p38 Mitogen-activated Protein Kinase Is Involved in Fas Ligand Expression*

(Received for publication, March 11, 1999, and in revised form, May 21, 1999)

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p38 mitogen-activated protein kinase (MAPK) is activated by T cell receptor engagement. Here we showed that T cell receptor activated p38α but not p38δ. Inhibition of p38α by the specific inhibitor SB 203580 prevented activation-induced cell death in T cells. SB 203580 had no effect on Fas-initiated apoptosis. Instead, SB 203580 preferentially inhibited activation-induced Fas ligand (FasL) expression. The inhibition on FasL expression by SB 203580 was correlated with the suppression on the FasL promoter activation. Overexpression of active MAPK kinase 3b, the activator of p38 MAPK, led to activation of FasL promoter and induction of FasL transcripts in T cells. Stress stimulation of T cells by anisomycin also induced FasL expression in a p38 MAPK-dependent manner. The induction of FasL expression in nonlymphoid cells such as 293T also required activation of p38 MAPK. Our results suggest that p38 MAPK is essential for FasL expression.

Fas (APO-1, CD95) is a 45-kDa membrane protein that triggers apoptosis when it interacts with Fas ligand (FasL)1 (for review see Ref. 1). The expression of Fas is low in resting T lymphocytes, whereas the expression of FasL is absent. T cell receptor engagement leads to increased expression of Fas and FasL. The subsequent Fas-FasL interaction is the major mechanism underlying activation-induced cell death of immature T cells (2–5). Fas and FasL are also up-regulated by various stress stimulation. Treatments with anisomycin, UV, γ-irradiation, or cytotoxic drugs induce the expression of Fas and FasL in T cells and tumor cells (6–12). Fas and FasL gene promoters have been extensively studied (12–21). Transcription elements including NF-AT, NF-κB, and AP-1 are identified on the FasL promoter (12, 13, 15–21).

MAPKs transduce extracellular signals into nucleus. Four groups of MAPKs have been identified in mammalian cells including extracellular signal-regulated kinase kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK, also known as SAPK), p38 MAPK (also known as RK and CSBP), and ERK5. The p38 MAPK was first identified for its activation in response to hyperosomolarity and endotoxic lipopolysaccharide (22–24). p38 MAPK is specifically activated by MKK3, MKK4, and MKK6 (25–29). Four members of p38 MAPKs have been described: p38α (22–24), p38β (30, 31), p38γ (also known as SAPK3 and ERK6) (32–34), and p38δ (also known as SAPK4) (35–37). Different tissue distribution is found among distinct p38 MAPK isoforms. For example, p38α and p38β are highly expressed in brain, and p38γ is predominantly expressed in muscle, whereas p38α and p38δ are the major isoforms in lymphoid tissue (30, 35, 37, 38). All four members of p38 MAPK are activated by MKK6, whereas p38α, p38γ, and p38δ are activated by MKK3 (34–37, 39). p38α and p38β are specifically inactivated by SB 203580, a pyridinyl imidazole drug, through binding in the ATP pocket (5, 40–42). In contrast, p38γ and p38δ are resistant to SB 203580 inhibition (31, 34–37).

In lymphocytes, p38 MAPK is stimulated by stimuli other than stresses. p38 MAPK is constitutively activated in freshly isolated thymocytes (43). p38 MAPK is activated in response to T or B cell antigen receptors and to IL-2 and IL-7 in lymphocytes (44–48). p38 MAPK is also shown to be activated in T helper 1 cells but not in T helper 2 cells when stimulated by TPA/ionomycin (49). In this study, we examined the role of p38 MAPK in activation-induced lymphocyte death. We observed that suppression of p38α by SB 203580 substantially prevented activation-induced cell death in T cells. The inhibition of activation-induced cell death by SB 203580 was attributed to a suppression of the FasL expression. The role of p38 MAPK was further demonstrated by the fact that activation of p38 MAPK by MKK3b increased FasL expression. Our results suggest the possibility that apoptosis induction may be enhanced by p38 MAPK activation through increased expression of FasL.

EXPERIMENTAL PROCEDURES

Reagents and Cell Lines—Concanavalin A, TPA, and A23187 were purchased from Sigma. SB 203580 was a gift of Dr. John C. Lee (SmithKline Beecham, King of Prussia, PA) and was subsequently purchased from Calbiochem (San Diego, CA). The active mutants of MKK3b (MKK3b(Glu189,Glu193)) and MKK6b (MKK6b(Glu207,Glu211)) were previously reported (30). CAT reporters containing AP-1 and NF-κB elements from the IL-2 promoter were previously described (50). kB-TATA-CAT containing two copies of the HIV-3 site (51) was a gift of Dr. Warren C. Greene (University of California, San Francisco, CA). Human FasL promoter (~453 to ~2 nucleotide) was isolated by PCR according to the method of Holtz-Heppelmann et al. (18) and was subcloned into the HindIII and Xhol sites of the pG2L-Base luciferase vector.
p38 MAPK-dependent FasL Expression

**RESULTS**

**p38 MAPK Is Essential for T Cell Activation**—T cell activation is accompanied by activation of p38 MAPK. Treatment of EL4 T cells with TPA/A23187 led to phosphorylation of p38 MAPK to an extent indistinguishable from stimulation with sorbitol or TNF-α (Fig. 1A). This was also confirmed by immunoprecipitation kinase assay using GST-AUF-2 (1–109) as substrate (Fig. 1B). Similar to anisomycin treatment, TPA/A23187 treatment significantly activated p38α in EL4 T cells. The p38 MAPK activation mediated by T cell activation was not limited to stimulation with TPA/A23187. Engagement of T cell receptor by TCR engagement was found in T cell hybridomas 10I and 9C12.7 (52) as well as in the purified splenic T cells (Fig. 1, not shown for 9C12.7 and splenic T cells). Both anisomycin and TCR-coupled p38α kinase activation was substantially inhibited by SB 203580 (20 μM) for 20 min, and the kinase activity of p38α and p38δ isolated by immunoprecipitation was measured using GST-AUF-2 as substrate.

As reported previously (37, 38) and confirmed in our immunoblots (not shown), p38α and p38δ are the major isoforms of p38 MAPK in T lymphocytes. p38δ is not inhibited by SB 203580.
The extent of inhibition was seen in EL4 and 10I T cells (not shown). The same effect was found at higher concentrations of SB 203580. The same inhibition was observed in T cells stimulated with anti-CD3 or anti-CD3 plus anti-CD28 (Fig. 1). Neither treatment of anisomycin activate p38α in T cells (not shown). On the contrary, hydrogen peroxide was an effective activator of both p38α and p38β in T cells (Fig. 1D). Hydrogen peroxide-activated p38α, but not p38β, was suppressed by SB 203580.

The induction of p38 MAPK activation was essential for TCR-mediated IL-2 production (46, 48). We observed that IL-2 secretion was suppressed by 45% with the addition of SB 203580 at concentrations as low as 0.625 μM in concanavalin A-stimulated splenic T cells (not shown). Further inhibition was found at higher concentrations of SB 203580. The same extent of inhibition was seen in EL4 and 10I T cells (not shown).

Because the concentrations of SB 203580 (10–20 μM) used in the present study have been reported to inhibit JNK activation in monocytes and neuronal cells (59, 60), we tested whether JNK was similarly suppressed in T cells. As a control, TCR-induced ERK activation was not affected by SB 203580 (Fig. 2). Anti-CD3-induced JNK activation was slightly enhanced in the presence of SB 203580. Therefore, the effect observed with SB 203580 was not due to an inhibition of JNK and ERK in activated T cells.

**Inhibition of p38 MAPK Prevented Activation-induced Cell Death but Had No Effect on Fas-initiated Apoptosis**—Because activation of p38 MAPK is essential for T cell activation, we investigated whether p38 MAPK was involved in activation-induced cell death. Activation of immature T cells such as T hybridomas by anti-CD3 induced cell death (52), as assessed by DNA fragmentation using fluorescence-activated cell sorter analysis (Fig. 3A). SB 203580 by itself did not trigger significant cell death during the time course of experiments (18–24 h). However, as previously reported (61), prolonged incubation with SB 203580 (≥24 h) did trigger apoptosis in T cells. All cell death analyses were thus conducted within 24 h period. Activation-induced cell death in T cell hybridoma 10I was suppressed by SB 203580, with a reduction in hypoploid fraction from 45 to 15% (Fig. 3A). The extent of inhibition decreased with reduced concentrations of SB 203580 (Fig. 3B), yet antagonism on activation-induced death was evident with 5 μM SB 203580. A similar inhibitory effect of SB 203580 was also observed in 9C12.7 and reactivated splenic T cells (not shown).

Previous reports have demonstrated that Fas-initiated apoptosis was completely resistant to SB 203580 (62, 63). This is also confirmed by the fact that SB 203580 did not prevent apoptosis triggered by anti-Fas antibody (Jo2) in activated splenic T cells (Fig. 3C). Therefore, SB 203580 inhibited activation-induced cell death but not Fas-initiated cell death. The activation-induced death process that was antagonized by SB 203580 apparently must be at the stage prior to Fas-FasL ligand interaction. How TCR activation-induced Fas and FasL expression was modulated by SB 203580 was next examined.

**p38 MAPK Was Essential for Activation-induced Fas/FasL Expression**—Resting T cells express low level Fas. The activation by anti-CD3 triggered a significant increase of the cell surface Fas (Fig. 4A) and the Fas transcript (Fig. 4B). The expression of surface Fas on T cells was partially inhibited in the presence of 10 μM SB 203580 (Fig. 4A, bold curve). A more prominent inhibition was observed with Fas mRNA (Fig. 4B), suggesting the requirement of p38 MAPK for Fas expression. SB 203580 did not completely suppress anti-CD3-induced Fas expression, in which both Fas mRNA and surface Fas expression remained abundant (Fig. 4).

Surface FasL protein and FasL mRNA was absent in resting T cells, and significant induction of surface FasL expression and FasL mRNA synthesis was observed followed TCR engagement (Fig. 4). In contrast to Fas, anti-CD3-triggered FasL mRNA increase was largely suppressed by SB 203580, suggesting that p38 MAPK may play a more critical role in FasL expression. Together, the suppression on activation-induced cell death by SB 203580 in 10I cells (Fig. 3) was due to an effective inhibition on FasL expression and a partial suppression on Fas expression. A preferential suppression on FasL expression by SB 203580 was also found in 9C12.7 cells (not shown).

Because inhibition of p38 MAPK only partially interfered with TCR-induced Fas expression, we tested whether the remaining Fas molecules still mediated apoptosis. Anti-Fas antibody Jo2 was unable to trigger apoptosis in resting T cells because the surface Fas expression was low (Fig. 3D). The Fas expression was up-regulated by stimulation with anti-CD3 for 12 h in the presence of SB 203580. The cells were then removed from stimulation and treated with Jo2 in the continued presence of SB 203580. Despite the fact that SB 203580 inhibited activation-induced cell death in T cells, the remaining Fas molecules on SB 203580-treated T cells were still functional as apoptotic-initiating molecules (Fig. 3D). Therefore, the inhibitory effect of SB 203580 on activation-induced death must be due to a preferential inhibition of FasL expression.

**p38 MAPK Was Required for the FasL Promoter Activation**—To further examine the induction of FasL, FasL promoter (∼453 base pairs), which accounts for the inducibility (18, 21), was isolated by PCR and was subcloned into luciferase reporter pGL2. The pGL2-FasL construct contained the transcription elements including NF-κB, NF-AT, and AP-1. In this experiment, 9C12.7 cells were used because transfection efficiency of 10I cells was very low. Consistent with activation-induced FasL mRNA expression (Fig. 4), stimulation of 9C12.7 cells with anti-CD3 significantly activated the FasL promoter (Fig. 5A). As a control, no activation of pGL2 vector was detected in stimulated 9C12.7 cells. TCR-activated FasL promoter activity was largely inhibited by SB 203580 (Fig. 5A), suggesting that FasL promoter activation is dependent on p38 MAPK. There is a good correlation between the inhibition of FasL promoter activation and the suppression of FasL mRNA induction by SB 203580. Therefore, the inhibition on the FasL protein level and mRNA level by SB 203580 (Fig. 4) is partly attributed to a suppression on TCR-activated FasL promoter activity.

**Activation of p38 MAPK Pathway Induced FasL Promoter Activation and FasL Expression in T Cells**—The role of p38 MAPK was further elucidated by using the active forms of...
M KK3b and MKK6b (30), activators of p38 MAPK. Transfection of active MKK3b and MKK6b into EL4 cells led to activation of both p38α and p38δ (Fig. 6 A; not shown for MKK6b). Overexpression of MKK3b or MKK6b activated the FasL promoter in EL4 T cells (Fig. 5 B). Either MKK3b or MKK6b was less effective than TPA/A23187 in the activation of FasL promoter. For reasons that are unclear, MKK3b was a better activator of the FasL promoter than MKK6b (Fig. 5 B). In addition to the activation of FasL promoter, overexpression of active MKK3b resulted in a detectable FasL mRNA expression in EL4 T cells 24 h after transfection (Fig. 6 B). Either MKK3b or MKK6b was less effective than TPA/A23187 in the activation of FasL promoter. For reasons that are unclear, MKK3b was a better activator of the FasL promoter than MKK6b (Fig. 6 B).

In addition to the activation of FasL promoter, overexpression of active MKK3b resulted in a detectable FasL mRNA expression in EL4 T cells 24 h after transfection (Fig. 6 B). In contrast, MKK3b alone was insufficient to increase the expression of Fas transcripts, suggesting the requirement of additional signals for Fas expression. Unlike TCR-coupled FasL expression (Fig. 4 B), MKK3b-induced FasL was not reduced by SB 203580 treatment. Because p38δ was resistant to SB 203580 inhibition (Fig. 6 A), the difference in sensitivity to SB 203580 between TCR-induced and MKK3b-induced FasL expression was most likely due to the activation of p38δ by MKK3b. This was supported by the suppression of MKK3b-induced FasL expression through co-expression of the dominant negative form of p38δ (Fig. 6 C). The present results suggest that both p38α and p38δ contribute to the expression of FasL, yet the selective activation of p38α by TCR confers the sensitivity of the FasL expression to SB 203580 inhibition.

FasL Expression Was Induced by Anisomycin in T Cells—We also examined whether the induction of FasL expression was limited to TCR engagement. p38 MAPK is activated by stress stimuli such as anisomycin (Fig. 7 A). Anisomycin also stimulated FasL transcript expression in EL4 T cells 3 h after treatment (Fig. 7 B). Anisomycin-induced FasL expression was partly suppressed by SB 203580. The sensitivity to SB 203580 may be explained by the observation that endogenous p38δ was minimally activated by anisomycin in T cells. It may be noted that we failed to detect an induction of FasL expression in hydrogen peroxide-treated T cells because of the significant cell death early in the treatment.

FasL Expression Was p38 MAPK-dependent in 293T Cells—The involvement of p38 MAPK in FasL expression was not restricted to T cells. Treatment of 293T cells with anisomycin

**FIG. 3.** SB 203580 inhibited activation-induced cell death but not Fas-initiated cell death. A, 10I T hybridoma cells were stimulated with immobilized anti-CD3 antibody 2C11 (5 μg/ml) in the absence or presence of 20 μM SB 203580 for 18 h. DNA content was determined by staining with 50 μg/ml propidium iodide and analyzed by FACScan. Fractions of cells with sub-G DNA content were assessed using CELLFIT program (Becton Dickinson) and were considered as percentages of cell death. B, dose-dependent inhibition of activation-induced cell death by SB 203580. Anti-CD3-triggered apoptosis was determined in the presence of different concentrations of SB 203590 as described in A. Results indicate the means of duplicate experiments. The experiments have been repeated at least twice. C, Fas-initiated apoptosis in activated splenic T cells was resistant to SB 203580. Purified splenic T cells were activated with TPA/A23187 and cultured in IL-2 for 4 days. Viable T cells were isolated and treated with immobilized anti-Fas antibody Jo2 (10 μg/ml) in the absence or presence of 10 μM SB 203580 for 18 h and cell death was determined as in A. CTR, untreated cell control. D, SB 203580-treated activated T cells were still sensitive to apoptosis induced by soluble FasL. Unstimulated 10I cells (CTR) or 10I cells activated by 2C11 for 12 h in the presence of SB 203580 (10 μM) were treated with immobilized Jo2, and cell death was quantitated after another 12 h.
also led to activation of p38α (Fig. 8A) as well as expression of FasL (Fig. 8B). The effective inhibition of anisomycin-induced FasL expression by SB 203580 was correlated with a suppression of p38α activation. Therefore, p38 MAPK was also required for FasL induction in 293T cells.

DISCUSSION

p38 MAPK is activated by TCR engagement and TPA/A23187 (43, 45–48) as well as by stress stimuli in T lymphocytes. The stimulation of p38 MAPK by TCR was essential for IL-2 production (46, 48). In this study, we demonstrated that TCR engagement activated p38α (Fig. 1C) and inhibition of p38α suppressed activation-induced cell death in T hybridoma (Fig. 3A). Our observation that p38 MAPK is essential for activation-induced T cell death is consistent with the observation that IgM-induced apoptosis of human B lymphocytes requires p38 MAPK (64) yet is in direct contrast to a recent report that antigen receptor-induced apoptosis is not affected by SB 203580 (45). The latter observation was made on the restimulation of lymph node T cells and on anti-IgM-induced WEHI 231 cells. We do not know the cause of such discrepancy because we found an inhibition of activation-induced apoptosis by SB 203580 on restimulated splenic T cells similar to that on T cell hybridomas.

Even though p38 MAPK is activated through Fas engagement, inhibition of p38 MAPK does not interfere with Fas-mediated apoptosis (Fig. 3, C and D, and Refs. 45, 51, 63, and 65). Instead, the inhibition on activation-induced cell death was at the stage of activation-induced FasL and Fas expression (Fig. 4). Suppression by SB 203580 was greater on FasL ex-
pression than on Fas expression. In addition, the residual surface Fas still mediated apoptosis in SB 203580-treated T cells (Fig. 3D), further supporting the idea that suppression on

activation-induced apoptosis was due to the predominant inhibition of FasL expression. The prime role of p38 MAPK in the induction of FasL is also illustrated by the overexpression of MKK3b leading to expression of FasL in T cells (Fig. 6B). In contrast, despite the participation of p38 MAPK in Fas expression (Fig. 4), activation of p38 MAPK alone did not increase Fas expression (Fig. 6B).

p38 MAPK-dependent FasL expression is attributed to a requirement for p38 MAPK for FasL promoter activation, as demonstrated by the sensitivity of TCR-induced FasL promoter activation to SB 203580 (Fig. 5A) and by the induction of FasL promoter by MKK3b and MKK6b (Fig. 5B). The transcriptional elements necessary for FasL expression, including AP-1, NF-kB, and NF-AT, have recently been identified (12, 15–21). TCR-induced NF-AT activation is inhibited by SB 203580 (45). The contribution of p38 MAPK in TNF-α-induced NF-κB activation has been documented (66, 67). On a preliminary study, we also observed that the activation of the analogous NF-κB and NF-AT elements were partially inhibited in presence of SB 203580. Analysis of the NF-AT and NF-κB elements from FasL promoter are currently being conducted. It is likely that the inhibition of FasL expression by SB 203580 may be attributed to a specific inhibition of NF-AT and NF-κB.

We have also observed a preferential activation of p38α, but not p38δ, by TCR engagement (Fig. 1C). In addition, p38δ was stimulated by hydrogen peroxide but not by anisomycin in T cells. Therefore, similar to that previously reported (36, 37), p38α and p38δ are differentially regulated in T cells. Interestingly, the induction of FasL was no longer sensitive to SB 203580 when both p38α and p38δ were activated by MKK3b (Fig. 6B). This is consistent with the known resistance of p38δ to SB 203580 (35–37). A role of p38δ in FasL expression was supported by the inhibition of FasL expression through the co-transfection of p38δ dominant negative mutant (Fig. 6C). Hence both p38α and p38δ contribute to FasL expression, and the sensitivity of TCR-induced FasL expression to SB 203580 is a consequence of the selective activation of p38α in T cells. Our results may serve as another example that the use of pyridinyl imidazole inhibitor is highly dependent on the isoforms of the p38 MAPK that are activated.

2 S.-C. Hsu, unpublished observation.
p38 MAPK is known to be activated by stress stimuli such as TNF-α, IL-1, UV, and γ-irradiation. Interestingly, Fas and FasL are up-regulated by stress stimulation including γ-irradiation, UV irradiation, and cytotoxic drugs (6–12). In this study, we also demonstrate that activation of p38 MAPK by anisomycin induced FasL expression (Fig. 7). This result suggests that, in addition to TCR-coupled signaling, p38 MAPK also mediates the induction of FasL by stress signals.

It may be noted that the present result does not imply that p38 MAPK alone accounts for FasL expression under physiological and pharmacological stimulation. Stimulation with anti-CD3 or anisomycin activated kinases other than p38 MAPK. The activation of FasL expression by p38 MAPK was performed through overexpression of MEKK1/MEKK6 at levels that were likely unphysiological. In addition, MKK3/6 activates signaling pathways that are p38 MAPK-independent (62, 63). Therefore, the present study cannot be used to argue against the requirement of other signal pathways for FasL expression. Furthermore, overexpression of MKK1 has been shown to induce JNK-dependent FasL expression (11, 15, 60). A recent study also indicates that ERK is required for activation-induced FasL expression (68). A full activation of FasL expression under physiological condition likely requires the coordination of p38 MAPK with other activation signals. We are currently investigating the integration of different activation signals in the induction of FasL.

Recent studies suggest that p38 MAPK is involved in a number of apoptotic processes. Activation of p38 MAPK is critical for apoptosis induced by nerve growth factor deprivation in PC12 cells (69). p38 MAPK mediates apoptosis induced by withdrawal of insulin in primary neuron culture (70). Inhibition of p38 MAPK activation prevents glutamate-induced apoptosis in rat cerebellar granule cells (71). Opposite effect on apoptosis between p38α and p38β have also been found (61, 72). Results from the present study suggest that p38 MAPK mediates FasL expression. It will be interesting to examine whether the above-mentioned apoptosis involves Fas-FasL interaction. The increased expression of FasL mediated by MKK3/6 may also explain why MKK3/6 activation enhances Fas-mediated apoptosis (62).

Acknowledgments—We thank Dr. Daniel Olive for helpful suggestions. Dr. Tse-Hua Tan for anti-INHAK antibody, and Dr. Warren Greene for αB-TATA-CAT. We also thank Douglas Platt for editorial correction of the manuscript.

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