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Differential Role of the JNK and p38 MAPK Pathway in c-Myc- and s-Myc-Mediated Apoptosis

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The s-Myc is similar to c-Myc in its ability to induce apoptosis requiring caspase activation. However, s-Myc is distinct from c-Myc in that it has activity to suppress tumor growth and does not require wild-type p53 to induce apoptosis. These facts suggest differential regulation between s-Myc and c-Myc. Here we showed that s-Myc-mediated apoptosis triggered by UV was not inhibited by the inactive form mutant JNK (APF), though c-Myc-mediated apoptosis was. Moreover, we found that JNK did not affect the transactivation activity of s-Myc, but stimulated that of c-Myc. In contrast, both Myc-mediated apoptosis and caspase-3-like protease activation were suppressed by kinase-negative MKK6 and an inactive form mutant p38(AGF). Our results indicate that s-Myc does not require the JNK signaling unlike c-Myc during UV-triggered apoptosis, but the MKK6/p38MAPK pathway might regulate common apoptotic machinery for both s-Myc and c-Myc upstream of caspase.

The Myc proteins c-Myc and s-Myc are transcription factors harboring the basic helix-loop-helix and leucine zipper motifs. Dimerization with Max is a prerequisite for the functional activities of the Myc proteins (1). Because the activity of c-Myc and s-Myc as a sequence-specific transcription factor through association with Max is needed for apoptosis accompanying caspase activation (2–4), and this apoptosis is suppressed by *bcl*-2/-xL, *ras* and IGF-1, both c-Myc and s-Myc will activate common apoptosis-executing machinery such as the caspase-9/Apaf-1/cytochrome *c*/Bcl-2 system (5). However, s-*myc* does not have cell transformation activity (6), and can induce apoptosis in the presence of 10% FBS in which wild-type p53 is not required unlike c-*myc* (7). These findings also raise the possibility that the regulatory mechanism of s-Myc is in part distinct from that of c-Myc, especially upstream of caspase activation during apoptosis. Hence, elucidating the molecular mechanism of the regulation of c-Myc and s-Myc should provide new information on the complicated mechanism of apoptosis. Unfortunately, until now, it has been unknown what upstream signal(s) triggers the Myc-dependent mechanism for apoptosis induction.

Both c-Myc and s-Myc promote apoptotic cell death induced by various stimuli including serum deprivation, Fas, UV irradiation, or antitumor agents (8–11). Extracellular stresses, including UV, often activate the JNK/p38 MAPK pathway (12, 13). Previous studies have shown that JNK and p38 MAPK are involved in apoptosis caused by NGF withdrawal in PC12 cells, antitumor agents in U937 cells, anisomycin in Jurkat cells and glutamate in rat cerebellar granule cells (14–17). Importantly, our recent findings showed that the JNK pathway is involved in c-Myc-mediated apoptosis triggered by UV (18). Consequently, we analyzed the role of the JNK- and p38 MAPK pathway in both s-Myc- and c-Myc-mediated apoptosis triggered by UV. In the present study, we demonstrated that JNK selectively regulated both the transactivation activity and apoptosis-inducing ability of c-Myc, but not s-Myc. In contrast, we also found that the MKK6/p38 MAPK pathway was involved in both c-Myc and s-Myc-mediated apoptosis upstream of caspase(s). Collectively, our results suggest that the biological activity of s-Myc is unconnected with the JNK signaling during UV triggered apoptosis, but both Myc-dependent apop-

Abbreviations used: JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated kinase; MKK, MAPK kinase; UV, ultraviolet; EGFP, enhanced green fluorescence protein; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; MCA, α-(4-methyl-coumaryl-7-amide); AMC, 7-amino-4-methyl-coumarin.

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totic signals are under the control of the MKK6/p38 MAPK pathway.

MATERIALS AND METHODS

Plasmids and antibodies. pSRα/hp38, pSRα/HA-MKK6, pSRα/HA-KN-MKK6 and His-ATF2 were provided by Professor E. Nishida (Kyoto University, Kyoto). Human p38 MAPK cDNA from pSRα/hp38 was subcloned into pcDNA3 to obtain pcDNA3p38. pcDNA3p38(AGF) was generated by PCR-based site-directed mutagenesis in which Thr-180 and Tyr-182 were replaced with Ala-180 and Phe-182. pCR3JNK1 and pCR3JNK1(APF) were provided by Dr. H. Seimiya (Cancer Chemotherapy Center, Cancer Institute, Tokyo). pc3GF, pc3GFhcmyc, pc3GFmsmyc, pcDNA3EGFP, pGal4, pGal4/c-Myc (1–262), pGal4/s-Myc (1–151), pcDNA3LacZ, and pG5E1bCAT were described before (7, 10, 18). Anti-p38 MAPK (C-20-G), anti-JNK1 (C-17), anti-HA (Y-11) and anti-phospho-JNK (G-7) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and anti-phospho-p38 (Thr180/Tyr182) antibody was from New England Biolabs, Inc. (Beverly, MA).

Apoptosis assay. Rat-1/SM cells were established as described before (19). HA-tagged kinase-negative MKK6 (KN-MKK6) expressing Rat-1/SM cells were established as follows. Rat-1/SM cells were cotransfected by pSRα/HA-KN-MKK6 plasmid with pcDNA3. Transfected cells were selected using G418 (400 μg/ml) over 2 weeks culture. Expression of HA-KN-MKK6 protein was checked in cloned cells, and two independent clones, RSKN6-1 and -2 were selected. Rat-1, Rat-1/SM, RSKN6s, HeLa, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Cytosystems, Castle Hill, Australia). For the transient transfection assay, cells (HeLa 5×10^5 cells; Rat-1/SM 10^5 cells/60-mm dish) were seeded 24-h before and cotransfected by each plasmid DNA mix (5 μg) containing EGFP with SuperFect reagent (Qiagen, Hilden, Germany). For the analysis of transient apoptotic cells expressing EGFP, cells were either irradiated or not by UVC and their morphology was examined as described (10).

Activation of p38 MAPK. Phosphorylation of p38 MAPK was examined by Western blot analysis. In brief, Rat-1 and Rat-1/SM cells were irradiated by UVC (100 J/m^2) following incubation in the same medium. Then, cells were once washed in PBS and quickly frozen at a given time. Cells were lysed in SDS buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 10 mM NaF, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM PMSF) and sonicated. Equal amounts of protein (80 μg) were resolved by SDS–PAGE and transferred to nitrocellulose membrane (Protran BA85, Schleicher & Schuell). After blocking [5% low-fat milk/TBS–0.1% Tween 20 (TBST)], anti-phospho-p38 (Thr180/Tyr182) antibody diluted in 5% BSA/TBST, and secondary anti-rabbit IgG-HRP were used. Signals were detected by ECL detection system according to the manufacturer's instructions (Amersham Inc., Amersham, UK).

Kinase assay of JNK. For the immunocomplex kinase assay, cells were lysed in WCE buffer (25 mM Hepes–KOH, pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 0.5 mM PMSF). JNK was immunoprecipitated from equal amounts of protein and immunocomplex was washed in WCE buffer (1-ml $\times$ 3 times) followed by kinase buffer (25 mM Hepes–KOH, pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 1 mM Na_3VO_4, 2 mM DTT) (1-ml $\times$ one time). Kinase reactions were performed as described using GST-c-Jun as a substrate (15).

Activities of caspases. Caspase activity was measured as follows. In brief, Rat-1/SM cells ($1–2 \times 10^6$) were irradiated by UVC and harvested at a given time. Cells were lysed in Chaps buffer (10 mM Hepes–KOH, pH 7.5, 2 mM EDTA, 0.1% Chaps, 1 mM DTT, 0.1 mM PMSF), and caspase activities were measured using the fluorogenic tetrapeptidyl-substrates, YVAD-MCA, DEVD-MCA and VEID-MCA, as described (19).

CAT assay. COS-7 cells (4×10^5) were seeded into 60 mm dishes and after 24-h, pG5E1bCAT reporter plasmid (1 μg) and internal control pcDNA3LacZ vector plasmid (0.5 μg) were cotransfected with an effector plasmid such as pGal4, pGal4/c-Myc(1–262), or pGal4/s-Myc (1–151) (2 μg), and pCR3JNK1 (0.5, 1, or 1.5 μg) by SuperFect reagents as above. For the transfection, the amounts of total plasmid DNA was adjusted to 5 μg by adding pcDNA3. Two days later, cells were harvested and a CAT assay was done as described (7, 10). Transfection efficiency was normalized by the β-galactosidase activity derived from internal control pcDNA3LacZ plasmid. The CAT activities in the cell lysates were quantified with a BAS 2000 Bio-Image analyzer.

RESULTS

Activation of JNK and p38 MAPK in both parental and myc-expressing cells. Stress stimuli such as UV irradiation activate stress-activated kinases such as JNK and p38 MAPK (12, 13). First, we compared the activation of these kinase pathways in parental Rat-1 and s-*myc*-expressing Rat-1/SM cells. The activation of JNK after UV irradiation was investigated by immunocomplex kinase assay (Fig. 1A), and the activation of p38 MAPK by Western blot analysis using anti-phospho-p38 specific antibody (Fig. 1B). These experiments showed that JNK was transiently activated in both Rat-1 and Rat-1/SM cells at 10–30 min after UV irradiation, but subsequently reactivation in Rat-1/SM cells at 120–240 min was stronger than in Rat-1 cells. In contrast, the phosphorylation of p38 MAPK was also transiently activated in both Rat-1 and Rat-1/SM cells at 20–120 min after UV irradiation, but no reactivation was observed. Because the activation of JNK in apoptosis was reported to be induced by caspase activation in some cases (20), next we examined the activation of caspases during UV-triggered apoptosis in Rat-1/SM cells. A time course experiment showed that caspase-3- and -6-like proteases were activated at 60–120 min after UV irradiation in Rat-1/SM cells accompanying apoptosis (Fig. 1C). In Rat-1 cells which did not undergo apoptosis, little caspase activation was detected (data not shown). These results indicated that the early transient activation of JNK and p38 MAPK was independent of myc-expression, and was not related with caspase or with the apoptotic phenotype of cells, while the reactivation of JNK in Rat-1/SM cells was likely associated with apoptosis-induction. However, our preliminary results indicated that caspase-inhibitors such as zVAD-fmk and zAsp-CH_2-DCB could not suppress the reactivation of JNK (data not shown). Therefore, the early transient activation of JNK and p38 MAPK in these cells seems to be independent of both *myc* and the caspase-cascade, but the reactivation of JNK may be correlated with apoptosis induction in Rat-1/SM cells.

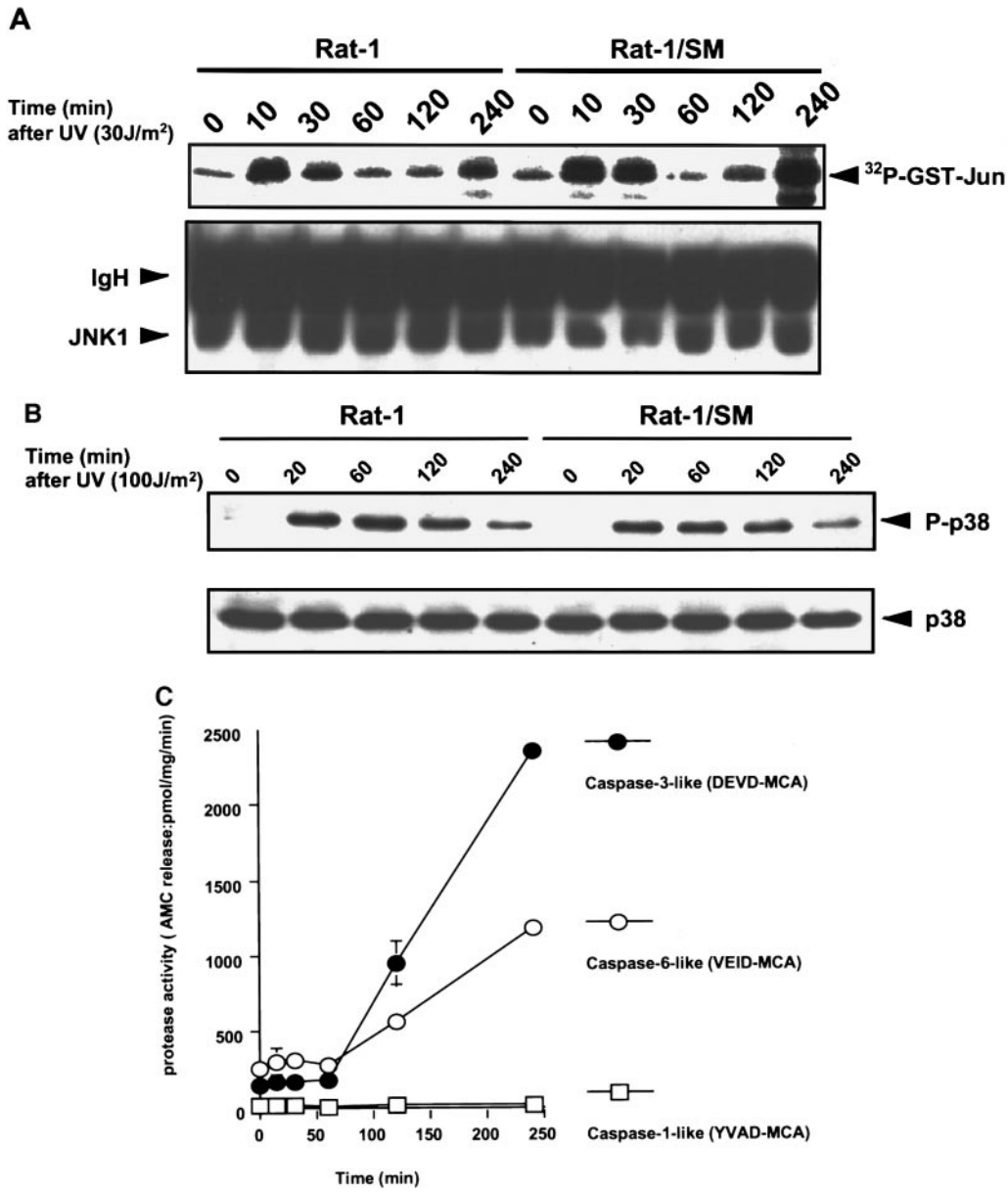

FIG. 1. Activations of the JNK/p38 MAPK pathway did not parallel caspase activation. (A) After UV irradiation (30 J/m^2), JNK1 was immunoprecipitated from equal amounts of cell lysate following immune-complex kinase assay with GST-c-Jun (1–92) as substrate. The upper figure shows the phosphorylation of GST-c-Jun and lower figure the immunoprecipitated JNK1 detected by Western blotting. (B) After UV irradiation (100 J/m^2), cell lysates were prepared at the indicated time and the extent of phosphorylation of p38 MAPK was assessed by Western blot analysis using phospho-p38 specific antibody. The upper figure shows the phosphorylated p38 MAPK and lower figure the expression level of p38 MAPK in the cells. (C) Caspase activities were examined at the indicated time after UV irradiation with tetrapeptide substrates, DEVD-MCA (caspase-3-like), VEID-MCA (caspase-6-like), and YVAD-MCA (caspase-1-like). Protease activity was demonstrated as a AMC-releasing activity. Data are the means and standard deviations from the results of two independent experiments done in duplicate.

Selective association of JNK in c-Myc-mediated apoptosis. Recently, we showed that JNK is involved in c-Myc-mediated apoptosis (18), and the results described above also suggest an association of JNK with s-Myc-mediated apoptosis triggered by UV in Rat-1/SM cells. Therefore, we studied the role of the JNK pathway in Rat-1/SM cells. Unexpectedly, we found that the expression of inactive form mutant JNK (APF) which acts in a dominant negative fashion, could not inhibit the UV triggered apoptosis in Rat-1/SM cells (Fig. 2A, left). For confirmation, we also examined the effect of JNK (APF) on s-Myc- and c-Myc-mediated apoptosis in HeLa cells. Surprisingly, in this study, we found that JNK (APF) selectively suppressed the c-Myc-mediated

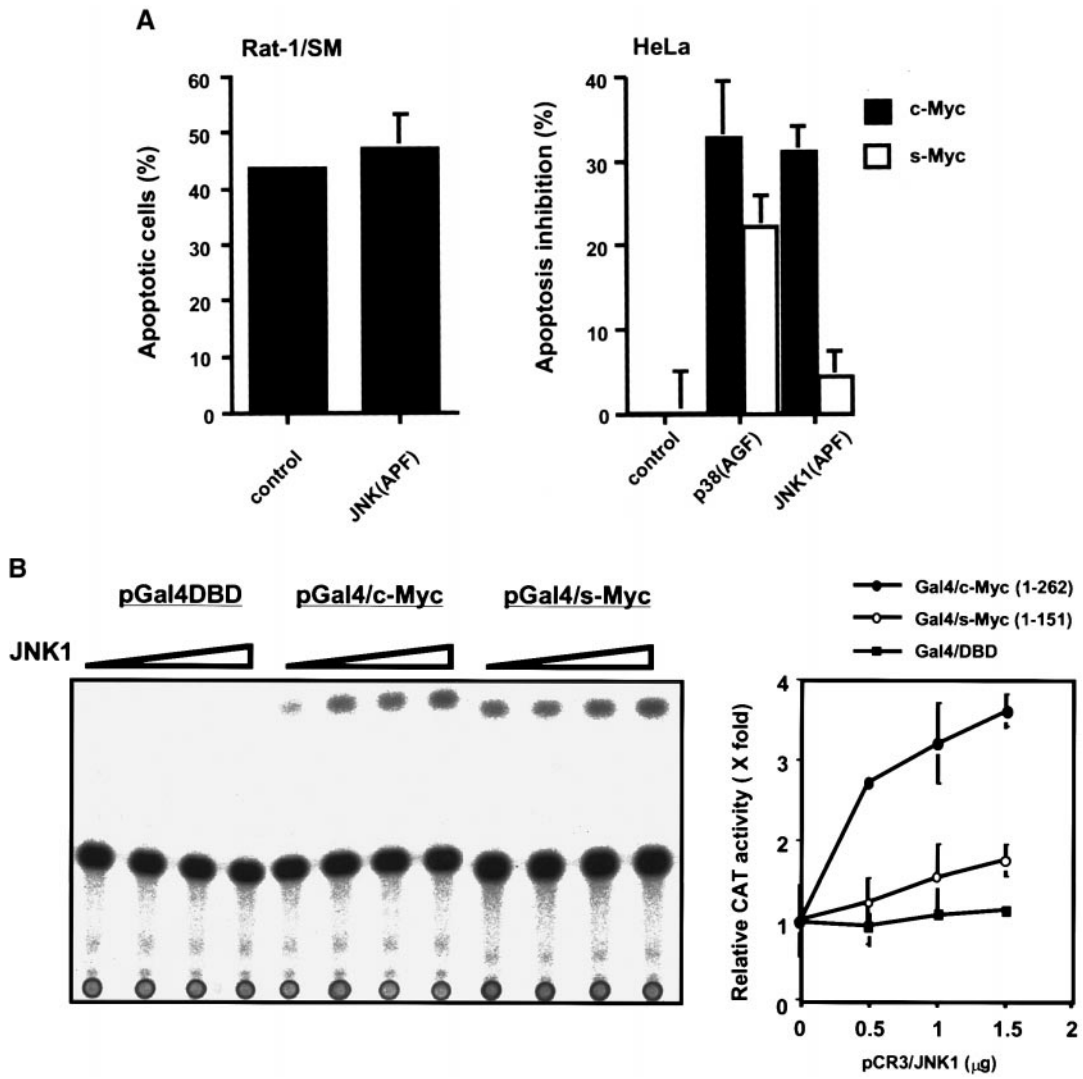

FIG. 2. Selective involvement of JNK in c-Myc-mediated apoptosis. (A) Control or dominant-negative JNK(APF)-expressing plasmids were cotransfected with pcDNA3EGFP into Rat-1/SM cells. Two days after transfection, cells were irradiated by UVC (30 J/m^2) following a 5-h incubation in the presence of 10% FBS, and % apoptotic cells calculated (left panel). JNK(APF)- and p38(AGF)-expressing plasmids were cotransfected with EGFP-carrying c-*myc* or s-*myc* expression vectors (pc3GFhcmyc and pc3GFmsmyc) into HeLa cells. Two days after transfection, cells were irradiated by UVC (100 J/m^2) following a 5-h incubation in the presence of 10% FBS, and the inhibition by JNK(APF) and p38 (AGF) were demonstrated (right panel). The apoptotic phenotype of EGFP-positive cells (more than 300 cells) was examined under a fluorescence microscope. Data are the means and standard deviations from the results of two independent experiments done in duplicate. (B) Transactivation activity of Myc was examined by CAT assay. pGal4 (1–147)-fused effector plasmid (2 μg) (pGal4DBD, pGal4/c-Myc and pGal4/s-Myc), pG5E1bCAT reporter plasmid (1 μg), and pcDNA3LacZ internal control plasmid (0.5 μg) was cotransfected with pCR3/JNK1 plasmid (0–1.5 μg) into COS7 cells. The total amount of plasmid was kept constant to 5 μg by adding pcDNA3 plasmid. The result of a representative CAT assay is shown (left panel), and the relative CAT activity normalized by the internal β-galactosidase activities is indicated (right panel). Data are the means and standard deviations from the results of two independent experiments done in duplicate.

apoptosis, but did not interfere with the s-Myc-mediated apoptosis, and in addition, p38 (AGF) suppressed both Myc-mediated apoptosis (Fig. 2A, right). Regarding the role of JNK, these results indicated that the JNK pathway is not involved in s-Myc-mediated apoptosis despite the correlated reactivation during apoptosis in Rat-1/SM cells (see Fig. 1A). Furthermore, we explored the selective association of JNK with c-Myc, and found that JNK affected the transactivation activity of c-Myc. CAT-assay showed that coexpression of JNK1 stimulated the transactivation activity of c-Myc in a dose-dependent manner, but that of s-Myc was little affected (Fig. 2B). These results indicated that JNK selectively regulates the transactivation activity of c-Myc, but not of s-Myc. Taken together, our observations strongly suggest a role for JNK as a selective regulator in the transactivation activity of c-Myc during apoptosis.

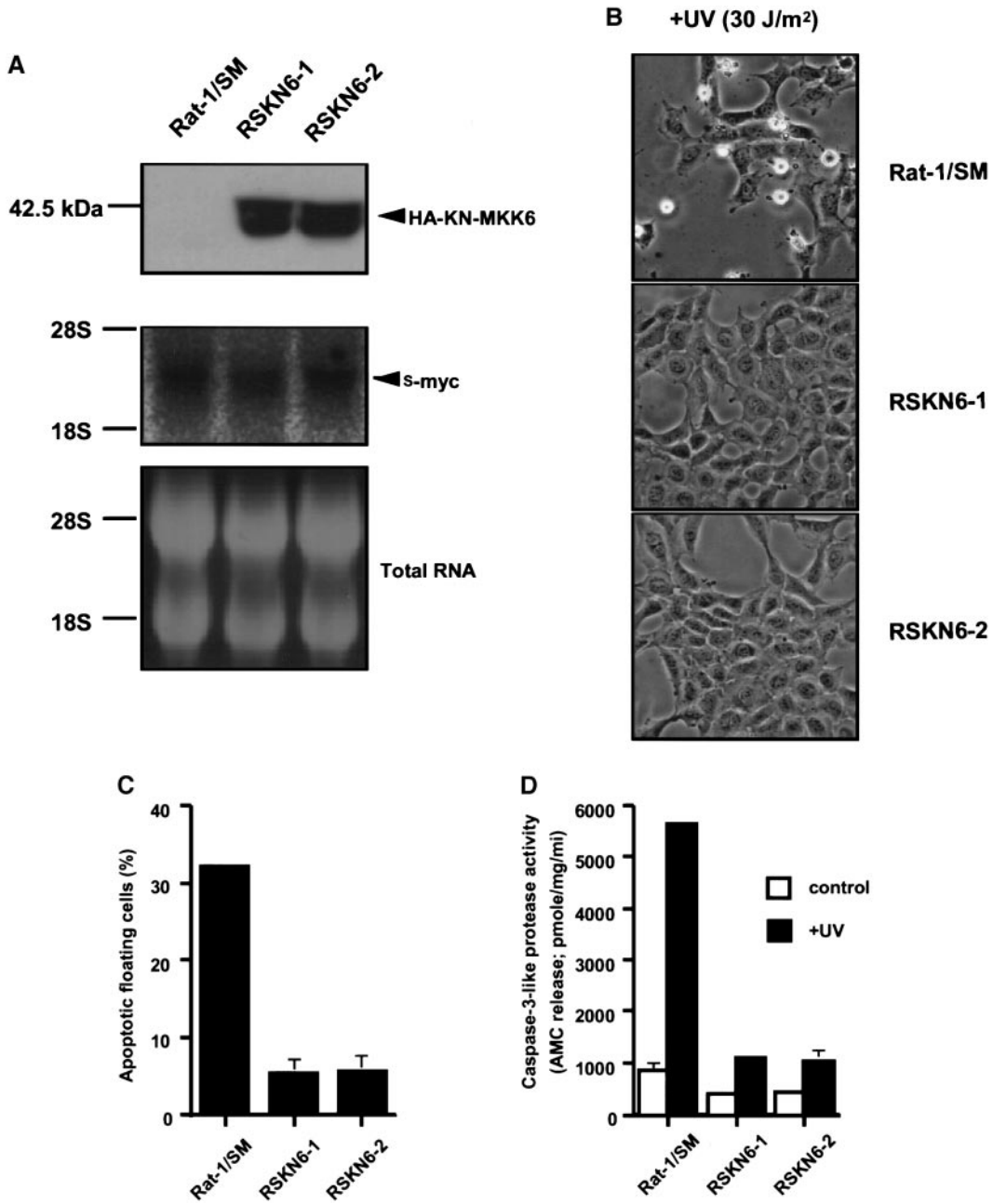

FIG. 3. Suppressive effect of kinase-negative (KN)-MKK6 on Myc-mediated apoptosis. (A) The expression of HA-KN-MKK6 in stable transfectants (RSKN6-1 and -2) was examined by Western blot analysis (upper panel). Equal amounts of proteins (30 μg) were resolved by SDS–PAGE, and the arrow indicates HA-tagged KN-MKK6 detected by anti-HA antibody. The expression of s-*myc* mRNA among the transfectants was also demonstrated by Northern blot analysis (middle panel). The lower panel shows the total amount of RNA used in northern blot analysis. (B) The cellular morphology of Rat-1/SM, RSKN6-1 and RSKN6-2 cells was photographed at 4-h ($\times$100) after UVC irradiation (30 J/m^2). (C) Apoptotic detached cells were counted 4-h after UV irradiation (30 J/m^2), and % floating cells is shown. (D) Caspase-3-like protease activation was analyzed after treatment of each cell as in C. Data are the means and standard deviations from the results of two independent experiments.

Involvement of MKK6/p38 MAPK pathway in Myc-mediated apoptosis. As shown in Fig. 2A, p38 (AGF) suppressed both c-Myc and s-Myc-mediated apoptosis triggered by UV. To clarify the role of the MKK6/p38 MAPK pathway, we established the kinase-negative (KN)-MKK6-expressing Rat-1/SM cell clones RSKN6-1 and -2. The expression of KN-MKK6 was demonstrated by Western blot analysis (Fig. 3A, top), and expression levels of s-*myc* in these cell clones were the same as in parental Rat-1/SM cells detected by northern blot analysis (Fig. 3A, bottom). We found that KN-MKK6-expressing cells were resistant to UV-induced apopto-

sis compared with Rat-1/SM cells (Fig. 3B), and this suppressive effect of KN-MKK6 was applied to apoptosis induction and caspase-activation. Consistent with apoptotic cell detachment (Fig. 3C), caspase-3-like protease activation by UV was also suppressed in the KN-MKK6-expressing cells (Fig. 3D). These observations indicated that the MKK6/p38 MAPK pathway regulated Myc-mediated apoptosis upstream of the caspase activation step triggered by UV irradiation. Additionally, we also observed that the expression of p38(AGF) could impair the UV, H_2O_2 and staurosporine-triggered apoptosis in Rat-1/SM cells in which p38 MAPK was activated (data not shown). Collectively, the MKK6/p38 MAPK pathway activated by selective toxic stimuli plays a positive role in Myc-mediated apoptosis, but is not controlled by Myc or by the caspase cascade.

DISCUSSION

The c-*myc* and s-*myc* sensitize cells to various apoptotic stress-stimuli including serum deprivation, UV irradiation and some anti-cancer drugs (21). However, the molecular mechanism for the regulation of s-Myc and c-Myc is little understood, and the role of stress signals in both c-Myc- and s-Myc-mediated apoptosis has not been elucidated. In the present study, for the first time, we examined the role of JNK- and the p38 MAPK pathways in Myc-mediated apoptosis triggered by UV, and demonstrated that JNK is a regulator for c-Myc and the MKK6/p38 MAPK pathway regulates Myc-mediated apoptosis upstream of caspase.

Here we showed that the JNK pathway was involved in the promoting the transactivation activity and the apoptosis inducing ability of c-Myc, but not s-Myc. Recently we also showed that JNK selectively phosphorylates c-Myc at Ser-62 and Ser-71, and interacts with c-Myc, but not s-Myc (18). In addition, Ser-62 and Ser-71 mutated c-Myc, which was not phosphorylated by JNK, lost its proapoptotic response to extracellular stimuli (18). These observations suggest that the phosphorylational modification by JNK would be required for the induction of proapoptotic gene(s) by c-Myc. In contrast, the JNK signaling as well as wild-type p53 is not required for the proapoptotic activity of s-Myc. Therefore, unlike c-Myc, s-Myc could induce proapoptotic gene(s) without the JNK signaling.

c-Myc acts in the cell transformation through the transcriptional regulation of target gene(s), while s-Myc showed a tumor suppressive effect following apoptosis induction (1, 22). Remarkably, recent finding showed that BIN1 protein, which suppressed the transformation by c-*myc*, is required for the proapoptotic ability of c-Myc through protein-protein interaction at N-terminal transactivation domain (23). From these observations, we hypothesize that the tumor suppressive activity of s-Myc is due to its strong apoptosis inducing ability, and that, without some another proapoptotic signal(s) and/or modulator protein(s), c-Myc could not exhibit enough proapoptotic activity to induce apoptosis, and so the cell proliferative effect of c-Myc would predominate during the cell transformation. Thus, the difference in the regulation of transcriptional activity by c-Myc and s-Myc may correspond to the distinct biological effect of c-Myc and s-Myc, and s-Myc might be unable to induce the expression of transformation associated genes.

Additionally, we demonstrated that the MKK6/p38 MAPK pathway affected the Myc-mediated apoptosis upstream of the caspase activation. c-Myc- and s-Myc-mediated apoptosis is likely mediated by similar mechanism(s) because anti-apoptotic factors such as *bcl*-2/xL, *ras*, and IGF-1 suppressed c-Myc- and s-Myc-induced apoptosis (24–26, our unpublished data). Our findings that the MKK6/p38 MAPK pathway regulates both c-Myc and s-Myc-mediated apoptosis would suggest the importance of common apoptotic machinery in Myc-mediated apoptosis. However, the target molecule(s) for the MKK6/p38 MAPK pathway has not been elucidated. Our preliminary observations indicated that p38 MAPK little interacts with c-Myc and did not phosphorylate a Myc-binding partner, Max protein (data not shown). As previously shown, the transactivation activity of both c-Myc and s-Myc through CACGTG sequence (E-box element) is essential for the induction of apoptosis (4), and Myc-mediated apoptosis will require a typical apoptotic machinery such as Apaf-1/Bcl-2,xL/caspase (27). Therefore, the MKK6/p38 MAPK pathway might regulate common downstream effector(s) controlled by both Myc and/or such apoptotic machinery.

Collectively, our study suggests that the JNK pathway is associated with the regulation of c-Myc itself during apoptosis, and that the MKK6/p38 MAPK pathway would play a role in c-Myc- and s-Myc-dependent signaling upstream of caspase-activation. Further explorations focused on the regulation of c-Myc and s-Myc are important to understand the complicated mechanisms involved in apoptosis and in transformation, and such study will offer new targets for cancer molecular therapy.

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