Activation of MST/Krs and c-Jun N-terminal Kinases by Different Signaling Pathways during Cytotrienin A-induced Apoptosis*

(Received for publication, June 2, 1999, and in revised form, December 21, 1999)

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We found that antitumor drugs such as cytotrienin A, camptothecin, taxol, and 5-fluorouracil induced the activation of a 36-kDa protein kinase (p36 myelin basic protein [MBP] kinase) during apoptosis in human promyelocytic leukemia HL-60 cells. This p36 MBP kinase, which phosphorylates MBP in an in-gel kinase assay, results from the caspase-3-mediated proteolytic cleavage of MST/Krs protein, a mammalian Ste20-like serine/threonine kinase. Herein the correlation between cytotrienin A-induced apoptosis and the activation of MST/Krs proteins was examined in human tumor cell lines, including leukemia-, lung-, epidermoid-, cervix-, stomach-, and brain-derived cell lines. In cytotrienin A-sensitive cell lines, we observed a strong activation of p36 MBP kinase by cleavage of the C-terminal regulatory domain of full-length MST/Krs proteins by caspase-3. When the kinase-inactive mutant form of MST/Krs protein was overexpressed in cytotrienin A-sensitive HL-60 cells, the cytotrienin A-induced apoptosis was partially inhibited. Because cytotrienin A also activated c-Jun N-terminal kinase, we examined the effect of the expression of dominant negative c-Jun on cytotrienin A-induced apoptosis. The expression of dominant negative c-Jun also partially inhibited cytotrienin A-induced apoptosis. Furthermore, coexpression of kinase-inactive MST/Krs protein and dominant negative c-Jun completely suppressed cytotrienin A-induced apoptosis. These findings suggest that the proteolytic activation of MST/Krs and c-Jun N-terminal kinase activation are involved in cytotrienin A-induced apoptosis in human tumor cell lines.

Apoptosis is induced by a wide variety of cellular stresses, including DNA damage, UV radiation, ionizing radiation, and oxidative stress (1, 2). It is morphologically distinct from necrosis in many of its characteristic changes as follows: DNA fragmentation, chromatin condensation, membrane blebbing, and cell shrinkage. Antitumor agents also induce apoptosis in some cancer cells both in vitro and in vivo, indicating that apoptosis plays a very important role in cancer chemotherapy (3, 4). However, the biochemical mechanism of apoptosis induction by antitumor agents is not yet fully understood.

Previous studies indicate that the apoptotic process is triggered by the activation of a caspase cascade (1, 5). Caspases are a family of cysteine proteases expressed ubiquitously in multicellular organisms as latent pro-enzyme forms. Activation of pro-caspases is an obligatory step in the execution of apoptosis, and this activation takes place by proteolytic cleavage. In the nematode Caenorhabditis elegans, three apoptosis-related genes have been cloned, ced-3, ced-4, and ced-9 (6). The former two genes are required for execution of the death program, whereas ced-9 prevents cell death. Mammalian homologues of Ced-3 and Ced-9 proteins have been identified as the interleukin-1β-converting enzyme cysteine protease (termed caspase-1) and Bcl-2, respectively (7, 8). The Ced-4 protein is homologous to the human protein Apaf-1, which participates in the activation of caspase-3 (9, 10). Caspase-3, an important effector caspase, is responsible for the cleavage of crucial substrates such as structural proteins, signaling proteins, and transcription-regulating proteins involved in the apoptotic process (1, 5).

In particular, the fact that caspases regulate the activity of several protein kinases indicates that protein phosphorylation/dephosphorylation mechanisms may play an important role in the initiation and progression of apoptosis. However, the role of individual targets in the common apoptotic signaling pathway triggered by anticancer agents is still uncertain.

Cytotrienin A (Fig. 1), a novel ansamycin antitumor agent, was isolated from Streptomyces sp. as an apoptosis inducer (11, 12). Recently, we have found that JNK1 and a 36-kDa kinase (termed p36 MBP kinase) are activated during the cytotrienin A-induced apoptosis in human promyelocytic leukemia HL-60 cells (13). This p36 MBP kinase is an active proteolytic product of the MST2/Krs1 and MST1/Krs2 protein kinases and were originally cloned by virtue of their homology to the budding yeast Ste20 kinase (13). Two other groups (14, 15) have also reported that MST1/Krs2 is cleaved by a caspase-3-like activity during Fas-induced apoptosis. Notably, Graves et al. (14) extensively examined the involvement of MST1/Krs2 together with mitogen-activated protein kinase cascade during apoptosis. It is possible that most apoptotic signals including the Fas-mediated signal and the cytotrienin-induced signal activate several pathways for apoptosis in a target cell. Unlike these apoptosis inducers, MT-21 (3-acetyl-4,5-dimethyl-5-oxo-2-pyrrolin-2-one), our synthetic apoptosis inducer, activates the signal from caspase-3, MST/Krs to JNK in a rather simple manner (16, 17). In the present study, we have investigated the caspase-mediated proteolytic activation of MST/Krs proteins in cytotrienin A-sensitive and -resistant human tumor cell lines.

* This work was supported in part by a Special Postdoctoral Researchers Program (to M. W.), by a grant for Multiobioprobes (RIKEN), and by a grant from the Ministry of Education, Science, Sports, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: JNK, c-Jun N-terminal kinase; MBP, myelin basic protein; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; MT-21, 3-acetyl-4,5-dimethyl-5-oxo-2-pyrrolin-2-one; ROS, reactive oxygen species; Z-Asp-CH₂-DCB, benzoyloxycarbonyl-Asp-CH₂O(O)₂,2,6-dichlorobenzene.

Printed in U.S.A.

This paper is available on line at http://www.jbc.org
were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5% Triton X-100, 50 mM NaCl, 0.5% Triton X-100). The final wash was performed in kinase buffer (20 mM HEPES (pH 8.0), 20 mM MgCl2, 50 mM NaCl, 0.05% Triton X-100). The kinase reaction was initiated by resuspending the pelleted beads in 30 μl of kinase buffer containing (γ-32P)ATP. SDS was removed from the gel; the protein was renatured, and a kinase assay was carried out by incubating the gel in buffer (40 mM HEPES (pH 7.5), 10 mM MgCl2, 2 mM dithiothreitol, and 0.1 mM EDTA) containing 20 μM (γ-32P)ATP. Gels were washed and dried, and the incorporated radioactivity was analyzed by autoradiography.

**Experimental Procedures**

**Materials—**Cytotrienin A was isolated from *Streptomyces* sp. RKB574, as described previously (11, 12). MBP was purchased from Sigma. Anti-Krs1 antibody, anti-Krs2 antibody, and anti-PARP antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Bcl-2 and Bax were purchased from Dako (Glostrup, Denmark) and MBL (Nagoya, Japan), respectively, and anti-caspase-3 antibody and γ-32P-ATP were from Transduction Laboratories (Lexington, KY) and ICN Biochemicals Inc. (Costa Mesa, CA), respectively. Camptothecin, etoposide, mitomycin C, doxorubicin, bleomycin, staurosporine, actinomycin D, colcemid, taxol, and 5-fluorouracil were purchased from Wako Pure Chemicals, Ltd. (Osaka, Japan). Cloning of human full-length MST1/Krs1 and MST2/Krs2 cDNA and constructions of expression vectors of kinase-inactive MST1/Krs1 and MST2/Krs2 were performed as described previously (17).

**Cell Culture—**HL-60 (human promyelocytic leukemia cells), U937 (human monoblastoid leukemia cells), K562 (human chronic myelogenous leukemia cells), Jurkat (human acute T-cell leukemia cells), and MKN74 (human stomach adenocarcinoma cells) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum. WI-38 (human normal lung fibroblast cells), A431 (human epidermoid carcinoma cells), HeLa (human cervix epithelioid carcinoma cells), and SH-SY5Y (human neuroblastoma cells) were maintained in RPMI 1640/10% fetal bovine serum and OPI medium supplemented with 20 μg/ml gentamicin. HL-60, WI-38, and HeLa cells were obtained from the RIKEN Cell Bank (Tsukuba, Japan). A549 and MKN74 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). Jurkat cells were supplied by Dr. M. Ishizuka (Institute of Chemotherapy, Shizuoka, Japan) and SH-SY5Y and SMS-KCN by Dr. H. Matsui (St. Marianna University, Tokyo, Japan) and Dr. A. Nakagawara (Chiba Cancer Res. Inst., Chiba, Japan), respectively. U937 cells transfected with dominant negative c-Jun were kindly provided by Dr. Nakaya (Showa University, Tokyo, Japan).

**Antitumor Agents Treatment—**Exponentially growing cells were exposed to various concentrations of antitumor agents. Following incubation for 24 h at 37 °C, 2 mM (2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt and 1-methoxy-5-methylphenazinium methyl sulfate were added into the culture medium and incubated for a further 2–3 h. Then the cell viability was determined by the measurement of ΔA570.

**Analysis of DNA Fragmentation—**DNA fragmentation assays were carried out as described previously (13, 17). The DNA ladders were visualized by UV illumination after ethidium bromide staining.

**Whole-cell Extracts and Western Blot Assay—**Preparation of whole-cell extracts and Western blotting analyses were performed as described previously (13). Bands were visualized by a Western blot Chemiluminescence Reagent (Pierce).

**In-gel Kinase Assay—**Cells treated/un-treated with various drugs were lysed in lysis buffer (20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 0.5% Triton X-100, 50 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 6 mM dithiothreitol, and 2% proline), and the protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad). To assay the total cell lysate, equal amounts of protein (50–100 μg) were electrophoresed in 10% SDS-polyacrylamide gels containing 0.5 mg/ml MBP as a substrate. Following electrophoresis, SDS was removed from the gel; the protein was renatured, and a kinase assay was carried out by incubating the gel in buffer (40 μM HEPES (pH 7.5), 10 mM MgCl2, 2 mM dithiothreitol, and 0.1 mM EDTA) containing 20 μM (γ-32P)ATP. Gels were washed and dried, and the incorporated radioactivity was analyzed by autoradiography.

**RESULTS**

**Antitumor Agent-induced Apoptosis and Cleavages of MST2/Krs1 and MST1/Krs2 in HL-60 Cells—**We previously reported that MST/Krs proteins consisting of MST1/Krs2 and MST2/Krs1 were activated during cytotrienin A-induced apoptosis (13). To characterize the relationship between the antitumor agent-induced apoptosis and the activation of MST/Krs proteins, we examined the effects of antitumor agents including cytotrienin A, camptothecin, etoposide, mitomycin C, doxorubicin, bleomycin, staurosporine, actinomycin D, colcemid, taxol, and 5-fluorouracil on proteolytic activation of MST/Krs proteins in HL-60 cells. As shown in Fig. 2A, a DNA ladder pattern typical of internucleosomal fragmentation, which is considered to be an early event in apoptosis, was detected upon treatment with all the antitumor agents tested here. In addition, the activation of p36 MBP kinase upon proteolysis of MST2/Krs1 and MST1/Krs2 was induced by these antitumor agents (Fig. 2B). In the case of colcemid treatment, the induction of DNA fragmentation was slightly weaker than other agents, and the activation of p36 MBP kinase also was weak. These results suggest that the proteolytic activation of Krs proteins may be closely related to the apoptotic program induced by antitumor agents, despite the differences in their modes of action.

**Effect of Cytotrienin A on Human Tumor Cell Lines—**We examined 10 human tumor cell lines in addition to one human normal cell line for cytotrienin A sensitivity as shown in Table I. Exponentially growing cultures of human tumor cell lines were exposed to various concentrations of cytotrienin A, and the effects of cytotrienin A on cell viability were evaluated by a colorimetric method. The sensitivity to cytotrienin A-induced apoptosis in T-cell leukemia, Jurkat cells, was approximately the same as the promyelocytic leukemia HL-60 cell line, which is extremely sensitive to cytotrienin A. In monoblastoid leukemia U937 and myelogenous leukemia K562 cells, cytotrienin A showed apoptosis-inducing activity with an IC50 of 50 ng/ml, whereas in stomach adenocarcinoma MKN74 cells, it showed weaker activity with an IC50 of 1000 ng/ml. In contrast, six other cell lines, including human normal lung fibroblast WI-38 cells, were found to be largely resistant to cytotrienin A treatment.

**Expression of MST2/Krs1, MST1/Krs2, Caspase-3, Bcl-2, and Bax in Human Tumor Cell Lines—**To investigate whether the expression of MST/Krs proteins is correlated with cytotrienin A sensitivity, we examined MST/Krs protein levels by
Western blotting using specific antibodies (Fig. 3A). The MST/Krs proteins were abundantly expressed in the cytotrienin A-sensitive cell lines HL-60, U937, K562, and Jurkat as shown in Table I. However, cytotrienin A-resistant cell lines such as WI-38 and A549 expressed lower levels of MST/Krs proteins. Next, we examined whether the proteolytic activation of MST/Krs proteins by cytotrienin A is correlated with cytotrienin A sensitivity. Proteolytic activation and p36 MBP kinase activity were detected by an in-gel kinase assay using MBP as a substrate. As shown in Fig. 3B, the activation of MST/Krs proteins by cytotrienin A was easily detected in the cytotrienin A-sensitive cell lines such as HL-60, U937, and Jurkat. However, no activation of MST/Krs proteins by cytotrienin A was detected in cytotrienin A-resistant cell lines such as WI-38, A549, A431, HeLa, MKN74, SH-SY5Y, and SMS-KCN. Although MKN74 cells expressed high levels of MST/Krs, p36 MBP kinase activation was not observed, and MKN74 cells showed low cytotrienin A sensitivity. The sole exception to this correlation is K562 cells. K562 cells are cytotrienin A-sensitive, but no activation of p36 MBP kinase was detected upon cytotrienin A treatment. K562 cells have the Philadelphia chromosome creating a Bcr-Abl fusion that is known to inhibit apoptosis induced by some antitumor agents (18). It is possible that Bcr-Abl acts to inhibit the proteolytic activation of MST/Krs proteins in K562 cells but does not prevent cell death. We went on to examine the protein expression level of three other apoptosis-related molecules: caspase-3, Bcl-2, and Bax. HL-60, U937, and Jurkat were prepared from each cell line, and the amounts of MST2/Krs1, MST1/Krs2, caspase-3, Bcl-2, Bax, and actin proteins were analyzed by Western blotting using specific antibodies. B, after cells were treated with 300 ng/ml cytotrienin A for 2 h, p36 MBP kinase (MBPK) activity was measured by an in-gel kinase assay using MBP as a substrate.

### Table I

| Cell line | Origin            | IC50 (ng/ml) |
|----------|-------------------|--------------|
| HL-60    | Promyelocytic leukemia | 7            |
| U937     | Monoblastoid leukemia | 50           |
| K562     | Myelogenous leukemia | 50           |
| Jurkat   | T-cell leukemia    | 9            |
| WI-38    | Normal lung fibroblast | >3000        |
| A549     | Lung carcinoma     | >3000        |
| A431     | Epidermoid carcinoma | >3000        |
| HeLa     | Cervix epithelioid carcinoma | >3000        |
| MKN74    | Stomach adenocarcinoma | 1000         |
| SH-SY5Y  | Neuroblastoma      | 2000         |
| SMS-KCN  | Neuroblastoma      | 2000         |

ROS- and Caspase-mediated Activation of MST2/Krs1 and MST1/Krs2 in Human Tumor Cell Lines—We previously reported that activation of p36 MBP kinase required caspase activation via production of reactive oxygen species (ROS) (13). To investigate the role of caspase-3 activation and ROS production in cytotrienin A-sensitive and -resistant cells, we ex-
examined the effects of benzoyloxy carbonyl-Asp-CH$_2$O(CO)-2,6-dichlorobenzene (Z-Asp-CH$_2$-DCB), a synthetic inhibitor of PARP was detected by immunoblotting with an anti-PARP substrate of caspase-3. The 28-kDa proteolytic fragment of digestion of poly(ADP-ribose) polymerase (PARP), a known effect of Z-Asp-CH$_2$-DCB or \(\text{NAC}\) in the presence of 300 ng/ml cytotrienin A. After a 2-h incubation, p36 MBP kinase activity was measured by an in-gel kinase assay using MBP as a substrate. B, the amounts of MST2/Krs1, MST1/Krs2, and PARP proteins were analyzed by Western blotting using specific antibodies.

Form 4. Caspase-mediated activation of MST/Krs proteins in human tumor cell lines. A, affect of Z-Asp-CH$_2$-DCB and N-acetylcysteine on p36 MBP kinase activation induced by cytotrienin A (Cyt A). Cells were treated with 50 \(\mu\text{M}\) Z-Asp-CH$_2$-DCB (Z-Asp) or 3 mM N-acetylcysteine (NAC) in the presence of 300 ng/ml cytotrienin A. After a 2-h incubation, p36 MBP kinase activity was measured by an in-gel kinase assay using MBP as a substrate. B, the amounts of MST2/Krs1, MST1/Krs2, and PARP proteins were analyzed by Western blotting using specific antibodies.

forms of the proteins. When these kinase-inactive MST/Krs proteins were transiently expressed in HL-60 cells, the activation of p36 MBP kinase by cytotrienin A was almost completely inhibited (data not shown). We then went on to examine the effect of kinase-inactive MST/Krs proteins on the induction of apoptosis by cytotrienin A. As shown in Fig. 5A, DNA fragmentation was partially inhibited by the transient expression of either inactive MST2/Krs1 or MST1/Krs2. The kinase-inactive MST/Krs proteins were abundantly expressed, and almost completely inhibited activation of p36 MBP kinase, but these mutant proteins were unable to completely inhibit cytotrienin A-induced DNA fragmentation. This result showed that other MST/Krs-independent pathways were capable of mediating cytotrienin A-induced cell death. We previously reported that cytotrienin A induced the activation of JNK during apoptosis in HL-60 cells, a cytotrienin A-sensitive cell line (13). To confirm whether the JNK signal was also important for cytotrienin A-induced apoptosis, we examined the effect of expressing dominant negative c-Jun on DNA fragmentation, activation of p36 MBP kinase, and JNK activity in HL-60 cells transfected with kinase-inactive MST2/Krs1 and MST1/Krs2. After a 2-h incubation in the presence of 300 ng/ml cytotrienin A, cellular DNA extracted from the cells was analyzed by agarose gel electrophoresis and stained with ethidium bromide. B, dominant negative c-Jun expressing U937 cells transfected with kinase-inactive MST2/Krs1 and MST1/Krs2. After a 2-h incubation in the presence of 300 ng/ml cytotrienin A, cellular DNA extracted from the cells was analyzed as described in A, C, after the preparation as described in B, p36 MBP kinase (MBPK) activity was measured by an in-gel kinase assay using MBP as a substrate. D, after the preparation as described in B, JNK activity was measured by in vitro kinase assay using glutathione S-transferase-c-Jun-(1–79) as a substrate.

Coexpression of the Kinase inactive Form of MST/Krs Proteins and Dominant Negative c-Jun Blocks Cytotrienin A-induced Apoptosis—To demonstrate a role for the MST/Krs proteins in cytotrienin A-induced apoptosis, we constructed expression vectors for kinase-inactive MST2/Krs1 or MST1/Krs2. Following the method of Creasy et al. (19), kinase-inactive forms of MST2/Krs1 and MST1/Krs2 were constructed by mutating Lys-56 of MST2/Krs1 or Lys-59 of MST1/Krs2 to Arg, disrupting ATP binding. We expected that kinase-negative MST/Krs proteins would compete with endogenous MST/Krs proteins for substrates and function as dominant negative proteins in cytotrienin A-induced apoptosis.
A-induced activation of p36 MBP kinase or JNK activity (Fig. 5, C and D). Furthermore, in order to examine the coexpression of kinase-inactive MST/Krs proteins along with dominant negative c-Jun on cytotrienin A-induced apoptosis, we transiently expressed kinase-inactive MST/Krs proteins in U937 cells that are stably expressing dominant negative c-Jun. As shown in Fig. 5B, DNA fragmentation induced by cytotrienin A was almost completely inhibited by the coexpression of kinase-inactive MST/Krs proteins and dominant negative c-Jun. This treatment blocked cytotrienin A-induced activation of p36 MBP kinase, whereas cytotrienin A-induced activation of JNK was not inhibited (Fig. 5, C and D). Therefore, the kinase-inactive MST/Krs proteins could not inhibit cytotrienin A-induced JNK activation. These results suggest that cytotrienin A induces apoptosis by activating the MST/Krs proteins and JNK via two different signaling pathways.

**DISCUSSION**

In our previous studies (11, 12), we identified a novel anticancer drug, named cytotrienin A, which is an ansamycin with a unique 1-aminocyclopropane-1-carboxylic acid in the molecule. Moreover, cytotrienin A induces apoptosis in human leukemia HL-60 cells via activation of p36 MBP kinase, an active proteolytic fragment of MST/Krs proteins (13). The MST/Krs proteins are Ste20-related protein kinases and are classified in the germinal center kinase subfamily (20). The germinal center kinase subfamily of p21-activated protein kinase-like kinases, which play regulatory roles in diverse cellular phenomena such as morphogenesis, stress-response, and proliferation, comprises at least 3 distinct members (germinal center kinase, HPK1, NIK, MST1/Krs2, MST2/Krs1, KHS, SOK1, Spac1, and Nrk1p) (20). Very little is known about the regulation and function of these kinases, including the MST/Krs proteins. All of the antitumor agents that were used in the present study induced the activation of the MST/Krs proteins and DNA fragmentation in HL-60 cells (Fig. 2). As MST/Krs proteins might be a general component of apoptosis induction by antitumor agents, we investigated the relationship between the expression and proteolytic activation of MST/Krs proteins in response to anticancer agents, including cytotrienin A, in various human tumor cell lines.

In HL-60 and Jurkat cells, which are highly sensitive to cytotrienin A, robust expression and proteolytic activation of MST/Krs proteins by caspase-3 were observed (Table I and Figs. 3 and 4). In contrast, cytotrienin A-resistant cell lines such as WI-38, A549, A431, HeLa, SH-SY5Y, and SMS-KCN expressed a very low amount of MST/Krs proteins, and there was no detectable proteolytic activation of MST/Krs proteins (Table I and Figs. 3 and 4). These effects of cytotrienin A are not dependent on the doubling time of each tumor cell line (data not shown). Although MKN74 cells expressed high levels of MST2/Krs1 and showed resistance to cytotrienin A treatment, there was no detectable proteolytic activation of MST/Krs by caspases (Fig. 3B). K562 cells are also cytotrienin A-sensitive, but no activation of p36 MBP kinase was induced by cytotrienin A-treatment. K562 cells possess a Philadelphia chromosome with a translocation between chromosomes 9 and 22 that generates the Bcr-Abl tyrosine kinase by the fusion of Bcr sequences upstream of the second exon of c-Abl. Bcr-Abl is known to inhibit chemotherapeutic drug-induced apoptosis (18). Considering this, it may be possible that Bcr-Abl acts as an inhibitor of the signal that activates MST/Krs proteins and that there is a different signal for cytotrienin A-induced apoptosis not affected by Bcr-Abl. These results suggest that the proteolytic activation of MST/Krs proteins rather than expression level might correlate with apoptosis. There appears to be no correlation between the expression level of crucial components of the apoptotic program such as caspase-3, Bcl-2, and Bax (1, 21, 22) and the cytotrienin A sensitivity of the tested human tumor cell lines. To date, we have only examined the expression and activation of MST/Krs proteins in a limited number of cell lines in response to a small number of antitumor agents. A more systematic analysis of the expression and the activation of MST/Krs proteins should be helpful in studies aimed at finding new antitumor agents and testing the sensitivity of antitumor agents in human tumor cells.

MST/Krs proteins consist of two family members, MST2/Krs1 and MST1/Krs2. Both proteins undergo caspase-mediated cleavage during apoptosis. MST2/Krs1 and MST/Krs2 possess consensus recognition sites for caspase-3, 319DELD322S and 323DEMD326S, respectively (13–15, 17, 19, 23–25). As shown in Fig. 4, A and B, a synthetic inhibitor of caspase-3, Z-Asp-CH2-DCB, blocked proteolytic activation of both MST2/Krs1 and MST1/Krs2 proteins in cytotrienin A-sensitive human tumor cell lines, indicating that caspase-3 activation is necessary for the activation of p36 MBP kinase. In addition, we have shown that in both HL-60 and Jurkat cells, ROS induced by treatment with cytotrienin A play an important role in the caspase-mediated activation of p36 MBP kinase (Fig. 4, A and B). These results indicate that MST/Krs proteins are responsive to oxidative stress. Apoptosis induced by MT-21, a synthetic apoptosis inducer, is also blocked by N-acetylcysteine, a radical scavenger (17), supporting our previous studies. SOK-1, another germinal center kinase family member, is directly activated via autophosphorylation triggered by ROS, but the kinase is not activated by growth factors, alkylating agents, cytokines, or environmental stresses such as heat shock and osmotic stress (26). Although MST/Krs proteins are also activated by ROS, the mechanism of activation of MST/Krs proteins is apparently different from that of SOK-1. Therefore, MST/Krs activation may be an important event in response to oxidative stress in human tumor cell lines.

To demonstrate a role for MST/Krs in cytotrienin A-induced apoptosis, we constructed expression vectors for kinase-inactive MST/Krs proteins by mutating a conserved Lys residue in an ATP binding pocket as Creasy et al. (19) reported previously. We expected that the overexpression of kinase-inactive MST/Krs proteins could compete with endogenous MST/Krs proteins and function in a dominant negative fashion. As a result, the overexpression of kinase-inactive MST/Krs proteins may inhibit cytotrienin A-induced apoptosis. Indeed, the overexpres-
sion of the kinase-inactive MST/Krs proteins partially sup-
pressed cytотriенин A-induced apoptosis (Fig. 5A). On the other
hand, the overexpression of the dominant negative c-Jun also
partially suppressed cytотriенин A-induced apoptosis (Fig. 5B).
We have previously shown that overexpression of kinase-inac-
tive MST/Krs proteins inhibited apoptosis induced by treat-
ment with MT-21 and that MST/Krs proteins also act upstream
of JNK during MT-21-induced apoptosis (17). If both MST/Krs
and JNK were located on the same signal cascade during cyto-
triенин A-induced apoptosis, the cotransfection of kinase-inac-
tive MST/Krs proteins partially sup-
pressed DNA fragmentation (Fig. 5B). However,
strong inhibition of DNA fragmentation (Fig. 5B) would also show
positive MST/Krs and dominant negative c-Jun would also show
the production of kinase-inactive MST/Krs proteins by cytotriенин A-induced apoptosis, the cotransfection of kinase-inac-
tive MST/Krs and dominant negative c-Jun was also shown to
inhibit DNA fragmentation (Fig. 5B). However, this linear model is contradicted by the following data: the
activation of MST/Krs (p36) by cytotriенин A treatment was
almost completely suppressed by the overexpression of kinase-inac-
tive MST/Krs (Fig. 5D). In addition, we previously reported that a caspase
inhibitor, Z-Asp, suppressed MST/Krs activation but not JNK
activation (13). Therefore, it is more likely that JNK activation
by cytotriенин A is independent of the caspase-MST/Krs signal
and that two different signals from MST/Krs and JNK are
required for a full induction of apoptosis by cytotriенин A. We
previously reported that radical scavengers such as N-acetyl-
cysteine and reduced glutathione suppressed cytotriенин A-in-
duced activation of JNK and MST/Krs induced by cytotriенин A (13).
Therefore, two different signaling pathways may be di-
vided downstream of ROS and upstream of the caspase cas-
cade. Considering these results, both the activation of JNK and the caspase-mediated cleavage of MST/Krs proteins appear to
be important for the induction of cell death by antitumor
agents (Fig. 6). This schematic model incorporates the previous
reports that MST/Krs proteins function upstream of JNK (13,
14). It suggests that there are several signaling pathways for
JNK activation in response to stimulants.

Acknowledgments—We are grateful to Dr. K. Nakaya for the domi-
nant negative c-Jun expressing U937 cells and to Eric C. Griffith for
critical reading of the manuscript.

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J. Biol. Chem. 2000 275:8766-8771.
doi: 10.1074/jbc.275.12.8766

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