Bcl-xL Blocks Activation of Related Adhesion Focal Tyrosine Kinase/Proline-rich Tyrosine Kinase 2 and Stress-activated Protein Kinase/c-Jun N-terminal Protein Kinase in the Cellular Response to Methylmethane Sulfonate*

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The stress-activated protein kinase/c-Jun N-terminal protein kinase (JNK) is induced in response to ionizing radiation and other DNA-damaging agents. Recent studies indicate that activation of JNK is necessary for induction of apoptosis in response to diverse agents. Here we demonstrate that methylmethane sulfonate (MMS)-induced activation of JNK is inhibited by overexpression of the anti-apoptotic protein Bcl-xL but not by caspase inhibitors CrmA and p35. By contrast, UV-induced JNK activity is insensitive to Bel-xL. The results demonstrate that treatment with MMS is associated with an increase in tyrosine phosphorylation of related adhesion focal tyrosine kinase (RAFTK)/proline-rich tyrosine kinase 2 (PYK2), an upstream effector of JNK and that this phosphorylation is inhibited by overexpression of Bcl-xL. Furthermore, overexpression of a dominant-negative mutant of RAFTK (RAFTK K-M) inhibits MMS-induced JNK activation. The results indicate that inhibition of RAFTK phosphorylation by MMS in Bcl-xL cells is attributed to an increase in tyrosine phosphatase activity in these cells. Hence, treatment of Bcl-xL cells with sodium vanadate, a tyrosine phosphatase inhibitor, restores MMS-induced activation of RAFTK and JNK. These findings indicate that RAFTK-dependent induction of JNK in response to MMS is sensitive to Bcl-xL but not to CrmA and p35, by a mechanism that inhibits tyrosine phosphorylation and thereby activation of RAFTK. Taken together, these findings support a novel role for Bcl-xL that is independent of the caspase cascade.

The cellular response to certain stress inducers includes cell cycle arrest and, in certain cases, lethality. However, the intracellular signals that control the induction of these events are mainly unclear. Whereas p53 has been implicated in promoting apoptosis induced by ionizing radiation (IR), other studies have demonstrated that Bcl-2 and Bcl-xL inhibit apoptosis in response to diverse agents (3, 4). The induction of apoptosis by diverse stimuli is associated with activation of aspartate-specific cysteine proteases (caspases) (5) and cleavage of poly(ADP-ribose) polymerase (PARP) (6), protein kinase C δ (PKCδ) (7), and other proteins (8). Importantly, the finding that cleavage of these proteins is blocked by overexpression of Bcl-xL (8, 9) has indicated that the Bcl-2-related family of anti-apoptotic proteins functions upstream to the activation of caspases. More direct evidence for involvement of interleukin-converting enzyme (ICE)-like proteases in apoptosis comes from studies utilizing the cowpox virus protein CrmA (10) and the baculovirus protein p35 (11), which are direct inhibitors of at least certain members of the caspase family.

Methylmethane sulfonate (MMS) is a monofunctional alkylating agent and is a potent inducer of cellular stress leading to chromosomal aberrations, point mutations, and cell killing (12–14). Previous studies have shown that reactivity of MMS with the ring nitrogens of the purine bases, particularly N-7 of guanine, correlates with induction of cell killing (12). Other studies have shown that treatment of a variety of cell types with MMS is associated with induction of JNK and p38 MAPK (15), but the signaling mechanisms involved in this response are unclear.

The stress-activated protein kinase (SAPK) (also known as c-Jun N-terminal kinase (JNK)) is induced by tumor necrosis factor (TNF), anisomycin, and interleukin-1 (16, 17). JNK is also activated by UV light, osmotic shock, IR, and a variety of other DNA damaging agents (18–22). Upstream regulators of JNK include GCK, MLK-3, SPRK, and hematopoietic protein kinase-1 (HPK-1) (23–26). Other studies have shown that JNK is activated by immediate upstream kinases such as SEK1/ MKK4 and/or MKK7 (27, 28). The finding that certain DNA damaging agents, but not TNF, induce JNK by a c-Abl-dependent mechanism has also supported distinct upstream effectors to JNK activation (20, 22, 29).

Many of the signals activating JNK also induce apoptosis. Accordingly, JNK has been implicated as a potential mediator of the apoptotic pathway in response to IR, TNF, certain other

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‡ The abbreviations used are: IR, ionizing radiation; SV, sodium vanadate; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal protein kinase; PARP, poly(ADP-ribose) polymerase; proline-rich tyrosine kinase; RAFTK, related adhesion focal tyrosine kinase; MMS, methylmethane sulfonate; PKCδ, protein kinase C δ; TNF, tumor necrosis factor; GST, glutathione S-transferase; ICE, interleukin-converting enzyme; PYK2, proline-rich tyrosine kinase.

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inducers (30), and trophic factor deprivation (31). By contrast, other studies have shown that TNF-induced apoptosis is independent of JNK activation (32). Therefore, the role of JNK in mediating apoptosis may be inducer- and cell type-specific.

Activation of the related adhesion focal tyrosine kinase (RAFTK) has been shown to be upstream to JNK in the response to TNF or UV in certain cell types (33). RAFTK (also known as PYK2 and Ca2+-dependent tyrosine kinase (CADTK)) is involved in signaling upstream to the extracellular-regulated kinase (ERK) pathway (34–36). RAFTK is a close relative of the pp125 FAK tyrosine kinase and is activated by various extracellular signals that increase intracellular calcium concentrations (34, 36). Moreover, RAFTK can tyrosine phosphorylate and thereby modulate the action of ion channels. Thus, RAFTK may function as an intermediate that links various calcium signals with both short and long term responses in neuronal cells (34, 36).

In this study, we demonstrate that MMS-induced activation of JNK in U-937 cells is blocked by overexpressing Bel-xL and not CrmA or p35. By contrast, Bel-xL had no apparent effect on UV-induced JNK activity. We also demonstrate that, in contrast to UV, MMS-induced activation of JNK is RAFTK-dependent and that tyrosine phosphorylation of RAFTK is sensitive to Bel-xL. Moreover, treatment of U-937/Bel-xL cells with sodium vanadate (SV) restores MMS-induced tyrosine phosphorylation of RAFTK and activation of JNK.

MATERIALS AND METHODS

Cell Culture and Reagents—Human U-937/neo, U-937/Bel-xL, U-937/CrmA, and U-937/p35 cells (37, 38) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. PC12 cells were grown in DMEM containing 10% horse serum, 5% heat-inactivated fetal bovine serum, and antibiotics. Cells [1 × 10⁶/ml] were seeded 24 h before treating with 1 mM MMS (Sigma) with or without 200 μM SV (Sigma). Cells were also treated with 40 J/m² UV (UV Stratalinker™ UV Stratagene).

Cell Proliferation Assays—Cells were treated with 1 mM MMS with or without 200 μM SV. The initial number of cells seeded was 1 × 10⁵/ml. After indicated times, the numbers of live cells was determined by trypan blue exclusion.

Immune Complex Kinase Assays—Cells were washed with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM SV, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 10 μg/ml leupeptin and aprotinin) as described (21). Lysates were incubated with anti-JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4 °C and then for 45 min after addition of protein A-Sepharose. The immune complexes were washed three times with lysis buffer and once with kinase buffer and reassembled in kinase buffer containing [γ−32P]ATP (6000 Ci/mmol; NEN Life Science Products) and GST-Jun (39).

Preparation of lysates and immunoblotting for PARP were performed as described using the C-2–10 anti-PARP monoclonal antibody (41). Preparations of lysates were treated with 1 mM MMS for 1 h or 40 J/m² UV for 15 min. Total cell lysates were immunoprecipitated with anti-JNK antibody, and in vitro immune complex kinase reactions were performed using GST-Jun fusion protein as substrate. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, stained with Coomassie Blue, and analyzed by autoradiography. E, different clones of U-937/Bel-xL cells were treated with 1 mM MMS for 1 h. mms-treated U-937 cells were used as control. In vitro immune complex kinase assays in anti-JNK immunoprecipitates were performed as described above.

Transient Transfections—PC12 cells were transiently transfected with vector or Flag-RAFTK (K-M) with pEBG-SAPK using LipofectAMINE (Life Technologies, Inc.). After transfection, cells were treated with MMS, and total cell lysates were subjected to incubation with GST protein adsorbed on GSH beads. The protein precipitates were analyzed by GST-Jun immune complex kinase assays as described above. Total cell lysates were also analyzed by immunoblotting with anti-GST-SAPK.

Measurement of Total PTPase Activity—U-937 or U-937/Bel-xL cells were treated with 200 μM SV and harvested after 3 h. Cells were lysed in phosphate-free 50 mM Tris-HCl, pH 7.5, buffer using a sonicator. The PTPase activity in whole cell lysate was measured by a nonradioactive photometric enzyme immunoassay kit (Boehringer Mannheim). The reactions were performed directly in the wells of a microtiter plate with GST protein adsorbed on GSH beads. The protein precipitates were analyzed by GST-Jun immune complex kinase assays as described above. Total cell lysates were also analyzed by immunoblotting with anti-GST-SAPK.

RESULTS AND DISCUSSION

To determine the involvement of Bel-xL in MMS- and UV-induced apoptosis, we assessed cleavage of the 116-kDa PARP protein to an 85-kDa fragment in response to these agents. As expected, MMS-treatment of U-937 cells resulted in PARP cleavage (Fig. 1A). Similar findings were obtained when U-937 cells were treated with UV, and there was no detectable cleav-
age of PARP when U-937/Bcl-xL cells were treated with MMS or UV (Fig. 1A). Previous studies have shown that PKCδ undergoes caspase-3-mediated proteolytic cleavage in an apoptotic pathway induced in response to diverse forms of stress (7, 44). To further determine the activation of caspase-3 by MMS or UV, cell lysates from MMS- or UV-treated U-937 and U-937/Bcl-xL cells were analyzed by immunoblotting with PKCδ. Similar to PARP cleavage, the results demonstrate that MMS or UV-treatment of U-937 cells results in PKCδ cleavage (Fig. 1B). Moreover, there was no detectable cleavage of PKCδ when U-937/Bcl-xL cells were treated with MMS or UV (Fig. 1B). We also compared the proliferation of U-937 and U-937/Bcl-xL cells in the presence of varying concentrations of MMS. At lower MMS concentrations (25–50 μM), there was little if any effect of MMS on proliferation of either cell type. At higher concentrations of MMS (1 mM), in contrast to U-937/Bcl-xL, approximately 50% of U-937 cells were dead (based on trypan blue exclusion) by 12–16 h. Moreover, more than 75% U-937 cells were dead when treated with 1 mM MMS for 24–36 h. These findings indicate that overexpression of Bcl-xL significantly blocks MMS-induced cell death. Moreover, overexpression of Bcl-xL also blocked MMS- and UV-induced internucleosomal DNA fragmentation, a hallmark of apoptosis (data not shown). Taken together, these findings indicate that overexpression of Bcl-xL is associated with inhibition of apoptosis in the response to MMS and UV. Whereas JNK activation has been associated with induction of apoptosis, we assayed anti-JNK immunoprecipitates for phosphorylation of GST-Jun. The results demonstrate increased JNK activity and no change in JNK immunoprecipitates for phosphorylation of GST-Jun. The analysis of anti-JNK immunoprecipitates from these clones also demonstrated inhibition of JNK activation by Bcl-xL (Fig. 1E). Thus, whereas both MMS- and UV-induced apoptosis is inhibited in U-937/Bcl-xL cells, only MMS-induced JNK activity is blocked by Bcl-xL. These findings suggest that Bcl-xL functions upstream to the activation of JNK by MMS, but not UV.

Previous studies have shown that Bcl-xL functions upstream in activation of caspase 3 (7, 44). Because MMS- and UV-induced PARP cleavage is inhibited in U-937/Bcl-xL cells, we asked whether this event is also sensitive to CrmA or p35. There was no detectable cleavage of PARP in MMS-treated U-937/CrmA or U-937/p35 cells (Fig. 2A). Similar results were obtained when cell lysates were analyzed for cleavage of PKCδ (data not shown). However, the finding that p35, but not CrmA, blocks UV-induced cleavage of PARP (Fig. 2B) indicated that UV and MMS activate caspasases by different mechanisms. Analysis of U-937/CrmA and U-937/p35 cells for MMS- or UV-induced JNK activity demonstrated that, in contrast to Bcl-xL, overexpression of CrmA or p35 in U-937 cells has no detectable effect on activation of JNK in response to these agents (Fig. 2C). The inhibition of JNK by MMS in U-937/Bcl-xL, but not in U-937/CrmA or U-937/p35 cells, suggested that activation of JNK by MMS is upstream to caspase activation. To further assess this issue, U-937 cells treated with MMS for various times were analyzed for activation of JNK and cleavage of procaspase-3. The results demonstrate that whereas JNK is activated by MMS at 1 h, cleavage of procaspase-3 is detected only after 4–6 h (Fig. 2D). Taken together, these findings indicate that in U-937 cells treated with MMS, Bcl-xL functions upstream of JNK activation by a mechanism either upstream from or independent of caspase inhibition. In this context, previous studies have shown that caspasases act both upstream and downstream to JNK activation depending upon cell type and inducer (45–47).

Previous studies have shown that activation of JNK by TNF, MMS, or UV is induced by c-Ab1-independent mechanisms (20, 22, 29). In this context, other studies have shown that the RAFTK tyrosine kinase plays a key role as an upstream regulator of the JNK pathway in response to UV in PC12 cells (33). RAFTK is activated by phosphorylation on tyrosine (33). Treatment of U-937 cells with MMS, but not UV, resulted in increased tyrosine phosphorylation of RAFTK (Fig. 3A). There was also no detectable tyrosine phosphorylation of RAFTK following treatment with agents, such as cis-platinum and IR, that activate c-Ab1 (data not shown). Whereas Bcl-xL blocks MMS-induced JNK activation and MMS induces tyrosine phosphorylation of RAFTK, we asked whether Bcl-xL functions upstream to RAFTK. The results demonstrate that Bcl-xL blocks MMS-induced tyrosine phosphorylation of RAFTK (Fig. 3B). By contrast, inhibition of caspases by overexpressing CrmA or p35 had no effect on RAFTK tyrosine phosphorylation (data not shown). RAFTK is also expressed in PC12 neuroblastoma cells (34, 43). To determine the role of RAFTK in MMS-induced activation of JNK in PC12 cells, total lysates from control and MMS-treated PC12 cells were subjected to immunoprecipitation with anti-RAFTK, and the protein precipitates were analyzed by immunoblotting with anti-Tyr(P). The results demonstrate that, similar to U-937 cells, treatment of PC12 cells with MMS is associated with increases in tyrosine phosphorylation of RAFTK (Fig. 4A). Furthermore, JNK activation is also increased in response to MMS in these cells (Fig. 4B). To further confirm a direct role for RAFTK in MMS-induced activation of
JNK, PC12 cells were transiently transfected with a dominant negative mutant of RAFTK (RAFTK K-M), treated with MMS, and assayed for activation of JNK. As a control, PC12 cells expressing the empty vector were also treated with MMS. The results demonstrate that treatment of PC12 cells expressing RAFTK K-M mutant, but not the empty vector, with MMS is associated with a significant inhibition of JNK activity (Fig. 4C). Other studies have shown that the protein-tyrosine kinase c-Src functions upstream to JNK in response to MMS (48). Because c-Src is activated by a RAFTK-dependent mechanism (35), these results together indicate that RAFTK acts as an upstream activator of the JNK signaling pathway in the cellular response to MMS.

Tyrosine phosphorylation of proteins has been implicated as playing critical roles in regulating cell death and survival. Previous studies have shown that treatment of certain cell types with tyrosine phosphatase inhibitor, SV, is associated with the regulation of apoptosis (49, 50). Because the results of the present study demonstrate that overexpression of Bcl-xL inhibits tyrosine phosphorylation of RAFTK, we sought to determine whether a tyrosine phosphatase is involved in this regulation. To compare the overall tyrosine phosphatase activity in U-937 and U-937/Bcl-xL cells, we measured the background tyrosine phosphorylation of proteins in these cell types and total tyrosine phosphatase activity. U-937 and U-937/Bcl-xL cells treated with SV were also assayed for total tyrosine phosphatase activity. The results demonstrate that overexpression of Bcl-xL in U-937 cells is associated with an increase (approximately 3-4-fold) in total tyrosine phosphatase activity, and as expected, treatment with SV inhibited this activity to basal levels (Fig. 5A). In concert with the increase in phosphatase activity, background tyrosine phosphorylation of proteins in U-937/Bcl-xL cells is significantly lower than that in U-937 cells (Fig. 5B). To determine the role of a tyrosine phosphatase in the regulation of RAFTK activity by MMS, cells were treated with SV with or without MMS, and anti-RAFTK immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P).

The results demonstrate that treatment with SV increases MMS-induced tyrosine phosphorylation of RAFTK in U-937 cells (Fig. 5C). Importantly, treatment of U-937/Bcl-xL cells with SV restores tyrosine phosphorylation of RAFTK in response to MMS (Fig. 5D). Taken together, these findings indicate that MMS-induced tyrosine phosphorylation and activation of RAFTK is regulated by a tyrosine phosphatase in U-937 cells. The 4-fold higher basal activity of tyrosine phosphatases in U-937/Bcl-xL cells contributes to inhibition of MMS-induced tyrosine phosphorylation of RAFTK.

To determine whether treatment of cells with SV affects MMS-induced JNK activity, U-937 and U-937/Bcl-xL cells were treated with SV with or without MMS, and anti-RAFTK immunoprecipitates were analyzed by immune complex kinase assays. The results demonstrate that treatment with SV significantly increases MMS-induced JNK activity in U-937 cells (Fig. 6A). More importantly, treatment of U-937/Bcl-xL cells with SV restored JNK activation in response to MMS (Fig. 6B). In this context, a recent study has suggested that activation of JNK by MMS and UV is mediated by distinct upstream modulators and that the MMS-response may be regulated by a tyrosine phosphatase (15). Moreover, activation of RAFTK by MMS is regulated by a tyrosine phosphatase and functions upstream to the activation of caspases. Taken together, the findings of our study indicate that MMS induces tyrosine phosphorylation of RAFTK and contributes directly to the activation of JNK.

To determine whether inhibition of phosphatase activity by SV also affects MMS-induced cell death, U-937 and U-937/Bcl-xL cells were treated with SV with or without MMS, and

[Fig. 3: Activation of RAFTK by MMS. A, U-937 cells were treated with 1 mM MMS for 30 min or 40 J/m² UV for 15 min. Total cell lysates were immunoprecipitated with anti-RAFTK antibody. The proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by immunoblotting with anti-Tyr(P). B, U-937/Bcl-xL cells were treated with 1 mM MMS for 30 min. Anti-RAFTK immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P).]

[Fig. 4: RAFTK-dependent activation of JNK by MMS. A, PC12 cells were treated with 1 mM MMS for 30 min. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-Tyr(P) antibody. B, total cell lysates from control and MMS-treated PC12 cells were subjected to immunoprecipitation with anti-JNK antibody. The protein precipitates were analyzed by in vitro immune complex kinase assays as described. C, PC12 cells were transiently transfected with vector or Flag-RAFTK K-M. The cells were also cotransfected with pEG-SAPK. Forty-eight hours after transfection, cells were treated with 1 mM MMS and harvested after 30 min. Cell lysates were incubated with GST, and the protein precipitates were analyzed by in vitro immune complex kinase assays (top panel). GST-protein precipitates were also analyzed by immunoblotting with anti-GST-SAPK (middle panel). The fold activation in JNK activity is expressed as the mean ± S.D. of three independent experiments (bottom panel).]

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cell death was assessed by trypan blue exclusion. The results demonstrate that treatment with SV increases MMS-induced cell death in U-937 cells (not shown). More importantly, SV treatment of U-937/Bcl-xL cells, which are resistant to MMS, was associated with approximately a 30% increase in cell death in response to MMS (Fig. 6C). Taken together, these findings suggest that inhibition of phosphatase activity by SV potentiates cell death in response to MMS.

Control of apoptosis and progression of cell cycle are closely linked processes, acting to preserve homeostasis and developmental morphogenesis. Proteins that regulate apoptosis, such as Bcl-2, have also been implicated in control of the cell (51–53). In this context, previous studies have shown that the hypoxia-induced genes involved in cell cycle regulation are p53, p21, and Bcl-2 (51). Moreover, it has been shown that overexpression of Bcl-2 in breast cancer cells is associated with prolongation of the cell cycle, particularly at the G1/S boundary (54). By contrast, overexpression of Bcl-xL in U-937 cells is associated with more rapid growth kinetics as compared with parent cells. Thus, Bcl-2 and Bcl-xL may function differently in control of cell cycle progression. Nevertheless, the role of Bcl-xL in regulating the cell cycle, which is different from its anti-apoptotic function, could contribute the levels and/or activation of phosphatases observed in these cells.

CrmA is a member of the serpin family that inhibits ICE by forming an active site-directed complex (10). CrmA expression blocks apoptosis induced by TNF or activation of Fas (55). Other studies have shown that p35 inhibits the proteolytic activity of Ced-3, ICE, CPP32, Ich-1, and Ich-2 but not granzyme B (11). The present studies demonstrate that in contrast to UV, MMS-induced cleavage of PARP, PKCδ, and internucleosomal DNA fragmentation is blocked by overexpression of CrmA (Fig. 2 and data not shown). These findings suggest that MMS induces apoptosis by a mechanism that is distinct, at least in part, from that induced by UV. Recent work has shown that whereas induction of apoptosis by TNF and Fas is sensitive to CrmA, DNA damage-induced apoptosis is dependent on activation of caspases that are CrmA insensitive (56). Thus, a CrmA-sensitive caspase is necessary for induction of apoptosis by only certain stress inducers that include TNF, Fas, and MMS.

The activation of a CrmA-sensitive or -insensitive caspase appears nonetheless to be independent of JNK activation, because neither CrmA nor p35 affects the induction of this kinase by diverse classes of inducers. Other studies have shown that a caspase-mediated cleavage of MEKK-1, a kinase that acts upstream to activation of JNK (57), is necessary for anoikis (46, 58). The present findings demonstrate that MMS, but not UV, induces JNK activity in U-937 cells by a RAFTK-dependent mechanism. Previous work has shown that whereas IR-, cisplatinum- and ara-C-induced activation of JNK is c-Abl-dependent, MMS and UV induce JNK by c-Abl-independent mechanisms (16, 18, 25). The present finding that MMS-induced, but not UV-induced, JNK involves activation of RAFTK provides further support for distinct signaling pathways in the responses to diverse stress causing agents. We also show that

Fig. 5. Pretreatment of cells with SV potentiates MMS-induced increases in tyrosine phosphorylation of RAFTK. A, U-937 or U-937/Bcl-xL cells were treated with 200 μM SV for 3 h. Total tyrosine phosphatase activity was measured as described in text. B, total lysates from U-937 or U-937/Bcl-xL cells were analyzed by immunoblotting with anti-Tyr(P) antibody. C, U-937 cells were treated with MMS with or without SV. Total cell lysates were subjected to immunoprecipitation with anti-RAFTK and analyzed by immunoblotting with anti-Tyr(P). D, U-937/Bcl-xL cells were treated with MMS with or without SV and anti-RAFTK immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) antibody.

Fig. 6. Pretreatment of cells with SV restores MMS-induced activation of JNK and cell death. A, U-937 cells were treated with MMS with or without SV. Total cell lysates were subjected to immunoprecipitation with anti-JNK and analyzed by in vitro immune complex kinase assays. B, U-937/Bcl-xL cells were treated with MMS with or without SV, and anti-JNK immunoprecipitates were analyzed by in vitro immune complex kinase assays. C, U-937 and U-937/Bcl-xL cells were treated with 1 mM MMS with or without 200 μM SV. The initial number of cells seeded was 1 × 10⁷/ml. After 12 h of treatment, the number of dead cells were determined by trypan blue exclusion. The results expressed as percentage of dead cells (mean ± S.D.) of three independent experiments.
Bcl-xL blocks MMS-induced tyrosine phosphorylation of RAFTK and activation of JNK. The effects of Bcl-xL on a RAFTK → JNK pathway are selective for MMS-induced signals because Bcl-xL had no effect on UV-induced JNK activity. MMS is a monofunctional alkylating agent that alkylates DNA and damages membrane proteins (14). By contrast, the UV response is initiated in the cytoplasm by a Ha-Ras-dependent mechanism (16, 59). Thus, sensitivity of JNK activation to Bcl-xL could be dependent on subcellular localization of the stress signal and whether RAFTK is involved as an upstream effector. Although Bcl-xL blocks activation of caspases, the effects of Bcl-xL on the RAFTK → JNK pathway are independent of p35 expression and thereby caspase activity. Recent studies have shown that overexpression of RAFTK is associated with induction of apoptosis in 293 cells (60). Moreover, the finding that inhibition of JNK significantly blocks ionizing radiation- or TNF-induced apoptosis has supported a direct role for JNK in apoptosis (30). The present findings demonstrate that in contrast to CrmA or p35, Bcl-xL blocks MMS-induced activation of RAFTK, JNK, and induction of apoptosis. Thus, the RAFTK → JNK pathway is upstream to caspase activation in the cascade of MMS-induced apoptosis.

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