Induction of Apoptosis by SB202190 through Inhibition of p38
Mitogen-activated Protein Kinase*

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p38, a subfamily of the mitogen-activated protein kinase, regulates gene expression in response to various extracellular stimuli. The pyridinyl imidazoles like SB202190 are specific inhibitors of p38α and p38β and have been widely used in investigation of the biological functions of p38. Here we show that SB202190 by itself was sufficient to induce cell death, with typical apoptotic features such as nuclear condensation and intranucleosomal DNA fragmentation. SB202190 stimulated the activity of CPP32-like caspases, and its apoptotic effect was completely blocked by the protease inhibitor benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone and expression of bel-2. In addition, SB202190 was able to potentiate apoptosis induced by Fas(APO-1) ligation or UV irradiation. Expression of p38β attenuated the apoptotic effect of SB202190 and the cell death induced by Fas ligation and UV irradiation. In contrast, expression of p38α induced cell death mildly. These results indicate that SB202190 induces apoptosis through activation of CPP32-like caspases and suggest that distinct members of the p38 subfamily of mitogen-activated protein kinase have different functions in apoptosis.

Apoptosis is an active process of programmed cell death in multicellular organisms (1), characterized by cellular shrinkage, membrane blebbing, nucleus condensation, and intranucleosomal DNA fragmentation (2). It has been shown that apoptosis is essential in many aspects of normal development and required for maintaining homeostasis (3–5, 49). Various death signals like Fas(APO-1) ligand and UV irradiation induce apoptosis through a common proteolytic pathway that consists of a group of cysteine proteases (caspases) (6, 11, 12). Caspases catalyze a controlled proteolysis of proteins, including caspases themselves and their downstream substrates such as poly(ADP-ribose) polymerase, protein kinase C, and MEKK1 (7–10). Overexpression of caspases induces apoptosis and, conversely, specific caspase inhibitors such as fluoro-methyl ketone-derived synthetic peptides (YVAD, VAD, and DEVD) block apoptosis (6). On the other hand, apoptosis can be suppressed by various survival signals like growth factors (3) and death antagonists like Bel-2 (13). Bel-2, the product of oncogene bel-2, blocks apoptosis induced by a variety of death signals in many types of cells, although the mechanisms by which bel-2 antagonize apoptosis are still under investigation (5, 14–16, 50). A fine balance between the death and survival signaling pathways appears to dictate the life or death of cells.

p38 is a distant member of the MAPK1 family. The p38 subfamily of MAP kinase consists of p38α, p38β, p38γ, and p38δ (17–26) and regulates gene expression in response to various extracellular stimuli like tumor necrosis factor-α and interleukin-1 (17–19). p38 is activated by dual specificity MAP kinase kinases, including MKK3, MKK6, and JNK1, that phosphorylate p38 on threonine 180 and tyrosine 182 (27–31). p38 in turn stimulates the activity of several transcription factors including ATF2, CHOP, and MEF-2C and other protein kinases such as MAPKAPK 2, MAPKAPK 3, and MNK1 (19, 31, 51–59). The MAP kinase kinase kinase of the p38 pathway may include Tak1, Ask1, and MLK (32–36), although the physiological relevance of these MAP kinase kinase kinases has yet to be determined.

The p38 pathway has been implicated to play a critical role in apoptosis (37). It was shown that many apoptotic signals were able to stimulate p38 activity and that activation of p38 was correlated with the induction of apoptosis in several types of cells (33, 37–39). Expression of constitutively activated MKK3 or MKK6 (both are p38 activators) was able to induce apoptosis in T lymphocyte Jurkat cells (40, 41). Surprisingly, it was found that p38 was not involved in MKK3 or MKK6-induced apoptosis, suggesting that MKK3 and MKK6 may induce apoptosis through activation of their unknown substrates (40, 41). An important question remained regarding the role of p38 activation in apoptosis.

The pyridinyl imidazole compounds were found to act as specific inhibitors of p38α and p38β (18, 20) but not p38γ and p38δ (21–26) through competition with ATP for the same binding site on the p38 kinase (42, 43). Crystal structural and mutagenesis analysis revealed that a single residue difference between p38 and other MAP kinases like JNK or extracellular signal-regulated protein kinase determines the specificity of the pyridinyl imidazole compounds like SB202190 and SB203580 (42, 43). These specific p38 inhibitors have been widely used in investigation of the physiological functions of p38 (44–46). Here we show that SB202190 was sufficient to induce apoptosis through activation of DEVD-sensitive caspases, and p38β inhibits apoptosis, whereas p38α induces apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—T lymphocyte Jurkat cells were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKAPK, MAP kinase-activated protein kinase; zVAD, benzoylcarbonyl-Val-Ala-Asp; DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; mAb, monoclonal antibody; GST, glutathione S-transferase; JNK, c-Jun NH2-terminal kinase; MEK1, MAP kinase kinase 1; MEKK1, MAP kinase kinase kinase 1.

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units/ml penicillin, and 100 mg/ml streptomycin. The Jurkat cells stably transfected with pSFFV-human bcl-2 (Jurkat/bcl-2) or the control plasmid pSFFV-neo (Jurkat.neo) (a gift from Dr. Stanley J. Korsmeyer, University of Washington) were cultured in the presence of G418 (0.2 mg/ml). HeLa cells were grown in Dulbecco’s modified Eagle’s medium with the supplements.

cDNA Constructs—Recombinant adenovirus encoding M2-Flag tagged p38α and p38β (a gift from Dr. Jiahuai Han, The Scripps Research Institute) has been described previously (40). GEX2T-hsp27 (a gift from Drs. Author Yee and Steve Pelech, (University of British Columbia, Vancouver, Canada) was expressed and purified on glutathione-agarose, as described (28).

Cell Viability Assays—Cells were treated with specific p38 inhibitors SB202190 (a gift from Dr. Alexander S. Kiselyov, Amergen), SB203580, the inactive derivative SB202474 (Calbiochem), or a specific MEK1 inhibitor PD098059 (New England Biolabs, Inc.) as described in the figure legends. The expression of M2-p38α and M2-p38β was monitored by Western blot analysis using anti-M2 monoclonal antibody (Kodak) as described (28).

Caspase Activity Assays—Jurkat.neo or Jurkat/bcl-2 cells (10⁶ cells) were treated with or without SB202190 (50 μM), PD098059 (50 μM) in the presence or absence of caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp (zVAD)-fluoromethylketone for 24 h. The cells were then harvested in lysis buffer and clarified by centrifugation. Endogenous MAPKAPK 2 was immunoprecipitated with anti-MAPKAPK 2 polyclonal antibody (Upstate Biotechnology Inc.) for 3 h at 4°C. The activity of the immune complex was assayed at 30°C for 30 min in 30 μl of kinase buffer (28) in the presence of 1 μCi [γ-32P]ATP (10 Ci/mmol) with GST-hsp27 as a substrate. The reactions were terminated with Laemmli sample buffer. The proteins were resolved by 13% SDS-polyacrylamide gel electrophoresis followed by autoradiography. The phosphorylated proteins were quantitated by a PhosphorImager.

Fig. 1. Inhibition of MAPKAPK 2 activity by the specific p38 inhibitor SB202190. Jurkat cells were preincubated with or without different concentration of SB202190 for 30 min and then treated with anti-Fas mAb (100 ng/ml) (A), or left alone (B), as indicated. After 2 h, cells were harvested, and endogenous MAPKAPK 2 was immunoprecipitated with anti-MAPKAPK 2 (Upstate Biotechnology Inc.) from cell extracts (150 μg). The activity of MAPKAPK 2 was measured in immune complex kinase assays with GST-hsp27 (5 μg) as a substrate.

Fig. 2. The specific p38 inhibitor SB202190 induces cell death in Jurkat cells. A, time course of cell death induced by SB202190. Jurkat cells were serum-starved for 24 h and then treated with the specific p38 inhibitor SB202190, the inactive inhibitor SB202474 (50 μM each), or the specific MEK1 inhibitor PD098059 (100 μM) or left untreated, for various period of times as indicated. The cell viability was monitored by trypan blue exclusion. B, SB202190 induces cell death in a dose-dependent manner. Jurkat cells were serum-starved and then treated with different concentration of SB202190 or PD098059 or left untreated for 24 h. The cell death was measured by propidium iodide staining. C and D, the same as A and B, except that the cells were HeLa cells.
The Specific p38 Inhibitor SB202190 by Itself Induces Apoptosis—It was shown that p38 was activated in Fas ligation-induced apoptosis of Jurkat cells (40, 41), but the activation of p38 was not required for the induction of cell death (40, 41). Therefore, p38 may not be involved in the apoptosis, or it may be a cellular stress response that is designed to protect cells from death. To test these possibilities, we first examined the effect of the specific p38 inhibitor SB202190 on p38 activity in Jurkat cells.

We next determined the effect of inhibition of p38 by SB202190 on cell death. Jurkat cells were serum-starved and then treated with SB202190 or left untreated for various times as indicated (Fig. 2). The cell viability was measured by trypan blue exclusion. As early as 14 h after incubation with SB202190, the cell death already reached half the maximum rate (Fig. 2A). The ability of SB202190 to induce cell death was shown in a dose-dependent manner, as measured by flow cytometry, using propidium iodide as an indicator (Fig. 2B). In addition, SB202190 induced cell death in HeLa cells (Fig. 2, C and D). Another specific p38 inhibitor SB203580 also induced cell death (data not shown). The inactive derivative SB202474, however, did not induce cell death (Fig. 2, A and C).
The results suggest that the effect of SB202190 may be related to its inhibition on p38. In contrast, the specific MEK1 inhibitor PD098059 did not induce cell death, even at a much higher concentration (100 μM).

The cell death induced by SB202190 in Jurkat cells had typical apoptotic appearances, including nucleus condensation as measured by staining with H33258 (Fig. 3A) and DNA fragmentation as measured by less than 2N DNA assays (Fig. 3B). SB202190-induced cell death in HeLa cells also had typical apoptotic appearance such as DNA fragmentation as measured by less than 2N DNA assay (data not shown). This indicates that the cell death induced by SB202190 was because of apoptosis, rather than nonspecific cytotoxicity.

**SB202190-induced Apoptosis Is Mediated by Activation of CPP32-like Caspases and Is Blocked by bcl-2**—Activation of CPP32-like caspases is essential for induction of apoptosis by various death signals in many types of cells including Jurkat cells (6). It is possible that SB202190 may induce apoptosis in Jurkat cells through activation of CPP32-like caspases. To test this possibility, we determined whether SB202190 was able to stimulate CPP32-like caspase activity.

Jurkat cells were treated with SB202190 or PD098059 or left untreated. After 24 h, cells were harvested, and the activity of CPP32-like caspases in cell extracts was measured by cleavage of the fluorescent peptide DEVD-AMC, which is a specific substrate of CPP32-like caspases (47). The cleavage of DEVD-AMC was significantly increased in cells treated with SB202190 but not in the control or cells treated with PD098059 (Fig. 4A). In addition, the DEVD-AMC cleavage induced by SB202190 was completely blocked by the caspase inhibitor zVAD-fluoromethylketone (Fig. 4A). Expression of bcl-2, which is able to block activation of CPP32-like caspases in Jurkat and many other types of cells (57), also blocked SB202190-induced cleavage of DEVD-AMC (Fig. 4A). Therefore, SB202190 may induce Jurkat cell death through activation of CPP32-like caspases.

We then tested whether activation of CPP32-like caspases is essential for SB202190-induced apoptosis. Jurkat cells that were stably transfected with expression vectors encoding bcl-2 (Jurkat/bcl-2) or the empty neomycin gene (Jurkat/neo) were treated with SB202190 in the presence or absence of zVAD, as indicated (Fig. 4B). SB202190 was able to induce cell death in control Jurkat/neo cells (Fig. 4B), but it failed to do so in zVAD-treated Jurkat/neo cells and in Jurkat/Bcl-2 cells as measured by H33258 staining and by less than 2N DNA assays (Fig. 4B). These results indicate that activation of CPP32-like caspases was required for SB202190-induced apoptosis and that Bcl-2 acted at or upstream of CPP32-like caspases to inhibit the apoptosis.

**SB202190 Synergistically Promotes Cell Death Induced by Fas Ligation and UV Irradiation**—It was shown that Fas ligation-induced apoptosis in Jurkat cells was mediated by activation of caspases including CPP32-like caspases (11), and so was SB202190 (Fig. 4). It is possible that SB202190 may stimulate the same apoptotic pathway as the Fas pathway. To test this possibility, we determined whether SB202190 potentiates Fas-like death pathway.

Cells were treated with various amounts of anti-Fas mAb in the presence or absence of SB202190. The death of cells was analyzed by flow cytometry, using propidium iodide as an indicator.
ng/ml anti-Fas mAb, although to lesser extent (Fig. 5, B and C).

UV irradiation was able to stimulate apoptosis in HeLa cells, probably through the Fas pathway (12). We then tested whether SB202190 potentiates UV irradiation-induced apoptosis. Indeed, SB202190 augmented the apoptotic effect of UV irradiation in HeLa cells in a synergistic manner (Fig. 5D). These results suggest that SB202190 may utilize the same death pathway as did Fas ligand.

SB202190-induced Apoptosis Is Attenuated by p38β but Augmented by p38α—SB202190 is a specific p38 inhibitor, and its apoptotic effect was only slightly blocked by the protein synthesis inhibitor cycloheximide (data not shown). Therefore, SB202190 may induce apoptosis, at least in part, through inhibition of p38 activity. To test this possibility, we determined the effect of overexpression of p38 on SB202190-induced apoptosis.

HeLa cells were infected with recombinant adenovirus encoding p38α, p38β, or control green fluorescence protein. After 24 h, the cells were treated with SB202190 or left untreated as indicated (Fig. 6). Infection of p38β significantly attenuated SB202190-induced cell death (Fig. 6, A and B), whereas infection of p38α augmented the apoptotic effect of SB202190 (Fig. 6B). In fact, expression of p38α by itself induced cell death mildly (Fig. 6B). Therefore, SB202190 may induce cell death through its inhibition on p38β.

We then tested whether expression of p38 was able to affect other forms of apoptosis. In HeLa cells, expression of p38β significantly attenuated cell death induced by anti-Fas mAb or UV irradiation (Fig. 6C). In contrast, expression of p38α augmented the apoptotic effect of UV irradiation and anti-Fas mAb (Fig. 6C). Therefore, p38β may function as a suppressor of apoptosis, whereas p38α may act as a mediator of apoptosis. Inhibition of p38β by SB202190 could increase cell susceptibility to apoptotic signals.

**DISCUSSION**

Our results indicate that the specific p38 inhibitor SB202190 is sufficient to induce cell death in Jurkat and HeLa cells. SB202190-induced cell death had typical apoptotic features including nucleus condensation and DNA fragmentation, suggesting that the cell death was because of apoptosis, rather than nonspecific cytotoxicity. This conclusion is further supported by the finding that SB202190-induced cell death was mediated by activation of CPP32-like caspases and was blocked by expression of bcl-2. Therefore, SB202190 may function as an apoptotic inducer.

The mechanisms by which SB202190 induces activation of CPP32-like caspases have yet to be determined. We speculate that SB202190 may stimulate the activity of CPP32-like caspases through inhibition of p38β. Expression of p38β was able to attenuate the apoptotic effect of SB202190 (Fig. 6). In addition, expression of p38β suppressed the cell death induced by UV irradiation and anti-Fas mAb as well (Fig. 6C), both of which are mediated by activation of caspases including CPP32-like caspases. Further investigation should reveal whether p38β directly or indirectly down-regulates CPP32-like caspases.

SB202190 is a specific inhibitor for both p38α and p38β (18, 20). Interestingly, the apoptotic effect of SB202190 was attenuated by p38β but augmented by p38α. This raises the question of how SB202190 could induce cell death if it inhibits two isoforms of p38 that have opposing effects on cell death. One
possible explanation is that in resting cells the anti-apoptosis effect of p38β might be a dominant one in comparison with the pro-apoptotic effect of p38α. Inhibition by SB202190 may shift the balance between cell death and survival toward death. SB202190 may not be able to completely inhibit overexpressed p38, so that its apoptotic effect was attenuated by overexpressed p38β but augmented by overexpressed p38α, which by itself mildly induced cell death. We cannot exclude out the possibility that SB202190 may inhibit other unknown targets to fully execute its apoptotic effects. It was reported that in vitro, SB202190 was able to inhibit JNK2 activity at concentrations over 10 μM (31), but it is not known whether SB202190 can do so in vivo. It will be of interest to identify other SB202190 target proteins, because they may be involved in cell survival as well.

The role of p38 in apoptosis has been investigated extensively but remains contradictory. It was reported that p38 might be a dominant one in comparison with the anti-apoptotic proteins (40, 41). Our results indicate that the role played by individual p38 isoforms.

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REFERENCES

1. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–243
2. Wyllie, A. H., Kerr, J. F. R., and Currie, A. R. (1980) Int. Rev. Cytol. 78, 251–306
3. Tompsett, C. B. (1995) Science 267, 1456–1462
4. Jacobson, M. J., Weil, M., and Raff. M. C. (1997) Cell 91, 347–354
5. Rinkenberger, J. L., and Korsmeyer, S. J. (1997) Curr. Opin. Genet. Dev. 7, 589–596
6. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
7. Lazebnik, Y. A., Kaufmann, S. H., and Vousden, K. H. (1997) Cell 91, 3–11
8. Tewari, N., Quan, L., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., and Greenberg, M. E. (1995) Science 267, 773–776
9. Emoto, Y., Manome, Y., Meinhardt, G., Kisaki, H., Kharbanda, S., Robertson, J., Barnes, M. J., Kamei, T., Ohara, H., and Stemkowski, P. S. (1995) Nature 370, 23668–23674
10. Hengartner, M. O. (1996) Cell 84, 273–276
11. Cauda, A., Crazzini, M., and de Vito, C. (1996) Science 270, 1027–1037