosphate-buffered saline. Their dot-blot light scatter profiles were analyzed by flow cytometry with the use of an FACScan flow cytometer (BD Biosciences, San Jose, CA). Shrunken cells with relatively high side-scatter and low forward-scatter properties were considered to be apoptotic and enumerated as a percentage of the total population. For both MKK3- and MKK6-expressing cells, the GFP-positive cells were analyzed for apoptosis by determining cell shrinkage.

### *Analysis of Mitochondrial Transmembrane Potential ($\Delta\Psi m$)*

$\Delta\Psi$m was evaluated by staining cells ($10^6$) with $DiOC_6(3)$ at a final concentration of 40 nM (stock solution 1 $\mu$M in ethanol) for 15 min at 37°C. The fluorescence emitted by cells was analyzed with an FACScan flow cytometer (BD Biosciences) with the use of the FL1 channel.

### *Concomitant Analysis of $\Delta\Psi m$ and Caspase-3 Activity in a Single Cell*

The cell-permeable fluorogenic substrate (Phiphilux-$G_2D_2$) and $DiOC_6(3)$ were used to monitor both caspase-3 activity and $\Delta\Psi$m in a single cell. Cells ($10^6$) were stimulated by incubation with TGF$\beta$ (1 ng/ml) for 48 h. They were collected by centrifugation and resuspended in 50 $\mu$l of Phiphilux-$G_2D_2$ substrate solution supplemented with 5% fetal calf serum. Cells were incubated in a 5% $CO_2$ incubator at 37°C for 45 min. They were then incubated with $DiOC_6(3)$, at a final concentration of 40 nM, in a 5% $CO_2$ incubator at 37°C for another 15 min. The cells were pelleted and resuspended in 500 $\mu$l of Phiphilux dilution buffer (OncoImmunin), and fluorescence emission was immediately determined with the use of the FL-1 ($\Delta\Psi$m) and FL-2 (caspase-3 activity) channels in an FACScan flow cytometer (BD Biosciences).

### *Assay of Caspase-8 Activity*

The caspase-8 activity was determined with the use of a colorimetric caspase assay (R & D Systems). Briefly, cells treated with 1 ng/ml TGF$\beta$ for various periods of time were collected and lysed according to the manufacturer's instructions. Caspase 8 colorimetric substrates (IETD-pNA or AEVD-pNA) were added to the cell lysate and assays were performed in a 100-$\mu$l volume in 96-well flat-bottomed plates. Absorbance was measured on a microplate reader at a wavelength of 405 nm after 1 h of incubation at 37°C and was standardized with the use of free colorimetric substrate. The results are expressed as fold-increase in the caspase activity in stimulated cells with the use of unstimulated cells as the reference.

### *Western Blot Analysis*

Cells were lysed by incubation in modified Laemnli buffer (60 mM Tris, pH 6.8, 10% glycerol, and 2% SDS, without $\beta$-mercaptoethanol and bromophenol blue) and sonication for 30 s on ice. The samples were centrifuged for 5 min at 15,000 $\times$ *g*. The supernatants were boiled for 5 min and frozen at −80°C or used immediately. Aliquots of the supernatants were assayed for protein concentration (microBCA protein assay; Pierce, Rockford, IL). $\beta$-Mercaptoethanol and bromophenol blue were added and cell lysate proteins (20 $\mu$g/lane) were resolved by SDS-PAGE. Proteins were then electroblotted onto 0.45-$\mu$m pore-size nitrocellulose filters, and the filters were blocked by incubation for 1 h with 5% nonfat milk in Tris-buffered saline, 0.1% Tween 20. The filters were then incubated for 1 h at room temperature or overnight at 4°C with anticaspase-8 mAb (clone 5F7; Upstate Biotechnology, Lake Placid, NY), FADD mAb (StressGen Biotechnologies), Bid antibody (R & D Systems) or anticleaved caspase-9 antibody (New England Biolabs, Beverly, MA), anti-caspase-3 antibody (polyclonal rabbit anti-caspase-3 antiserum; PharMingen, San Diego, CA), phospho-p38 mAb (New England Biolabs) or p38 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were washed three times for 10 min, in Tris-buffered saline, 0.1% Tween 20 and incubated for 1 h with peroxidase-labeled anti-mouse or anti-rabbit immunoglobulins. Blots were developed with the use of the enhanced chemiluminescence detection system (Pierce).

## RESULTS

### *TGF$\beta$-induced $\Delta\Psi m$ Loss Is Caspase-dependent*

We previously reported that TGF$\beta$-mediated apoptosis in the Burkitt's lymphoma cell line (BL41) was dependent on caspase-3 activation. To characterize upstream events involved in both caspase-3 activation and apoptosis induced by TGF$\beta$, we first studied mitochondrial transmembrane potential, which is an important marker of mitochondria involvement during the apoptotic process. TGF$\beta$-induced cell shrinkage (61 vs. 3% in control cells after 48 h of stimulation), as assessed by cell dot-blot light scatter profiles and flow cytometry, was associated with a loss of $\Delta\Psi$m, as quantified by staining with $DiOC_6(3)$ (71 vs. 3% in control cells) (Figure 1A). Similar results were obtained when BL41 cells were activated with either recombinant TGF$\beta$ or porcine TGF$\beta$ in the presence or the absence of fetal calf serum during the first 60 min of the stimulation. In addition, the loss of $\Delta\Psi$m observed in the presence of TGF$\beta$, was considerably reduced in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (50 $\mu$M), whereas loss of $\Delta\Psi$m mediated by ionomycin, which is known to be caspase-independent, was not affected by zVAD-fmk (Figure 1B). These observations suggest that TGF$\beta$ causes a reduction in mitochondrial transmembrane potential via a caspase-dependent pathway.

### *TGF$\beta$ Promotes Caspase-8 Activation and Bid Cleavage*

Caspase-8 has been reported to regulate mitochondria activation in various models (Muzio *et al.*, 1996; Li *et al.*, 1998). We then investigated whether various specific inhibitors (IETD-fmk and AEVD-fmk) of caspase-8 modulated apoptosis and the loss of $\Delta\Psi$m induced by TGF$\beta$ (Figure 2A). These two inhibitors caused dose-dependent inhibition of both cell shrinkage and loss of the $\Delta\Psi$m. Inhibition was observed at an inhibitor concentration of 10 $\mu$M and was more marked at a concentration of 75 $\mu$M. In our experimental conditions, inhibition was stronger with AEVD-fmk than with IETD-fmk and was similar for concentration of 75 $\mu$M AEVD-fmk and 100 $\mu$M zVAD-fmk. The increase in apoptosis induced by TGF$\beta$ was similarly inhibited: the increase of shrinkage and loss of $\Delta\Psi$m mediated by TGF$\beta$ were 5.7- and 6.2-fold, respectively, but only 2.4- and 3.6-fold in the presence of IETD-fmk (75 $\mu$M), 1.6 and 1.7-fold in the presence of AEVD-fmk (75 $\mu$M), and 1.4- and 1.5-fold in the presence of zVAD-fmk (75 $\mu$M), respectively (Figure 2A). The effects of these inhibitors were specific because the carrier dimethyl sulfoxide, used at the same concentration, had not effect on the loss of $\Delta\Psi$m and cell shrinkage induced by TGF$\beta$. To assess further the involvement of caspases sensitive to IETD-fmk and AEVD-fmk during TGF$\beta$-mediated activation, we tested whether cell lysates from TGF$\beta$-treated BL41 cells cleaved these two substrates. Indeed, we showed by

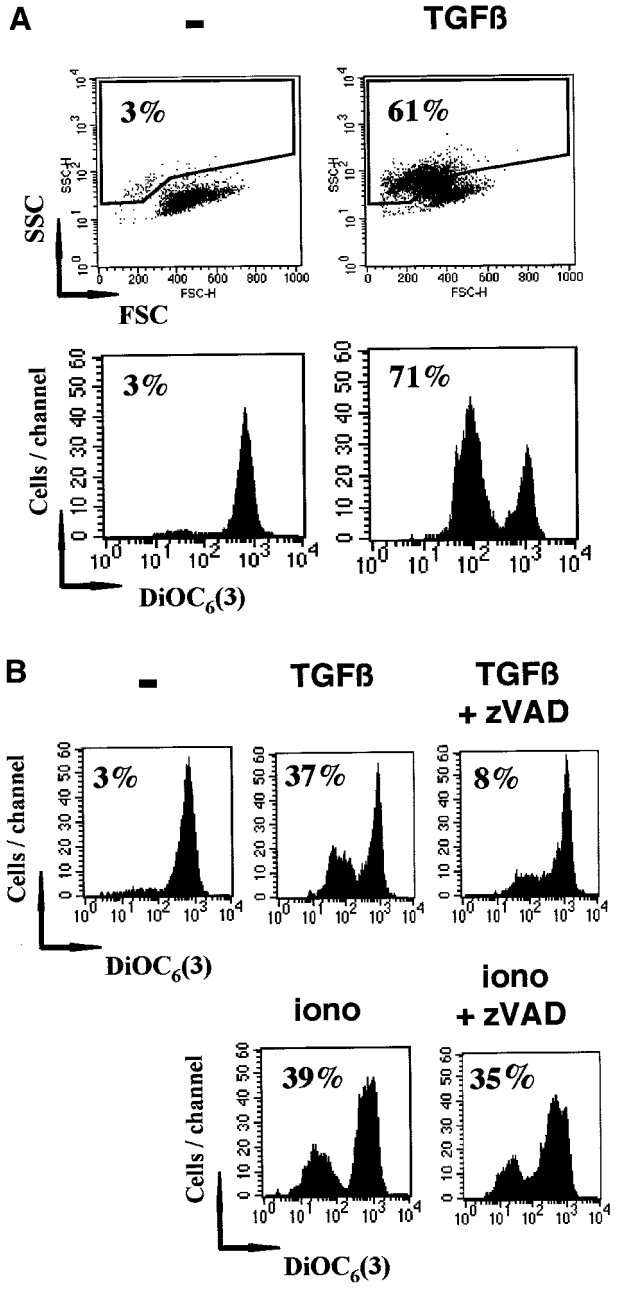


**Figure 1.** TGF$\beta$ induces the caspase-dependent loss of $\Delta\Psi$m. (A) BL41 cells were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml). Apoptotic cells were selected as shrunken cells with high side-scatter (SSC) and low forward-scatter (FSC) properties as assessed by flow cytometry. Apoptotic cells were counted and their number expressed as a percentage of the total cells. After staining with $DiOC_6(3)$, $\Delta\Psi$m was assessed by flow cytometry, and cells with low $\Delta\Psi$m were counted and their number expressed as a percentage of the total population. (B) BL41 cells were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml) alone (TGF$\beta$), or in combination with 100 $\mu$M zVAD-fmk (TGF$\beta$ + zVAD), or in the presence of ionomycin (10 $\mu$g/ml) alone (iono), or in combination with 100 $\mu$M zVAD-fmk (iono + zVAD). After staining with $DiOC_6(3)$ cells with a low $\Delta\Psi$m were counted as described above. The results are representative of at least three independent experiments.

enzyme-linked immunosorbent assay that both AEVD-pNA and IETD-pNA substrates were cleaved in vitro by lysates from TGF$\beta$-treated cells (Figure 2B). Caspase activity was already apparent at 24 h but was maximal after 48 h of TGF$\beta$ activation, showing kinetics similar to that for the decrease in $\Delta\Psi$m (Figure 2B). The activation of caspase-8 upon stimulation with TGF$\beta$ was directly demonstrated by the detection of cleaved p44/45 and p20 kilodalton fragments in Western blots of cell extracts obtained at various times after TGF$\beta$ treatment. The cleaved fragments were comparable with control cleaved caspase-8 forms observed in Jurkat cells stimulated with anti-Fas antibody (Figure 2C). The kinetics of appearance of the cleaved forms of caspase-8 was similar to that of caspase activity with the use of IETD-pNA and AEVD-pNA as substrates (Figure 2, B and C).

Caspase-8 activation may be monitored in the cell by following the cleavage of its natural substrate, Bid, which in turn mediates cytochrome *c* release and caspase-9 activation. Western blot analysis showed that TGF$\beta$ treatment of BL41 cells mediated the cleavage of Bid, as shown by the disappearance of the p22 uncleaved form of Bid and the production of p15 fragment (Figure 2D). We also observed caspase-9 activation, as shown by the disappearance of the p47 proform and the production of the cleaved fragment p37 (Figure 2D). We could not observe cleavage of caspase-10, but due to the lack of a reliable antibody specific for the cleaved fragments of caspase-10, we were unable to rule out the possibility that TGF$\beta$ also mediates caspase-10 activation.

### *Interactions between Caspase-8 and Caspase-3 Activation*

We next determined the sequence of events between caspase-8 and caspase-3 activation and the loss of $\Delta\Psi$m. For this, we first investigated whether AEVD-fmk, which inhibited the loss of $\Delta\Psi$m induced by TGF$\beta$ in BL41 cells more strongly than did IETD-fmk, also modulated caspase-3 activation. In the presence of TGF$\beta$, both shrinkage and the loss of $\Delta\Psi$m were associated with the appearance of the active fragments p44/45 and p20 of caspase-8 and the p19 and p17 fragments of caspase-3 (Figure 3). Morphological changes and the activation of caspase-8 and caspase-3 were completely prevented by zVAD-fmk. Inhibition of the loss of $\Delta\Psi$m in the presence of AEVD-fmk correlated with a decrease in the amount of the active cleaved fragments p44/45 and p20 of caspase-8 detected. Under these conditions, the activation of caspase-3 was also prevented. In particular, the cleavage of the caspase-3 proform into p20 and p19 was greatly reduced in the presence of AEVD-fmk. These observations are consistent with a pathway of caspase-3 activation involving upstream activation of caspase-8. We then investigated the possible relationship between the loss of $\Delta\Psi$m and caspase-3 activation. For this, BL41 cells, unactivated or activated by incubation for 48 h with TGF$\beta$, were stained with both $DiOC_6(3)$, to measure loss of $\Delta\Psi$m, and a cell-permeable fluorogenic substrate containing the specific caspase-3 sequence GDEVDG (PhiPhilux-$G_2D_2$), to detect caspase-3 activity in a single cell by flow cytometry (Figure 4). As expected, 97% of control cells were not shrunken, displaying intense $DiOC_6(3)$ labeling and no cleavage of PhiPhilux-$G_2D_2$. After TGF$\beta$ treatment, in the viable cell compartment (67% of total cells), most cells displayed a high

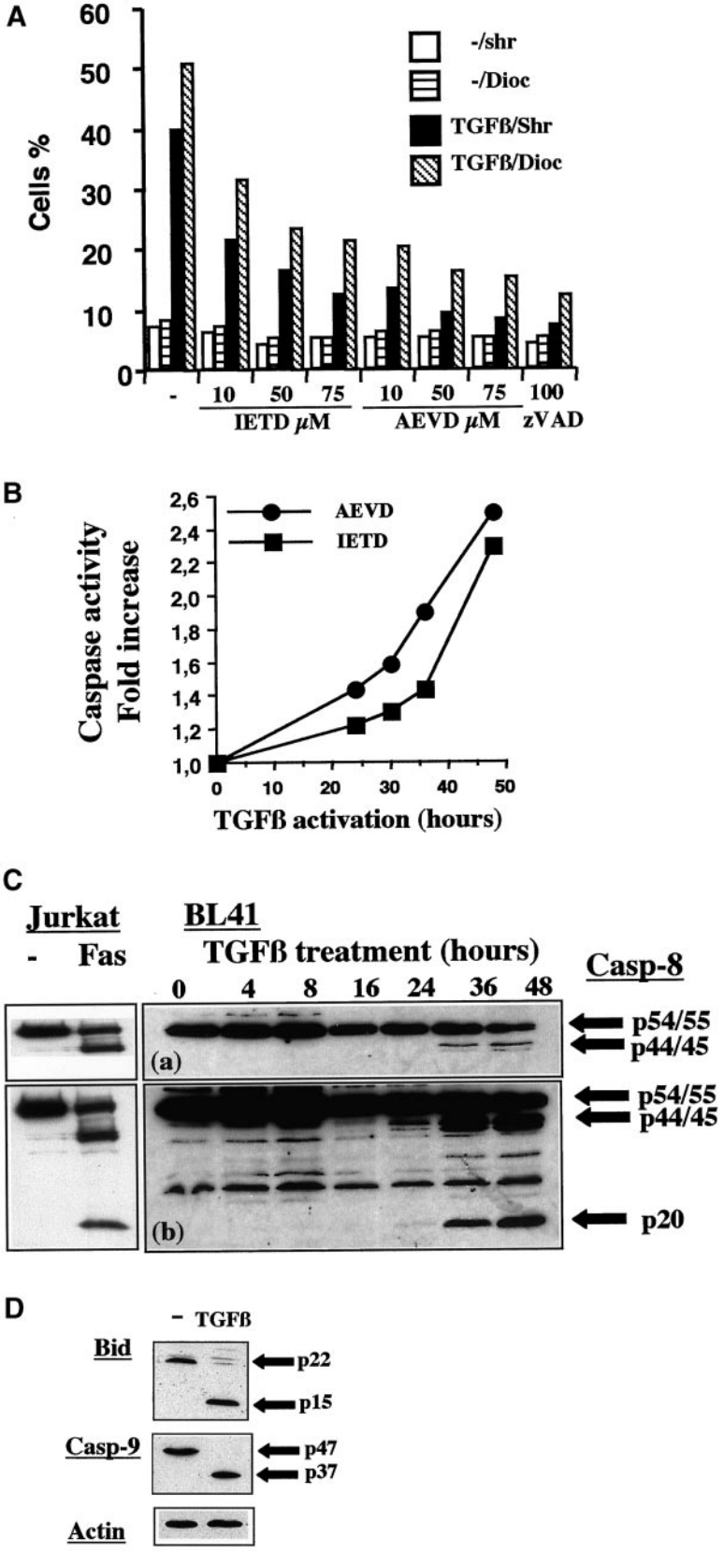


**Figure 2.** TGF$\beta$ promotes the caspase-8 dependent loss of $\Delta\Psi$m and apoptosis. (A) BL41 cells were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml) and various concentrations of IETD-fmk (10, 50, 75 $\mu$M), AEVD-fmk (10, 50, 75 $\mu$M), or ZVAD-fmk (100 $\mu$M). Cell shrinkage (Shr) and the loss $\Delta\Psi$m (Dioc) were assessed by flow cytometry, as described in Figure 1. (B) Cells were cultured with TGF$\beta$ (1 ng/ml) and caspase activity was determined at various times after TGF$\beta$ treatment, with the use of IETD-pNA and AEVD-pNA as substrates. Results are expressed as the ratio between caspase activity in TGF$\beta$-treated cells and that in control cells. (C) Cells were cultured with TGF$\beta$ (1 ng/ml) for various periods of time. Whole cell extracts were separated by SDS-PAGE and the various forms of caspase-8 were detected by immunoblotting with the use of anti-caspase-8 antibodies. A short (a) and a longer (b) exposure of the films is shown. As control for caspase-8 specificity, Jurkat cells were cultured for 24 h in the absence (−) or the presence of anti-Fas antibody (CH11 antibody, 1 $\mu$g/ml). (D) Cells were cultured without (−) or with TGF$\beta$ (1 ng/ml) for 48 h. Whole cell extracts were separated by SDS-PAGE and the levels of the various forms of Bid and caspase-9 were determined by immunoblotting, with the use of anti-Bid or anti-caspase-9 antibodies. Results are representative of three independent experiments. The amount of protein loaded in each lane was assessed by stripping the filter and reprobing it with an antibody specific for human actin.

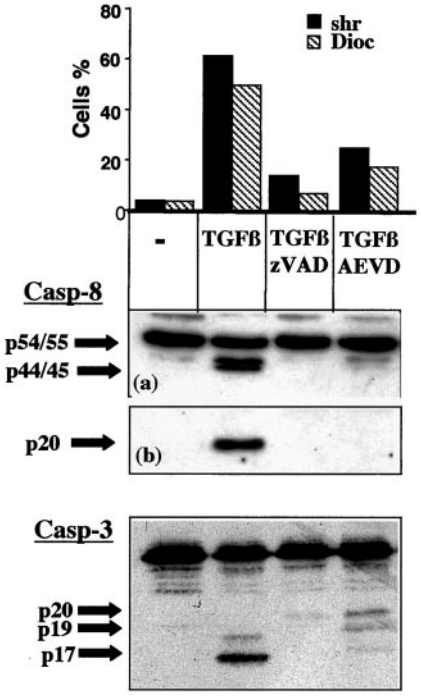


**Figure 3.** AEVD inhibits TGF$\beta$-mediated caspase-8 and caspase-3 activation. BL41 cells were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml) in association with ZVAD-fmk (50 $\mu$M), IETD-fmk (75 $\mu$M), or AEVD-fmk (75 $\mu$M). Cell shrinkage and loss of $\Delta\Psi$m were assessed by flow cytometry, as described in Figure 1. The cleavage of caspase-8 and of caspase-3 was assessed by immunoblotting. A short (a) and a longer (b) exposure of the films is shown for caspase-8. Results are representative of three independent experiments.

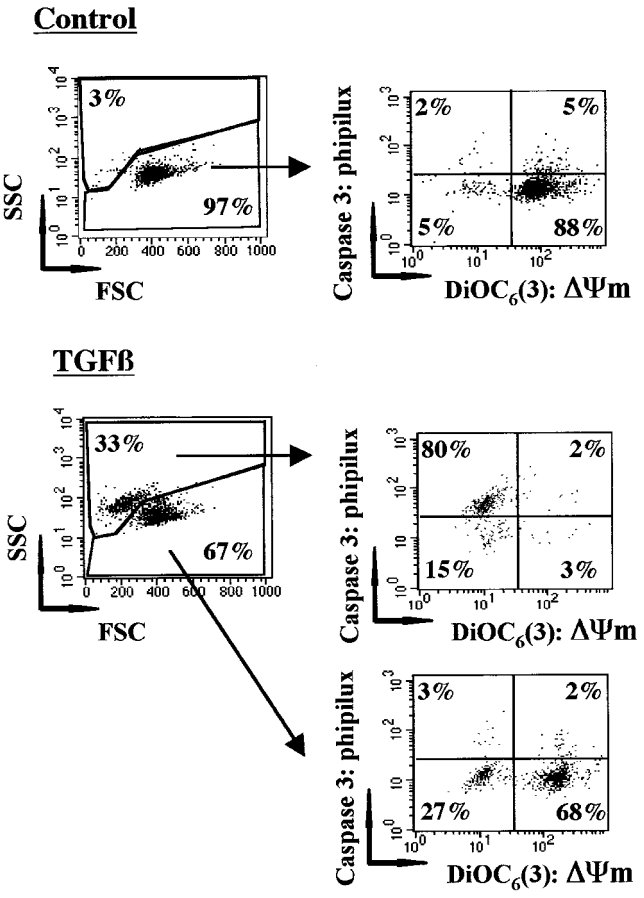


**Figure 4.** TGF$\beta$ mediates $\Delta\Psi$m loss and caspase-3 activation in the same cells. BL41 cells were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml) and were stained with $DiOC_6(3)$ and PhiPhilux $G_2D_2$. They were analyzed for FSC/SSC and gated on the basis of their FSC/SSC characteristics. The gated populations were analyzed for Phiphilux and $DiOC_6(3)$ staining. Results are representative of four independent experiments.

$\Delta\Psi$m with no caspase-3 activity (68%), with a smaller proportion of cells having low $\Delta\Psi$m with no caspase-3 activity (27%). This strongly suggests that caspase-3 activation and shrinkage occur in cells that have decreased $\Delta\Psi$m. In contrast, only 15% of TGF$\beta$-induced shrunken cells displayed a low $\Delta\Psi$m with no caspase-3 activity, with most cells (80%) displaying low $\Delta\Psi$m and caspase-3 activity. This is consistent with the finding that caspase-3 is activated only when $\Delta\Psi$m falls. Thus, our results show that 1) activation of caspase-8 and caspase-3 and loss of $\Delta\Psi$m are prevented by a caspase-8 inhibitor, and 2) caspase-3 is activated in cells exhibiting loss of $\Delta\Psi$m. Thus, these data (Figures 3 and 4) are consistent with a sequence of events involving caspase-8 activation, associated with loss of $\Delta\Psi$m and caspase-3 activation.

### *TGF$\beta$-mediated Caspase-8 Activation Is FADD-independent*

Many studies have demonstrated that caspase-8 activation is dependent on the oligomerization of caspase-8 by its association with the adaptor protein FADD, via the DEDs of the two molecules. This requires the interaction of FADD either directly or indirectly with surface receptors such as Fas or TNF-R, which possess death domains. Because no such death domains have been described in TGF$\beta$ receptors and because some Burkitt cell lines are sensitive to Fas-mediated apoptosis (Lens *et al.*, 1996), we investigated the possible involvement of death receptors in TGF$\beta$-mediated apoptosis. In our experimental conditions, the BL41 cells were insensitive to Fas-mediated apoptosis, as assessed with the use of the agonist antibody ligand CH11 antibody (up to 10 $\mu$g/ml)) in the presence or absence of TGF$\beta$. In addition, the antagonist ZB4 antibody, which inhibited CH11-mediated Jurkat apoptosis, did not affect the extent of TGF$\beta$-apoptosis in BL41 cells. Thus, the apoptosis and caspase-8 activation triggered by TGF$\beta$ are independent of the Fas pathway. Similarly, the TNF-R pathway was not involved in our experimental conditions because 1) the addition of TNF-$\alpha$ in the presence or absence of TGF$\beta$ did not affect the level of apoptosis, which was similar to that observed with TGF$\beta$ alone; and 2) we detected no TNF-$\alpha$ production upon TGF$\beta$-stimulation. Because receptors known to recruit FADD do not seem to be involved in TGF$\beta$-mediated apoptosis, we investigated more directly the possible involvement or re-

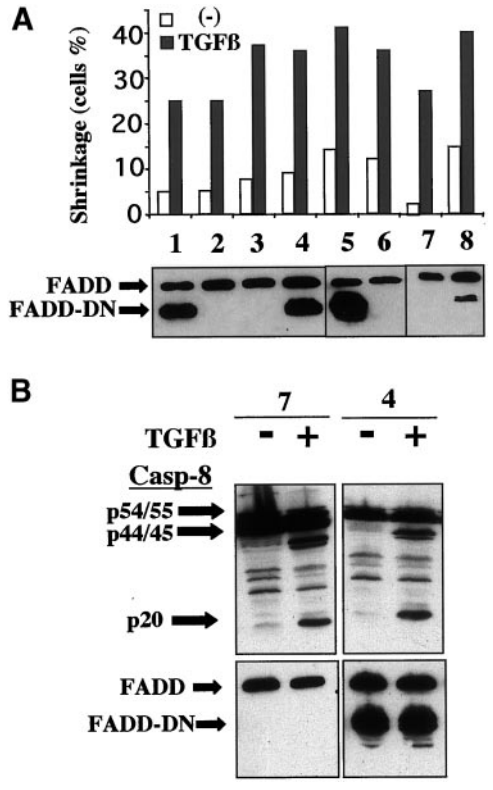


**Figure 5.** TGF$\beta$-mediated caspase-8 activation is FADD-independent. (A) Independent clones of BL41 cells producing either endogenous FADD only or various amounts of the truncated form of FADD were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml). Shrunken cells were counted by flow cytometry, as described in Figure 1. The production of FADD and FADD-DN was assessed by immunoblotting of the cell lysates obtained from each of the unstimulated clones. (B) After 48 h of incubation with or without TGF$\beta$, the cleavage of caspase-8 was determined by immunoblotting the cell lysates obtained from clones 7 and 4. The blot was reprobed with anti-FADD antibodies to determine FADD and FADD-DN levels.

quirement of the FADD molecule in our system. For this, we established stable transfectant clones of BL41 cells expressing a dominant negative mutant of FADD lacking the DED domain, which were therefore unable to associate with caspase-8 and as a consequence TNF-mediated apoptosis is blocked (Chinnaiyan *et al.*, 1995, 1996). Various clones of BL41, expressing either only endogenous FADD (Figure 5A, lanes 2, 3, 6, and 7) or various levels of the truncated FADD, which was detected as a band that migrated faster than the wild-type band (Figure 5A, lanes 1, 4, 5, and 8) were selected. We observed that TGF$\beta$ induced apoptosis in all clones, independently of the production of dominant negative FADD molecule, as assessed by cell shrinkage (Figure 5A) or decreases in $DiOC_6(3)$ staining. We also observed that TGF$\beta$-induced caspase-8 cleavage into the active fragments p44/45 and p20 occurred to a similar extent in cells producing or not producing FADD-DN (Figure 5B). Thus, TGF$\beta$-mediated caspase-8 activation and apoptosis are independent of the presence of the DED of FADD.

### *TGF$\beta$-mediated p38 Activation Regulates Caspase-8 Cleavage and Apoptosis*

To identify the pathway leading to caspase-8 activation upon TGF$\beta$-treatment of BL41 cells, we investigated regulation of the activation of members of the MAPK family. Indeed, various members of this serine/threonine kinase family have been shown to regulate both cell cycle progression and apoptosis and to be activated by TGF$\beta$ (Hartsough and Mulder, 1995; Yamaguchi *et al.*, 1995; Atfi *et al.*, 1997; Frey and Mulder, 1997; Hanafusa *et al.*, 1999; Tibbles and Woodgett, 1999). We previously reported that TGF$\beta$ induced both cell cycle arrest and apoptosis in BL41 cells (Schrantz *et al.*, 1999). We therefore investigated the possible role of these kinases in TGF$\beta$-mediated apoptosis in these cells. The involvement of ERK or p38 in the TGF$\beta$-mediated induction of apoptotic features was investigated in the presence of various inhibitors specific for the activation of p38 (SB203580 or PD169316) or ERK (PD98059 or U0126). In the presence of SB203580 (20 $\mu$M) or PD169316 (2 $\mu$M), both cell shrinkage and the decreasing of $DiOC_6(3)$ labeling were significantly inhibited (Figure 6A), whereas cell cycle arrest (assessed by quantification of cells arrested in G1) was not prevented. In addition, the ERK-specific inhibitors U0126 and PD98059 did not interfere with TGF$\beta$-mediated apoptosis (Figure 6A). Similar conclusions were reached when we quantified the inhibition of the fold-increase of apoptosis by TGF$\beta$ in the presence of various doses of inhibitors. Because the loss of $\Delta\Psi m$ was sensitive to p38 inhibitors, we investigated whether p38 also affected TGF$\beta$-induced caspase-8 activation. Indeed, the production of the cleaved fragments p44/45 and p20, observed upon TGF$\beta$-activation (Figure 6B, lane 2), was much reduced in the presence of the p38 inhibitors SB203580 (Figure 6B, lane 3) and PD169316 (Figure 6B, lane 4), whereas U0126 (Figure 6B, lane 5) and PD98059 (Figure 6B, lane 6) did not prevent the production of these active cleaved fragments. We demonstrated that TGF$\beta$ activated the p38 pathway by Western blot analysis with an antibody recognizing specifically the phosphorylated form of the protein. The active phosphorylated form (pp38) was detected after 1 h of stimulation, was present in a larger amount at 8 h, and remained present in a large amount for up to 24 h of treatment with TGF$\beta$ (Figure 6C). In this cell line, we detected no activation of the JNK pathway by TGF$\beta$, with the use of antibodies against the phosphorylated form of JNK or by in vitro JNK kinase assay. The importance of p38 in control of the early steps of TGF$\beta$-induced apoptosis was also demonstrated by the ability of the 2 p38 inhibitors SB203580 and PD169316 (Figure 6D, lanes 3 and 4, respectively) to prevent the cleavage of both Bid and caspase-9 observed in TGF$\beta$-treated cells (Figure 6D, lane 2 vs. control lane 1).

### *TGF$\beta$-mediated Apoptosis Is Dependent on Durable p38 Activation*

Because both TGF$\beta$-mediated caspase-8 activation and apoptosis were observed only after 24 h and were maximum after 48 h of stimulation, we used kinetic experiments to investigate the mechanisms underlying the TGF$\beta$-mediated

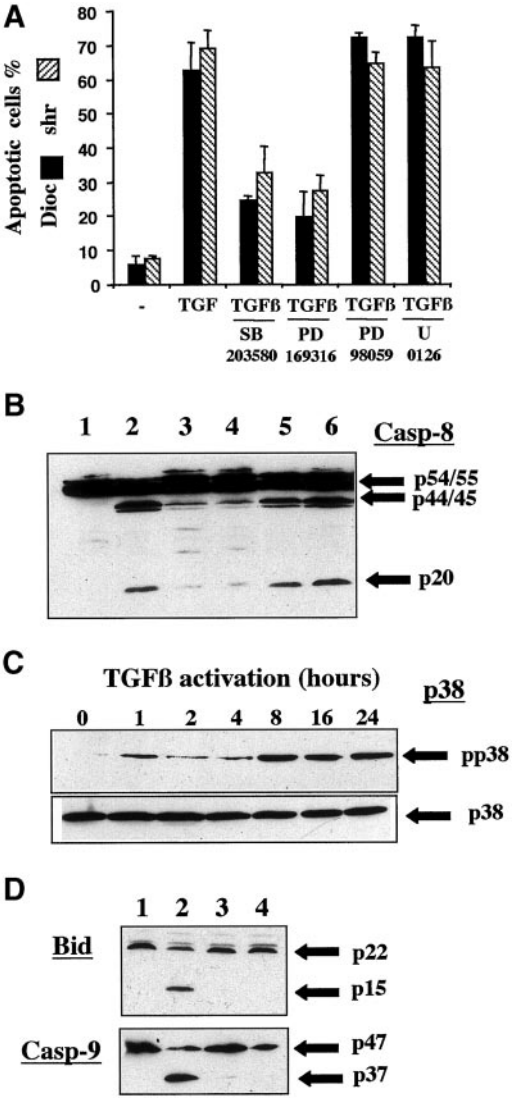


**Figure 6.** TGF$\beta$-mediated apoptosis and caspase-8 activation are p38-dependent. (A) BL41 cells were cultured for 48 h without (−) or with TGF$\beta$ (1 ng/ml) in the absence or presence of SB203580 (20 $\mu$M) or with PD169316 (2 $\mu$M), PD98059 (20 $\mu$M), or U0126 (10 $\mu$M). Shrunken cells and cells with low $\Delta\Psi$m were counted by flow cytometry, as described in Figure 1. (B) BL41 cells were cultured for 48 h without (lane 1) or with TGF$\beta$ (1 ng/ml) (lane 2), or with TGF$\beta$ and SB203580 (lane 3), TGF$\beta$ and PD169316 (lane 4), TGF$\beta$ and U0126 (lane 5), or TGF$\beta$ and PD98059 (50 $\mu$M) (lane 6). The cleavage of caspase-8 was determined by immunoblotting. (C) BL41 cells were cultured for the times indicated with TGF$\beta$ (1 ng/ml), and the p38 and phosphorylated forms of p38 (pp38) were determined by immunoblotting with the use of specific antibodies. (D) Cells were cultured for 48 h without (lane 1) or with TGF$\beta$ (1 ng/ml) (lane 2), or with TGF$\beta$ and SB203580 (lane 3), or TGF$\beta$ and PD169316 (lane 4). Whole cell extracts were separated by SDS-PAGE and the various forms of Bid and caspase-9 were detected by immunoblotting with the use of anti-Bid or anti-caspase-9 antibodies.

apoptosis. First, we determined whether a brief activation by TGF$\beta$ was sufficient to promote the apoptosis in BL41 cells.

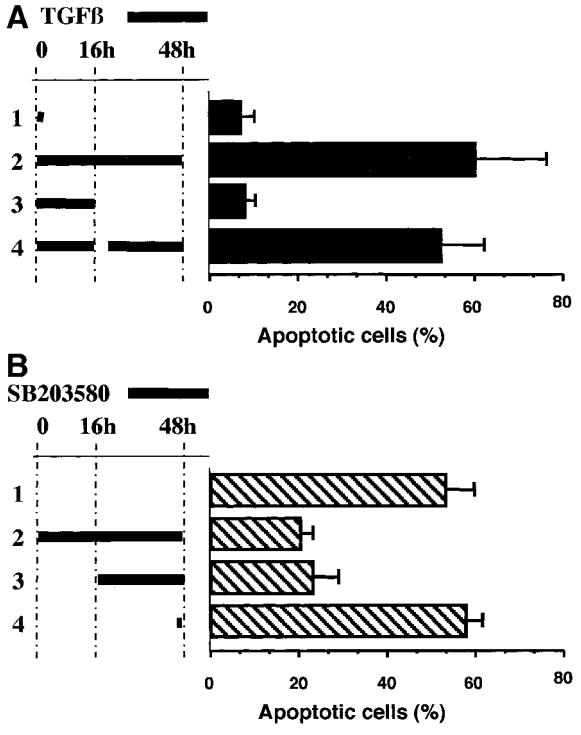


**Figure 7.** TGF$\beta$-mediated apoptosis is dependent on prolonged p38 activation. (A) BL41 cells were cultured for 48 h in the presence of TGF$\beta$ (1 ng/ml) for various times. Lane 1, for the first hour of culture; lane 2, during the whole culture. For lanes 3 and 4, cells were activated with TGF$\beta$ for the first 16 h of culture, harvested, washed, and cultured for an additional 30 h in the absence (lane 3) or the presence of TGF$\beta$ (lane 4). (B) BL41 cells were activated for 48 h with TGF$\beta$ (1 ng/ml) in the absence (lane 1) or the presence of SB203580 (20 $\mu$M) during the whole culture (lane 2), from 16 to 48 h (lane 3), or for the last hour of culture (lane 4). Shrunken cells were counted by flow cytometry, as described in the legend to Figure 1.

Activation of 1 h (Figure 7A, lane 1) or for 16 h (Figure 7A, lane 3) was not sufficient to promote an apoptotic response 48 h later: <10% of cells were apoptotic compared with 58% when TGF$\beta$ was present during all the entire culture period (Figure 7A, lane 2). Addition of TGF$\beta$ for the remaining time of culture (16–48 h) to cells activated with TGF$\beta$ for 16 h (Figure 7A, lane 4) resulted in a level of apoptosis comparable with that in the cultures activated with TGF$\beta$ throughout the culture period. Therefore, the apoptotic response was dependent on the continuous presence of TGF$\beta$. These data, associated with the observation that TGF$\beta$ promotes prolonged activation of p38 (Figure 6C), prompted us to investigate whether this long-lasting activation of p38 was necessary to mediate the TGF$\beta$-induced apoptosis observed in BL41 cells. The presence of the p38 inhibitor SB203580 during the last 32 h of a 48-h culture inhibited the TGF$\beta$-mediated apoptosis to a similar extent as the presence of the inhibitor SB203580 throughout the culture (Figure 7B). These data support the conclusion that TGF$\beta$-mediated apoptosis in BL41 cells is dependent on the continuous presence of TGF$\beta$ and on a prolonged activation of p38.

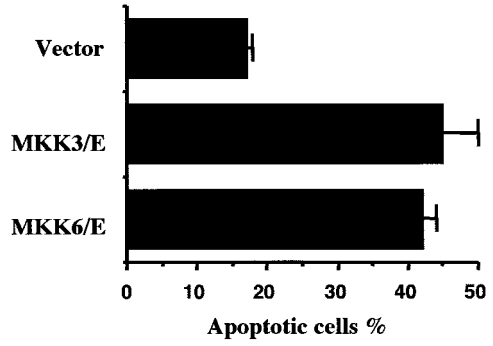


**Figure 8.** Overexpression of MKK3 and MKK6 can promote apoptosis in BL41 cells. BL41 cells were transiently transfected with the pcDNA-MKK3(b)E (MKK3/E) or pcDNA-MKK(b)/E (MKK6/E) or the corresponding empty vector (vector) and the pIRES.hrGFP vector. Eighteen hours after transfection, dead cells, due to the electroporation shock, were removed from the cultures by centrifugation through a Ficoll gradient. Viable cells were then cultured at 37°C for 24 h. and the positive FL1-gated cells, that is, the cells transfected with GFP and either MKK3 and MKK6, were tested for apoptosis by assessing cell shrinkage.

### *p38 Activation Can Promote Apoptosis in BL41 Cells*

We therefore investigated whether p38 activation was sufficient to induce apoptosis in BL41 cells. For this, we overexpressed, by transient transfection, two dominant active mutants of upstream activators of p38, MKK3(b)E or MKK6(b)E (Han *et al.*, 1996; Raingeaud *et al.*, 1996) together with a plasmid encoding GFP to allow the identification of transfected cells. Eighteen hours after transfection, dead cells were removed by centrifugation through Ficoll and viable cells were cultured for a further 24 h. Apoptotic cells (characterized as shrunken cells) were then quantified in the GFP-positive cells populations. Fewer than 18% of cells transfected with the empty vector were apoptotic, whereas 42 and 45% of those transfected with MKK3(b)E and MKK6(b)E, respectively, were apoptotic (Figure 8). Thus, activation of p38 can promote an apoptotic death of these cells and the involvement of p38 activation in TGFβ-mediated apoptosis in BL41 cells is confirmed.

## DISCUSSION

TGFβ-mediated apoptosis is a complex process associated with cell cycle arrest. We previously reported that, in contrast to TGFβ-mediated cell cycle arrest, TGFβ-induced apoptosis in Burkitt lymphoma B cells was dependent on caspase activation (Schrantz *et al.*, 1999). We showed that caspase-3 was activated and responsible for the control of various apoptotic features and Rb cleavage. Several studies have shown that caspase-3 may be activated by mitochondrial-dependent or -independent pathways (Porter and Janicke, 1999). Indeed, in type I Jurkat cells, Fas-activated caspase-8 may directly activate caspase-3, whereas in type II Jurkat cells, caspase-3 activation is dependent on a mitochondrial pathway associated with the caspase-8-dependent cleavage of Bid (Scaffidi *et al.*, 1999). In turn, cleaved Bid induces opening of the permeability transition pore and the release of cytochrome *c* into the cytoplasm, which controls the activation of caspase-9 (Schendel *et al.*, 1999). These different pathways leading to caspase-3 activation have been extensively explored with other stimuli, but the TGFβ-induced events involved in this apoptotic process upstream from caspase-3 remain poorly defined. We found that TGFβ induced caspase-8 activation, as shown by the appearance of the cleaved fragments p44/45 and p20, and the ability of cell lysates from TGFβ-activated BL41 cells to cleave in vitro colorimetric substrates specific for caspase-8 (IETD-pNA and AEVD-pNA). This caspase-8 activation was correlated with Bid cleavage. TGFβ also induced a decrease in mitochondrial membrane potential and the activation of caspase-9. All these events were inhibited by AEVD and IETD inhibitors, strongly suggesting that TGFβ-induced mitochondrial depolarization processes were mediated by Bid after its cleavage by caspase-8. In addition, flow cytometry analysis of $\Delta\Psi m$ and caspase-3 activity in the same cell indicated that caspase-3 activation did not occur in the absence of a loss of $\Delta\Psi m$. This correlates well with the reported pathway of caspase-3 activation, which is dependent on the mitochondria release of cytochrome *c*. The association of cytochrome *c* and ATP with cytoplasmic Apaf1 then leads to autocleavage and the activation of caspase-9, which is directly responsible for the cleavage of caspase-3 (Li *et al.*, 1997; Qin *et al.*, 1999). Indeed, in BL41 cells, this pathway is probably responsible for caspase-3 activation because TGFβ mediates the activation of both caspase-9 and caspase-3. Our data are consistent with the notion that, in Burkitt's lymphoma cells, the TGFβ-induced apoptotic response is based on the sequential activation of caspase-8, cleavage of Bid, and loss of $\Delta\Psi m$ associated with caspase-9 and caspase-3 activation.

It has been suggested that caspase-10, which is very similar to caspase-8, is recruited along with caspase-8 into apoptosis signaling complexes associated with the death receptor and that caspase-10 plays a functional role in death receptor-mediated apoptosis (Fernandes-Alnemri *et al.*, 1996; Vincenz and Dixit, 1997). However, the cleavage of this caspase was not demonstrated due to a lack of reliable antibodies. AEVD and IETD both inhibit caspase-10, although to a lesser extent than caspase-8 (Thornberry *et al.*, 1997; Garcia-Calvo *et al.*, 1998). Thus, the involvement of caspase-10 in TGFβ-activated pathways cannot be ruled out.

Recently, TGFβ has also been shown to activate caspase-8 in hepatoma cells as well as B cells, but the mechanisms involved in this activation are not well understood (Inman and Allday, 2000; Shima *et al.*, 1999). Caspase-8 activation is mainly associated with apoptosis mediated by members of the TNF-R family, which possess a death domain in their cytoplasmic region (Ashkenazi and Dixit, 1998). On ligand binding, these receptors may directly or indirectly recruit the FADD adaptor protein. Caspase-8 activation results in autocleavage of the oligomerized proforms after association with FADD via interactions between the DEDs of the two molecules (Boldin *et al.*, 1996; Muzio *et al.*, 1996). The mechanism leading to caspase-8 activation by TGFβ was indirect because TGFβ receptors lack death domains. Interestingly, TGFβ-induced loss of $\Delta\Psi m$ and apoptosis show a late kinetics and were maximal after 48 h of stimulation. This suggests that TGFβ-induced caspase-8 activation and apoptosis may be mediated by a biphasic process resulting from

the induction by TGFβ of ligands able to recruit FADD molecules via their death domains directly or indirectly. In agreement with Inman and Allday (2000), we found no evidence for the involvement of Fas/FasL or TNF/TNF-R interactions in our experimental conditions. This is consistent with the reported inhibition by TGFβ of CD95L-induced neutrophil apoptosis (Chen *et al.*, 1998; Genestier *et al.*, 1999). We therefore investigate directly the possible involvement of FADD in the TGFβ-induced activation of caspase-8, with the use of a dominant negative FADD molecule lacking the DED domain. Various clones producing only the endogenous FADD molecule or various amounts of the DED-truncated FADD-DN displayed similar patterns of apoptotic response and caspase-8 cleavage after TGFβ treatment. This suggests that TGFβ-mediated caspase-8 activation and apoptosis are independent of FADD. It is not clear whether TGFβ-induced caspase-8 activation is completely independent of FADD molecule or whether it could be mediated by FADD molecules devoid of DED domains. It has recently been reported that anticancer drugs induce apoptosis and caspase-8 cleavage in a FADD-independent manner, suggesting that death receptor activation is not a prerequisite for drug-induced caspase-8 activation (Wesselborg *et al.*, 1999). The nature of the adaptor molecules capable of mediating caspase-8 oligomerization and cleavage remains to be determined. Nevertheless, our data are consistent with the emerging hypothesis that caspase-8 activation is not restricted to death receptors.

One clue for the characterization of this FADD-independent caspase-8 activation pathway is related to the TGFβ-mediated activation of p38. Indeed, this member of the serine/threonine MAPK family has been implicated in the regulation of apoptosis mediated by various stimuli. Several groups have reported that TGFβ promotes p38 activation dependent on upstream activation of MKK6 and TGFβ-activated kinase 1, but the role of p38 in TGFβ-mediated apoptosis is still unclear (Yamaguchi *et al.*, 1995; Ichijo *et al.*, 1997). TGFβ also promotes activation of the JNK pathway in various cell types (Atfi *et al.*, 1997; Frey and Mulder, 1997). Although p38 was activated in our experimental conditions, we observed no activation of JNK, as assessed both by JNK phosphorylation and in vitro kinase assays, in TGFβ-treated BL41 cells. Although they differ between cell types and stimuli, p38 pathways are more frequently involved in the induction of the apoptotic response through different mechanisms. For instance, p38 has been reported to be involved in the induction of Fas-L, suggesting that one possible role for p38 is to activate, via the regulation of transcription factors such as activating transcription factor 2, the production of ligands of various members of the TNF-R family (Hsu *et al.*, 1999). Fas has also been reported to promote activation of p38, suggesting that p38 may play a more direct role in the triggering of the apoptotic response (Juo *et al.*, 1997). Zhuang *et al.* (2000) have recently reported that singlet oxygen-induced mitochondrial dysfunction, caspase-3 activation, and apoptosis are dependent on p38-mediated Bid cleavage. They reported that although singlet oxygen promotes both caspase-8 and Bid cleavage, the inhibition of p38 prevents the cleavage of Bid but has no effect on caspase-8 cleavage, suggesting that in their experimental conditions, p38-dependent Bid cleavage and mitochondrial activation were mediated by a caspase-8-independent pathway. p38 has also recently been shown to be involved in early events of cadmium-induced apoptosis upstream from the mitochondria (Galan *et al.*, 2000). We used two different inhibitors of the p38 pathway to show that the TGFβ-mediated caspase-8 activation and loss of $\Delta\Psi m$ were p38-dependent. Together with the observation that inhibition of the loss of $\Delta\Psi m$ was also prevented by caspase-8 inhibitors, our data are consistent with the notion that, on TGFβ stimulation, p38 controls the activation of caspase-8, which is responsible for mitochondrial activation. Further evidence that p38 acts upstream from the caspase cascade is provided by the observation that p38 phosphorylation was not prevented by zVAD-fmk. Thus, p38-induced caspase-8 activation has previously been shown to be mediated by death domain receptor signaling, but our results provide evidence for another p38-dependent pathway independent of these death receptors.

The implication of p38 in the apoptotic process triggered by TGFβ in BL41 cells was strengthened by the observation that transient overexpression of active forms of MKK3 or MKK6, which lead to p38 activation, promotes a significant amount of apoptosis in these cells. In addition, when BL41 cells were transfected with MKK3 or MKK6 only transfected cells (GFP-positive cells) were apoptotic, whereas nontransfected cells (GFP-negative cells) also present in the same culture were not apoptotic, which is in favor of the hypothesis that p38 regulates TGFβ-mediated apoptosis of BL41 through an intracellular rather than an autocrine pathway. Nevertheless, the exact contribution of p38 activation during more physiological stimuli, like the presence of TGFβ, remains to be elucidated. Indeed, although p38 activation is necessary to promote apoptosis in BL41 cells in the presence of TGFβ, as demonstrated by the effect of various p38 inhibitors, it is possible that the regulation of the full apoptotic process required cooperation between p38 and the Smads-mediated pathway. This type of cooperation between the Smads and transcription factors activated by members of the MAPK family (including ERK and p38) has been observed (Hanafusa *et al.*, 1999; Yue and Mulder, 2000), and our preliminary data also suggested cooperation between p38 and Smads during TGFβ-mediated BL41 activation. This raises the question of whether p38 directly modulates the phosphorylation states of adaptor molecules responsible for caspase-8 activation or could contribute to regulation by acting on the transcription of the genes encoding the regulatory molecules. Our preliminary data showing that the p38 inhibitor SB203580 inhibited a reporter gene that contains a TGFβ-inducible promotor are compatible with the hypothesis that TGFβ, through p38 activation, and thus probably activation of transcription factors, regulates the expression of a novel adaptor molecule, distinct from FADD. The further characterization of this (or these molecules) would then allow a better understanding of the precise role of p38 during TGFβ-mediated caspase-8 activation.

The activation of p38 by TGFβ appears to be biphasic or long lasting. Indeed, although p38 activation was detected as early as 1 h after TGFβ stimulation, maximum p38 phosphorylation was observed after 8 h and phosphorylation levels remained high until 24 h. Because caspase-8 activation and the loss of $\Delta\Psi m$ occurred only after 24 h of activation, the kinetics of p38 activation suggest that the apoptotic signaling induced by TGFβ was associated with this late p38

activation. Indeed, we observed that the presence of TGFβ during the first 24 h of stimulation was sufficient to promote cell cycle arrest, but only low levels of apoptosis. Maximum apoptosis at 48 h was observed only if cells were cultured continuously in the presence of TGFβ. This suggests that a first round of signaling, which may involve the regulation of cyclin-dependent kinase inhibitors or other pathways, occurs early during the first 24 h of incubation with TGFβ. Although, p38 was activated during this period, this pathway did not seem be involved in cell cycle control because p38 inhibitors SB203580 and PD169316 were not able to counteract the G1 accumulation of TGFβ-treated BL41 cells. In contrast, a late cell activation by TGFβ, involving p38 activation, seems to be required for caspase-8 activation and subsequent apoptosis because addition of SB203580 after 16 h of TGFβ stimulation prevented apoptosis measured after 48 h of stimulation. A similar biphasic pattern of activation of JNK by TGFβ human fibrosarcoma cells as well as activation of p38 and JNK mediated by TNF in hepatocyte cells has been reported previously but was not directly associated with caspase-8 activation (Hocevar *et al.*, 1999; Talarmin *et al.*, 1999), In addition, the requirement for long-lasting activation of JNK during apoptosis of Fas-activated human neuroblastoma cells and TNF-stimulated rat mesangial cells has also been reported (Goillot *et al.*, 1997; Guo *et al.*, 1998). Thus, the delayed activation of various members of the MAPK family plays a crucial role in determining their ability to regulate various biological activities. Indeed, biphasic activation has also been reported for ERK and the control of G1 progression and G1/S transition are directly regulated by late activation of ERK (Talarmin *et al.*, 1999). Our data are consistent with a TGFβ-mediated apoptosis pathway dependent on the late activation of p38. Although the exact mechanism by which p38 activates caspase-8 is unknown, the finding that this MAPK pathway is involved in caspase activation provides new insight into the cascade of events leading to apoptosis mediated by TGFβ.

In conclusion, our data link TGFβ-activated signal transduction pathways to the caspase cascade by providing evidence that the cleavage of caspase-8 by TGFβ is controlled by upstream activation of the MAPK p38. This p38-mediated activation of caspase-8 is mediated by a novel pathway that appears to be independent of FADD.

## ACKNOWLEDGMENTS

We thank F. Petit and J. Estaquier for their kindly collaboration and Drs V. Dixit and J. Han for the FADD-DN, MKK3(b) and MKK6(b) expression plasmids used in this study. This work was supported by INSERM and grants from the Association pour la Recherche sur le Cancer (ARC, Villejuif, France) and Fondation de France. N. Schrantz receives a fellowship from Foundation pour la Recherche Médicale.

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