

學位申請論文

李慶權

Studies on Protein Kinase That Is Activated by Caspase during Apoptosis

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1998

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Contents

1. Abstract.....	1
2. Introduction.....	2
3. Materials and methods.....	6
3.1. Purification of the 34 kDa protein kinase	6
3.2. Peptide sequencing of the protein kinase.....	7
3.3. Plasmid constructs	7
3.4. Cell culture and transient transfection.....	8
3.5. Panning of transfected cells.....	9
3.6. Annexin V-FITC staining	9
3.7. Analysis of DNA fragmentation by agarose gel electrophoresis.....	9
3.8. MTT and MTS assay	9
3.9. Western blot analysis.....	10
3.10. MST kinase assay	10
3.11. JNK1 kinase assay	11
3.12. <i>In vitro</i> caspase-3 assay.....	11
3.13. Preparation of monoclonal anti-MST antibody	12
4. Results.....	13
4.1. Identification of protein kinase activity induced during the Fas-mediated apoptosis.....	13
4.2. Purification of the 34 kDa protein kinase	19
4.3. Proteolytic activation of MST/Krs by caspase during apoptosis	25
4.4. Analysis of MST expression.....	33
4.5. Proteolytic cleavage of endogenous MST by Fas-stimulation	33
4.6. Proteolytic activation of MST by staurosporine.....	36
4.7. Effect of MST in Fas-mediated apoptosis.....	39
4.8. Effect of PAK2 in Fas-mediated apoptosis.....	42
5. Discussion.....	48
5.1. MST is physiological substrate of caspase	48
5.2. Activation mechanism of MST	49
5.3. The role of MST and PAK2 in apoptosis	51
6. References	54
7. Acknowledgment.....	58

1. Abstract

The Fas system has been extensively investigated as a model of apoptosis and the caspase cascade has been shown to be a characteristic mechanism of signaling of apoptosis. To investigate the involvement of kinase/phosphatase in Fas-mediated apoptosis, I undertook studies to identify protein kinases activated during apoptosis. A kinase activity of 34 kDa was activated after the stimulation of Fas on human thymoma-derived HPB-ALL cells. Partial amino acid sequencing of the purified kinase revealed it to be MST/Krs, a member of the protein kinases of yeast STE20 family. Overexpressed MST/Krs was activated by proteolytic cleavage and proteolytic activation was blocked by the caspase inhibitor, Z-VAD-FK or Ac-DEVD-CHO. A mutant MST with Asp → Asn replacement at a putative caspase cleavage site was resistant to either the proteolytic cleavage or the activation of the kinase. Endogenous MST was also cleaved to 34 kDa fragment by Fas-ligation and proteolytic cleavage of endogenous MST was blocked with Ac-DEVD-CHO. Further investigation revealed that proteolytic activation of MST occurred at least by two-step procedures, resulting in kinase-active 34 kDa fragment followed by generation of 40 kDa fragment from 60 kDa intact MST by the stimulation with staurosporine or agonistic anti-Fas antibody. These findings suggest that MST is physiological substrate of caspase and proteolytic cleavage is one activation mechanism of MST.

Overexpression of various forms of MST or PAK2, another STE20-related kinase reported to be activated by caspase, had no detectable effects on the Fas-mediated apoptosis in Jurkat cells, suggesting overexpression of MST or PAK2 is not sufficient to induce apoptotic changes. So the role of MST and PAK2 on apoptosis including other member of STE20 family should be further investigated.

2. Introduction

Apoptosis is a form of cell death resulting from the activation of a genetically determined cell suicide program (reviewed by Horvitz *et al.*, 1994; Jacobson *et al.*, 1997). Cells undergoing apoptosis show characteristic morphological features such as condensation of cytoplasmic and nuclear contents, blebbing of plasma membranes, fragmentation of nuclei, and ultimate breakdown into membrane-bound apoptotic bodies that are rapidly phagocytosed (Kerr *et al.*, 1972).

Genetic analysis of the nematode *Caenorhabditis elegans* has identified three genes that control the general apoptotic program. Two genes, *ced-3* and *ced-4*, are required for the apoptotic program (Yuan and Horvitz, 1990). *Ced-9*, which functions upstream of *ced-3* and *ced-4*, negatively regulates the apoptotic program by preventing activation of *ced-3* and *ced-4* (Hengartner *et al.*, 1992). Recent biochemical studies support these concepts, as CED-3 and CED-4 can physically interact (Chinnaiyan *et al.*, 1997; Irmeler *et al.*, 1997), most likely by virtue of their N-terminal domains, which both contain a motif designated a caspase recruitment domain (CARD) (Hofman *et al.*, 1997). Furthermore, CED-9 and CED-4 have been found to directly interact when expressed in yeast and in mammalian cells (Chinnaiyan *et al.*, 1997; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997).

The apoptotic program delineated in *C. elegans* is conserved in mammalian cells, which contain homologs of *ced-9* and *ced-3*. One of these homologs, *bcl-2*, can partially substitute for *ced-9* in preventing apoptosis in *C. elegans* (Hengartner and Horvitz, 1994). The other homologs, including caspase-3, encode cysteine proteases that are closely related to CED-3 (Yuan *et al.*, 1993; Fernandes-Alnemri, 1994; Xue *et al.*, 1996; Alnemri *et al.*, 1996). In mammalian cells, caspase-3 normally exists as a 32 kDa inactive precursor that is converted proteolytically to a

20 kDa and 10 kDa active heterodimer when cells are signaled to die (Nicholson *et al.*, 1995; Schlegel *et al.*, 1996; Wang *et al.*, 1996). Bcl-2, located on the outer membrane of mitochondria, prevents the activation of caspase-3 (Bloukakis *et al.*, 1996; Chiannaiyan *et al.*, 1996; Armstrong *et al.*, 1996; Erhardt and Cooper, 1996; Ibrado *et al.*, 1996; Monney *et al.*, 1996).

A mammalian homolog of CED-4, Apaf-1, was also found recently (Zou *et al.*, 1997). It has an amino-terminal CARD domain and a 320 amino acid central portion with 22% identity and 48% similarity to CED-4. The C-terminal half of Apaf-1 has twelve WD40 motifs that are believed to mediate protein-protein interaction. Using purified components, Zou *et al.* have shown that cytochrome c, dATP, the ced-4 homolog Apaf-1, and Apaf-3 are sufficient to activate pro-caspase-3, demonstrating that, in addition to ced-3 and ced-9, the ced-4 components of the apoptotic mechanism is highly conserved.

Fas antigen (Fas/CD95/Apo-1) (Yonehara *et al.*, 1989) is a type I-membrane protein belonging to the TNF receptor family (Itoh *et al.*, 1991), which includes TNF receptor 1 and 2 (TNFR1 and TNFR2), low affinity nerve growth factor receptor, CD27, CD30, CD40 and OX40 (Nagata and Golstein, 1995). Fas ligand (FasL) also belongs to the TNF family and is synthesized as a type-II membrane protein (Suda *et al.*, 1993). Ligation of Fas with FasL or with agonistic anti-Fas monoclonal antibody induce apoptotic cell death in various cells (Suda *et al.*, 1993; Yonehara *et al.*, 1989; Trauth *et al.*, 1989). Fas-mediated apoptosis plays pivotal roles in T and B cell homeostasis (Nagata and Golstein, 1995; Abbas, 1996; Nagata, 1997); activation-induced death of T cell, maintenance of immune privilege, and deletion of activated or autoreactive B cell. The intracellular domain of Fas contains the death domain required for induction of apoptosis (Nagata and Golstein, 1995).

The death domain consists of approximately 70 amino acids and is conserved among several proteins including TNFR, FADD/MORT1, and TRADD (Yuan, 1997). The downstream signals of Fas/FasL have been studied extensively. Triggering of Fas recruits FADD/MORT1 to Fas via interaction between the death domains of Fas and FADD (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995). Pro-caspase-8/MACH/FLICE is also recruited to Fas through interaction between death effector domains of FADD and pro-caspase-8, after which several caspases are activated (Boldin *et al.*, 1996; Muzio *et al.*, 1996).

Activation of the caspase cascade is an unique biochemical mechanism of apoptosis (Nagata, 1997). Caspases cause apoptosis when overexpressed in cells. Caspase inhibitors such as Z-VAD-FK and Ac-DEVD-CHO can block apoptosis, suggesting that the caspase cascade is an essential mechanism of apoptosis. Known molecular targets of caspases are growing in number; DFF (a heterodimeric protein, triggers DNA fragmentation in cell-free systems after proteolytic activation by caspase-3) (Liu *et al.*, 1997), Lamin (Takahashi *et al.*, 1996), poly(ADP-ribose) polymerase (PARP), sterol-regulatory element-binding protein (SREBP) (Wang *et al.*, 1996), 70 kDa peptide of U1 snRNP (Casciola *et al.*, 1994), protein kinase C δ (PKC δ) (Emoto *et al.*, 1995), and DNA-dependent protein kinase (DNA-PK) (Casciola *et al.*, 1995) are also cleaved by caspases. It is not known, however, whether the cleavage of these substrates plays a role in apoptosis.

Protein kinases and (or) phosphatases have been suggested to play a role in apoptosis although the caspase cascade is essential to apoptosis. FAP-1 (Sato *et al.*, 1995), a Fas-associated phosphatase, negatively regulates Fas-mediated apoptosis in some cells. PKC δ is proteolytically activated by various apoptotic stimuli. Staurosporine, a serine/threonine kinase inhibitor, induces apoptosis (Jacobsen *et al.*, 1996; Mehlen *et al.*,

1996). Butyrolactone I, a CDK-specific inhibitor, inhibits Fas-mediated apoptosis (Furukawa *et al.*, 1996). SAPK/JNK or p38 kinase activity increases during apoptosis (Cahill *et al.*, 1996) and activation of SAPK is reportedly necessary for the neuronal apoptosis (Xia *et al.*, 1995). A specific 34 kDa kinase becomes activated by various apoptotic stimuli (Cahill *et al.*, 1996; Lu *et al.*, 1996). Recently, PAK2 was reported to be activated by caspase-dependent cleavage and suggested to be involved in apoptosis (Rudel and Bokoch, 1997). p21-activated kinases (PAKs) are a serine threonine kinase whose activity is regulated by the small guanosine triphosphatases (GTPases) Rac and Cdc42 (Manser *et al.*, 1994 and Knaus *et al.*, 1995). PAKs regulate morphological and cytoskeletal changes in a variety of cell types (Sells *et al.*, 1997 and Brzeska *et al.*, 1997), implicating PAKs as downstream mediators of the effects of Rac and Cdc42 on the actin cytoskeleton. However, the biochemical role of kinase/phosphatase in apoptosis is still unknown.

To investigate the involvement of kinase/phosphatase in Fas-mediated apoptosis, I undertook studies to identify the kinase activated during apoptosis. A kinase activity of 34 kDa was identified by in-gel phosphorylation assay using histone as substrate. This protein kinase was purified and sequenced. The peptide sequencing result showed that 34 kDa kinase is identical to catalytic domain of MST1/Krs2 and MST2/Krs1, STE20 family kinases. MST1/MST2 was proteolytically activated by apoptotic stimuli and activation is blocked by caspase inhibitor, suggesting proteolytic activation of MST plays a role in generating apoptosis-inducing signals downstream of caspases.

3. Materials and methods

3.1. Purification of the 34 kDa protein kinase

Human thymoma-derived cell line, HPB-ALL was cultured to 5×10^5 cell/ml in 40 liter of RPMI 1640 (Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS), 20 mM HEPES (pH 7.3), 50 μ M 2-mercaptoethanol, 50 unit/ml penicillin, and 50 μ g/ml streptomycin. Cells were concentrated to a density of 2.5×10^7 cells/ml and stimulated with 1 μ g/ml anti-Fas mAb (CH-11) (Yonehara *et al.*, 1989), for 2 hr. After washing once with cold phosphate-buffered saline (PBS), cells were harvested and frozen in liquid nitrogen. Cell pellets were thawed on ice and suspended with 200 ml of 20 mM Tris-HCl (pH 7.5) containing 10% glycerol, 50 mM NaF, 10 mM β -glycerol phosphate, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μ g/ml aprotinin (buffer IA) and cells were homogenized in Dounce homogenizer with 10 strokes. After centrifugation for 1 hr at $100,000 \times g$ at 4°C, the supernatant was loaded onto SP Sepharose FF (40 ml). After adding NaCl to 0.15 M, 1/3 of the flow-through fraction was loaded onto HiPrep 16/10 Q Sepharose FF. After washing with buffer QA (buffer IA + 0.15 M NaCl), the column was eluted with a 200-ml linear gradient of 0.15 M to 0.5 M NaCl. The column was run three times and eluted fractions were assayed with in-gel kinase assay using histone (Sigma) as substrate as described previously (Kameshita and Fujisawa, 1989). The protein kinase fractions were pooled and 1/8 the volume of saturated ammonium sulfate in 20 mM Tris-HCl (pH 7.5) containing 50 mM NaF, 10 mM β -glycerol phosphate, 2 mM EDTA, 0.1 mM DTT, 0.1 mM vanadate, 0.1 mM PMSF and 5 μ g/ml aprotinin (buffer BA) was slowly added with gentle stirring and further incubated for 30 min on ice. After centrifugation for 30 min at $10,000 \times g$ at 4°C, 1/2 the volume of the supernatant was loaded onto

butyl Sepharose FF (7 ml). The column was eluted with buffer BA containing 50% ethylene glycol. The column was run twice and the kinase-active fractions were dialyzed against buffer QA. Then, the active fractions were loaded onto a Mono Q column and eluted with a 20 ml gradient of 0.15 M to 0.5 M NaCl. Activity fractions were concentrated to 2.5 ml by vacuum evaporation and resolved on Hiload 16/60 Superdex 75. Aliquots of the kinase fraction were subjected to two-dimensional electrophoresis and analyzed by silver staining and in-gel kinase assay.

3.2. Peptide sequencing of the protein kinase

Following the final Superdex 75 column chromatography, the kinase-active fractions were pooled and concentrated by vacuum evaporation. After trichloroacetic acid precipitation and washing with ice-cold acetone, precipitate was dissolved in 8 M urea, 65 mM DTT, 2% pharmalyte 3-10 (Pharmacia), 0.5% Triton X-100 and 0.1% SDS, and resolved by two-dimensional electrophoresis using Multiphor II system (Pharmacia) and Investigator (Millipore). Protein spots that comigrated with protein kinase activity in the in-gel kinase assay were recovered and partially digested with V8 protease (Boehringer Mannheim). Peptides were resolved on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (ABI). Resulting peptides were subjected to amino acid sequence analysis.

3.3. Plasmid constructs

MST cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) from human HPB-ALL total RNA by using LA-PCR (Takara, Tokyo). For cloning of MST2 primers, 5'-GTAGGATCCATGGAGCAGCCGCCGGCGCCT-3' and 5'-GTAGAATTCGGGAATTTACCTGGGCATGTACCATTGTCA-3' were

used. Primers, 5'-GTAGGATTCATGGAGACGGTACAG-3' and 5'-GTAGAATTCTGGCTAACAAACATGAGGC-3' were used for cloning of MST1. PCR products were double-digested with BamHI/EcoRI and subcloned into pBluescriptII-SK and confirmed by sequencing. FLAG-tagged full-length MST and C-terminal deletion spanning amino acid 1-299 (MST1) and amino acid 1-300 (MST2) were generated by PCR. The coding region was inserted into mammalian expression vector pME18S (Sakamaki *et al.*, 1992) as FLAG-tagged forms.

PAK2 cDNA was obtained by PCR from HPB-ALL cDNA library using sense primer, 5'-GGCCTCGAGATGTCTGATAACGGAGAACTG-3' and antisense primer, 5'-

GAAGTGCAGTTAACGGTTACTCTTCATTGC-3' Sequence was confirmed by sequencing. PCR product was double-digested with XhoI and PstI and subcloned into pME18S for mammalian cell expression. Point mutants of MST and PAK2 were obtained by Quick-Change site-directed mutagenesis (Stratagene) and confirmed by sequencing.

3.4. Cell culture and transient transfection

Jurkat and HPB-ALL cells were cultured in RPMI 1640 with 10% fetal calf serum (FCS), 20 mM HEPES (pH 7.3), 50 μ M 2-mercaptoethanol, 50 unit/ml penicillin and 50 μ g/ml streptomycin. KB cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, and 100 μ g/ml kanamycin. Jurkat (1×10^7 cells) cells, grown to 2×10^5 cells/ml and KB cells, grown in 10-cm dishes to 70% confluency were transfected with expression vectors encoding various forms of MST1 and MST2 by electroporation at 300 volt with a capacitance of 960 μ F using Gene Pulser (Bio-Rad). Cells were incubated for 48 hr at 37°C and then treated with various stimulants.

3.5. Panning of transfected cells

Jurkat cells were co-transfected with vectors encoding MSTs and a truncated form of IL3 receptor (tAic2A) (Itoh *et al.*, 1990). After 48 hr, transfected cells were collected, washed twice with PBS(-), suspended in RPMI 1640 containing 5 mM EGTA and applied on the non-coated culture dish that was pre-coated with 10 µg/ml monoclonal anti-Aic2C antibody (HC) (Yonehara *et al.*, 1990). Cells were incubated 37°C for 1hr and washed briefly with RPMI 1640 twice. Bound cells were recovered by pipetting and used for cell death analysis.

3.6. Annexin V-FITC staining

Annexin V-staining of cells was done according to the manufacture's instructions (Kamiya Biomedical Co.). Briefly, the cDNA-transfected and panned Jurkat cells (2×10^5 cells) as described above, were stimulated with CH-11 to induce apoptosis. Cells were collected, washed with PBS and resuspended in binding buffer. Cells were stained with 10 µl of annexin-FITC for 10 min and analyzed by flow cytometer.

3.7. Analysis of DNA fragmentation by agarose gel electrophoresis

Cells (1×10^6) were lysed in 100 µl of 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. Supernatant was mixed with 40 µg of RNase A and incubated at 37°C for 1 hr. After incubation with 40 µg of proteinase K at 37°C for 1 hr, the digested sample was precipitated with 20 µl of 5 M NaCl and 120 µl of isopropanol. The DNA was dissolved in 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and separated in 1.5% agarose gels.

3.8. MTT and MTS assay

Ten microliter of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (5 mg/ml in PBS) was added to $1-2 \times 10^5$ cells in 100 μ l medium. After 4 hr 37°C, the reaction was stopped by adding 100 μ l of 20% SDS/50% DMF (dimethyl fluoride). The absorbance (OD 590 nm) was measured using microplate reader. For MTS assay, cells were treated with 20 μ l of MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium, inner salt) solution and incubated cells for 1-4 hr at 37°C. Absorbance (OD 490 nm) was measured with microplate reader.

3.9. Western blot analysis

Cellular total proteins were (30 μ g) separated by SDS-PAGE and transferred to PVDF membrane (Millipore). The membranes were blocked in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 (TBST) with 5% skim milk at room temperature for 1 hr. Antibodies were applied in TBST containing 5% skim milk at appropriate dilutions for 1 hr.

The membranes were then washed with TBST and incubated for 1 hr with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) or rabbit anti-goat IgG (Organon Teknika Co.). The specific signals were detected on X-ray films using an enhanced chemiluminescence (ECL) detection system (Amersham).

3.10. MST kinase assay

Various forms of FLAG-tagged MST were immunoprecipitated from total cell lysate of transfected cells with 2 μ g of anti-FLAG M2 antibody (Kodak) and protein G-Sepharose (Pharmacia) for 2 hr at 4°C, and then washed extensively. For immunoprecipitation of endogenous MST, 2 μ g of monoclonal anti-MST antibody was used. Same amounts of

immunoprecipitates as analyzed by Western blot were subjected to in-gel kinase assay using 0.5 mg/ml of histone (Sigma), MBP (Sigma) or glutathione S-transferase (GST)-cJun (1-79) as substrates. For immune complex kinase assay, immunoprecipitates were incubated with 5 µg of substrate in 20 µl of kinase reaction buffer [40 mM HEPES (pH 7.5) with 20 mM MgCl₂, 20 mM β-glycerol phosphate and 0.1 mM vanadate] containing 25 µM ATP and 2.5 µCi of [γ -³²P] ATP for 20 min at 30°C. Reactions were terminated by adding 7 µl of 4 × Laemmli's sample buffer and boiling for 5 min. A portion of the sample (15 µl) was separated on a 12% SDS-polyacrylamide gel and analyzed with phosphoimage analyzer (Fuji, Japan).

3.11. JNK1 kinase assay

Fusion protein of GST and N-terminal region of c-Jun (spanning amino acid 1-79) was expressed in *E.coli* and purified with glutathione-Sepharose 4B (Pharmacia). Total cell lysate was incubated with protein G-Sepharose (Pharmacia) and 1 µg of goat anti-JNK1 (Santa Cruz). Immune complex kinase assay was performed as described above using 5 µg of GST-c-Jun (amino acid 1-79) as a specific substrate.

3.12. *In vitro* caspase-3 assay

cDNA of caspase-3/CPP32 that is deleted N-terminal 28 amino acid coding sequence was cloned into pQE30 vector, expressed as His-tagged form in *E. coli* and purified using His-Trap column (Pharmacia). Mutant form of MST1 and MST2 with the replacement of Lys→ Arg at the ATP binding region of kinase domain, was subcloned into pQE30 vector and purified as above. Recombinant MST1 and MST2 were treated with recombinant caspase-3 and incubated 37°C for 30 min and resolved

in 10% SDS-polyacrylamide gel. Proteolytic cleavage of MST was analyzed by western blotting using monoclonal anti-MST antibody.

3.13. Preparation of monoclonal anti-MST antibody

Mice were immunized with bacterially expressed His-tagged MST1 and MST2. His-tagged MST1/2 was purified with His-Trap column and further purified with Mono Q column. Fraction of high purity was dialyzed against PBS and used for immunization. Mice were immunized 3 times with 4 day interval by foot-pad injection. Lymphocytes from immunized mice were collected and fused with NS-1 myeloma cells and grown in ASF104 medium (Ajinomoto, Japan) supplemented with 10% FCS and HAT (hypoxanthine/aminopterin/thymidine) for the selection of fused cells. Two cloning procedures by serial dilution were carried out after the selection and finally all of the wells containing single clones were positive for anti-MST antibody production. Cloned hybridoma cells were grown in ASF104 medium with 10% FCS. Culture supernatant of the hybridoma cells without FCS was loaded onto protein G-sepharose column and eluted with 50 mM glycine-HCl (pH 2.3). Eluate was immediately neutralized with 1 M Tris-HCl (pH 9.0) and dialyzed against 20 mM Tris-HCl (pH 7.5).

4. Results

4.1. Identification of protein kinase activity induced during the Fas-mediated apoptosis

To investigate the involvement of protein kinase/phosphatase in Fas-mediated apoptosis, the change of kinase activity was analyzed using in-gel kinase assay, an useful method to detect changes of serine/threonine kinases. Human HPB-ALL thymoma cells that undergo apoptosis within 3 hr by Fas-ligation (Fig. 1A), were treated with 1 μ g of agonistic anti-Fas mAb (CH-11) and incubated for varying intervals at 37°C. Then, the changes of kinase activity were examined using various protein substrates. A histone kinase activity of 34 kDa was detected after 1 hr incubation with CH-11 which maximized after 2-3 hr (Fig. 1B). This kinase activity was also detected 90 min after Fas-ligation when myelin basic protein (MBP) was used as substrate (Fig. 2, lower panel). 34 kDa kinase activity was not detected when casein, GST-cJun, or MAP2 was used as substrate (Fig. 2, middle panel). No apparent 34 kDa kinase activity was detected when no substrate protein was co-polymerized in gel, indicating this kinase has no autophosphorylation activity (Fig. 2, upper panel). The kinetics of activation of the 34 kDa kinase correlated well with DNA ladder formation and the onset of morphological apoptosis (Fig. 1). Longer exposure to CH-11 showed decreased kinase activity, suggesting the loss of cell components by apoptosis. Kinase activity of 34 kDa was induced with CH-11 dose-dependently (Fig. 1C). These results indicate that activation of 34 kDa kinase occurs Fas-dependently with the formation of DNA ladder. To examine whether other histone kinases are activated by Fas-ligation, cytoplasmic extracts of Fas-stimulated and unstimulated HPB-ALL cells were analyzed by Q-Sepharose chromatography (Fig. 3). The 34 kDa histone kinase activity was eluted at 0.3 M NaCl from the CH-11-stimulated (Fig. 3, upper panel) but not

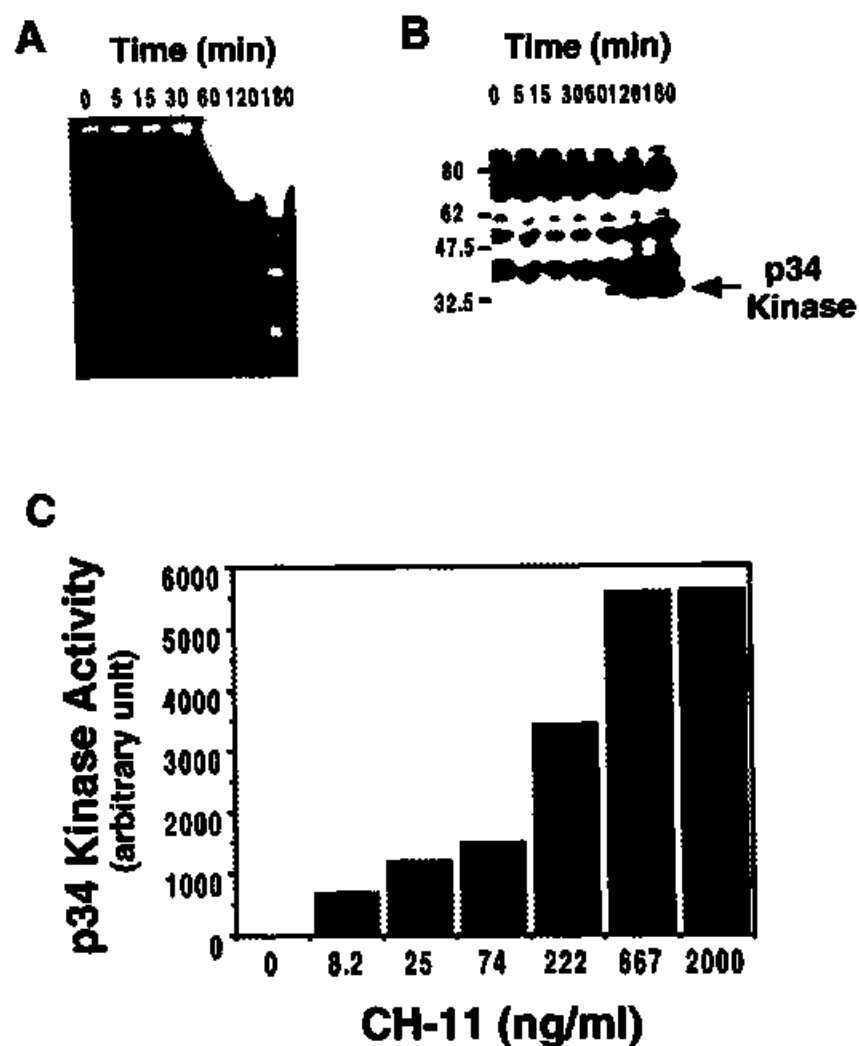


Fig. 1. Activation of 34 kDa protein kinase in Fas-induced apoptosis.

HPB-ALL cells were treated with 1 μ g/ml anti-Fas mAb (CH-11) and harvested at the indicated times. Cell lysate, prepared as described in "Materials and methods", was resolved on 1.5% agarose and DNA fragmentation was monitored by staining with ethidium bromide (A), or resolved on 12% polyacrylamide gel containing 0.2 mg/ml histone as substrate and analyzed by autoradiography (B). (C), HPB-ALL cells were treated with indicated amounts of CH-11 and harvested at 2 hr. Total cell lysate was subjected to in-gel kinase assay using histone as substrate and the kinase activity with 34 kDa was measured as described in "Materials and methods".

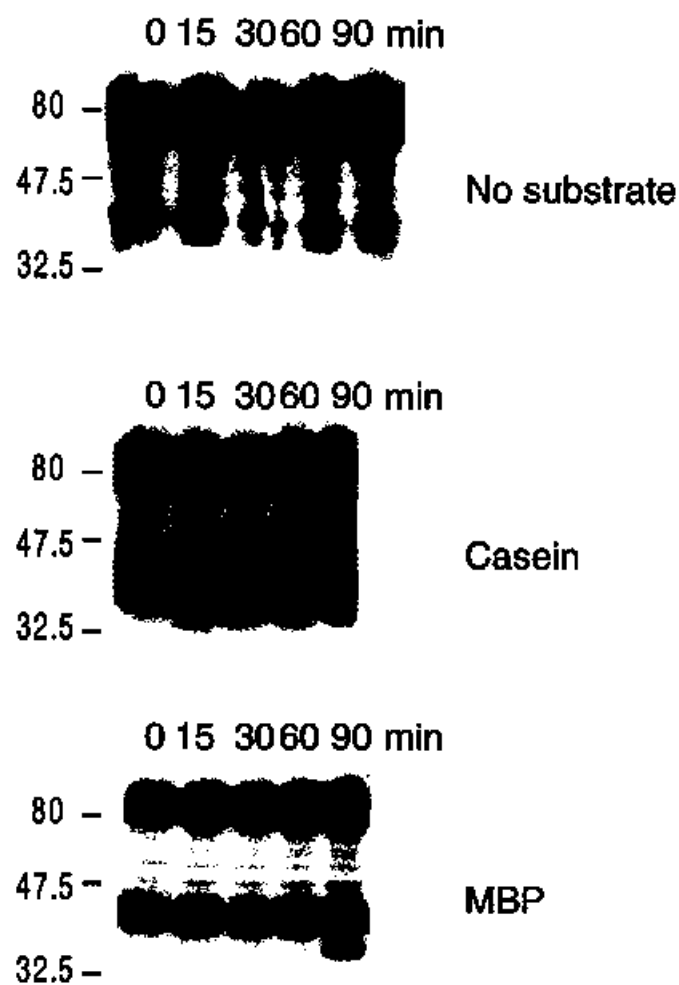


Fig.2. Substrate specificity of 34 kDa kinase

In gel-phosphorylation assay was performed as Fig. 1B using casein (0.5 mg/ml) (middle panel), MBP (0.2 mg/ml) (lower panel) or no substrate (upper panel).

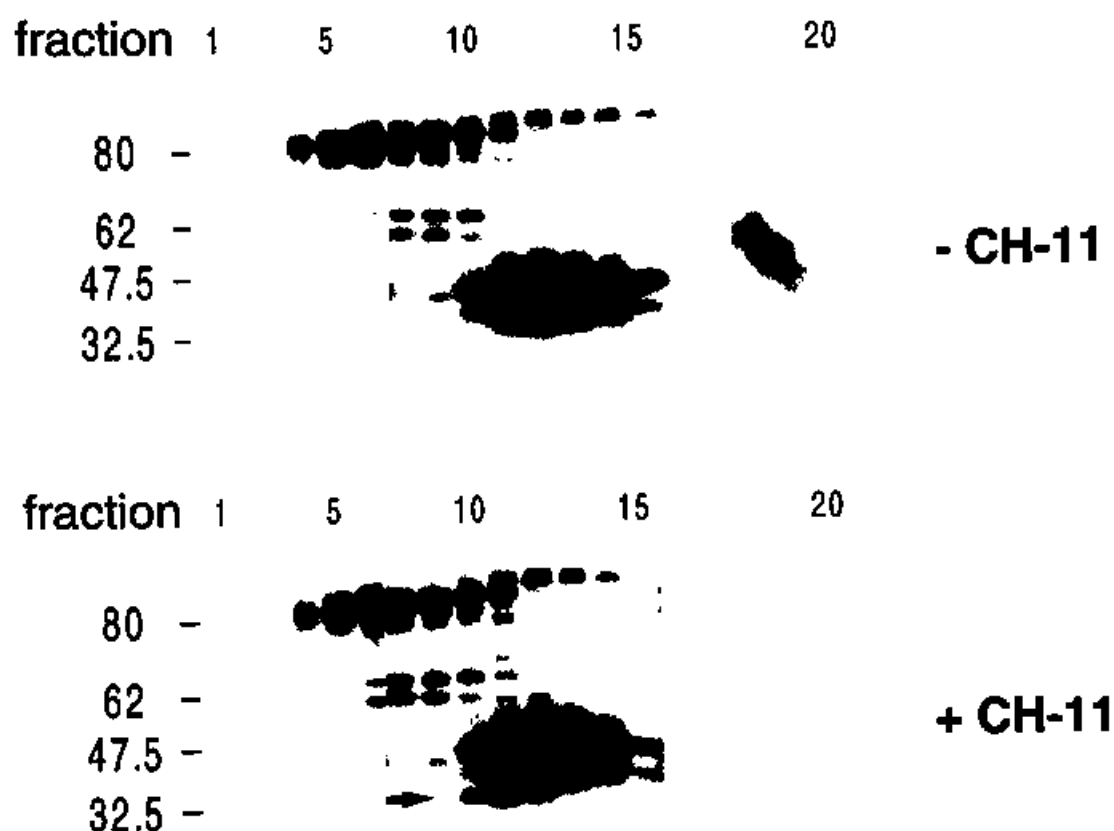


Fig. 3. Partial purification of p34 kDa kinase

HPB-ALL cells (1×10^8) were stimulated with CH-11 for 2 hr. Cytoplasmic extracts was loaded onto Q-sepharose column and eluted with 0 to 0.3 M NaCl gradient. Fractions from unstimulated (upper panel) or stimulated (lower panel) cells were separated by SDS-PAGE and subjected to in-gel phosphorylation assay using histone as substrate.

from unstimulated cytoplasmic extract. No significant change in other histone kinase activity was detected after the stimulation with CH-11.

To test whether the induction of 34 kDa histone kinase is a general event in apoptosis, the effect of other apoptotic stimuli on the activation of 34 kDa kinase was investigated using staurosporine, a serine/threonine kinase inhibitor, and synthetic ceramides, which induce apoptosis with unknown mechanisms (Fig. 4A). The activity of the 34 kDa protein kinase was also induced in HPB-ALL cells by the stimulation with staurosporine or C-2 ceramide in combination with cycloheximide (CHX) with a similar time course as CH-11, though C-2 ceramide or CHX alone did not induce the activity (Fig. 4B). The activation of the kinase coincided well with the induction of apoptosis, since staurosporine or C-2 ceramide plus CHX induced apoptosis while C-2 ceramide or CHX alone did not, in HPB-ALL cells (Fig. 4A and B).

The activation of 34 kDa kinase was markedly inhibited by the pretreatment with peptide inhibitor of caspase, Z-VAD-FK. Z-VAD-FK also inhibited the induction of apoptosis by CH-11, C-2 ceramide or staurosporine. A 60 kDa kinase activity was also induced by the treatment with staurosporine (Fig. 4B). However, this activity was not inhibited by Z-VAD-FK indicating that activation of this kinase is an upstream event of the activation of caspase and/or not involved in apoptosis. Ac-DEVD-CHO, another peptide inhibitor for caspase-3-like protease inhibited the activation of the 34 kDa kinase to a lesser extent than Z-VAD-FK, and the inhibition profiles correlated well with those of DNA ladder formation, implying that the 34 kDa kinase was activated at downstream of Ac-DEVD-CHO-sensitive caspase-3-like protease. In contrast, Ac-YVAD-CHO, an inhibitor for caspase-1-like protease, did not inhibit the activation of the kinase (data not shown) suggesting that Ac-YVAD-CHO-sensitive caspase-1-like protease is not involved in Fas-mediated apoptosis

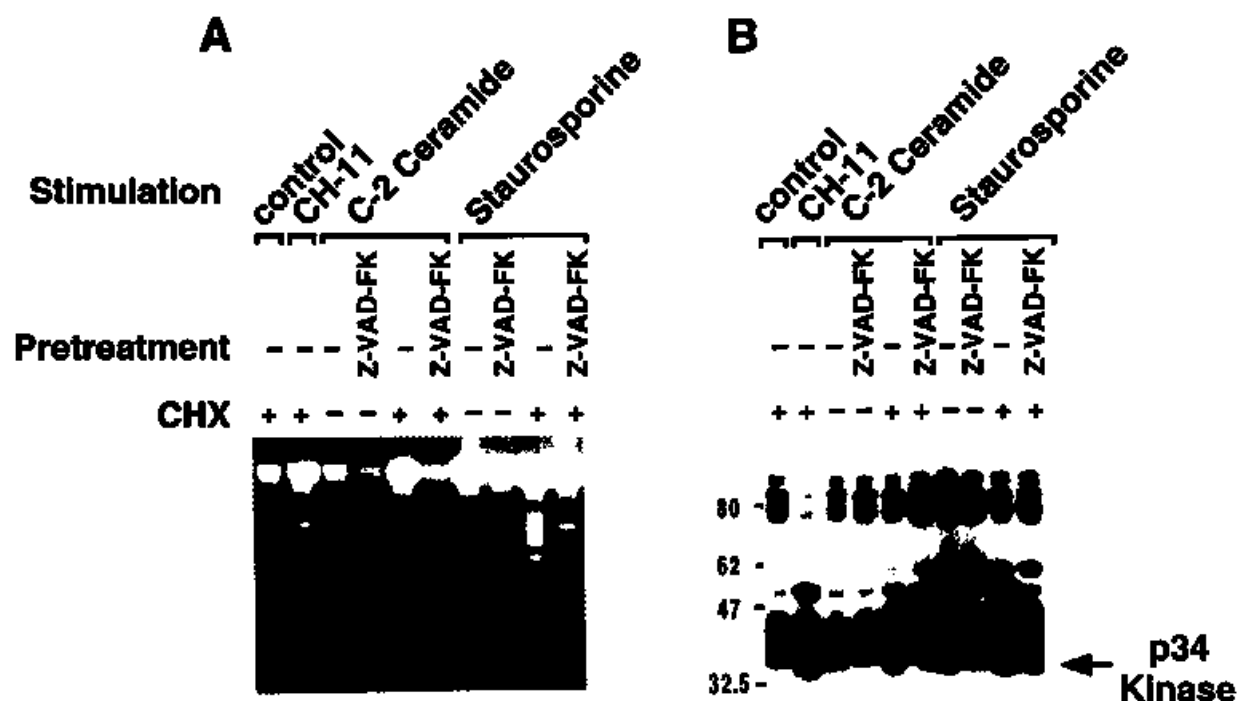


Fig. 4. The 34 kDa protein kinase is activated by various apoptotic stimuli and inhibited by caspase inhibitor. HPB-ALL cells were preincubated for 1 hr with (+) or without (-) cycloheximide (CHX, 25 μ g/ml), and with 1% DMSO (-) or Z-VAD-FK (25 μ M). Then CH-11 (1 μ g/ml), C-2 ceramide (100 μ M) or staurosporine (1 μ M) was added and incubated for 2 hr. The stimulated cells were resolved on 1.5% agarose gel (A) or subjected to in-gel kinase assay (B) as described in the legend to Figure 1.

in HPB-ALL cells. These results suggest that activation of the 34 kDa kinase is a general event occurring at the downstream of the activation of caspase.

4.2. Purification of the 34 kDa protein kinase

To investigate the role of 34 kDa histone kinase in Fas-mediated apoptosis, purification of this kinase activity was performed from HPB-ALL cells stimulated with CH-11. The protein kinase activity was purified with a stepwise column chromatography procedure; SP Sepharose, Q-Sepharose, butyl Sepharose, Mono Q and Superdex 75 gel filtration column chromatography. During the purification of 34 kDa kinase, it was reported that PKC δ was proteolytically activated by caspase on apoptosis and cleaved PKC δ phosphorylated histone (Emoto *et al.*, 1995). Then the possibility that p34 kinase is proteolytic fragment of PKC δ was investigated. MonoQ-purified 34 kDa kinase fraction did not react to anti-PKC δ polyclonal antibody. The 34 kDa protein kinase did not efficiently phosphorylate MBP (amino acid 4-14) and P5307, a good substrate for PKC δ (Fig. 5). These results suggest that the protein kinase with 34 kDa is not a cleaved form of PKC δ .

34 kDa histone kinase fractions from the final Superdex 75 column chromatography were analyzed by SDS-PAGE, and distribution of kinase activity and protein profile were observed by silver staining and in-gel kinase assay, respectively (Fig. 6A). The 34 kDa protein of doublet was eluted at the elution volume expected from the molecular weight (Fig. 6A). The p34 histone kinase activity with the same retention volume as 34 kDa protein in silver staining, was detected in in-gel phosphorylation assay indicating gel-filtrated p34 kinase exist in monomer (Fig. 6B). No histone kinase activity of other molecular weight was detected in in-gel

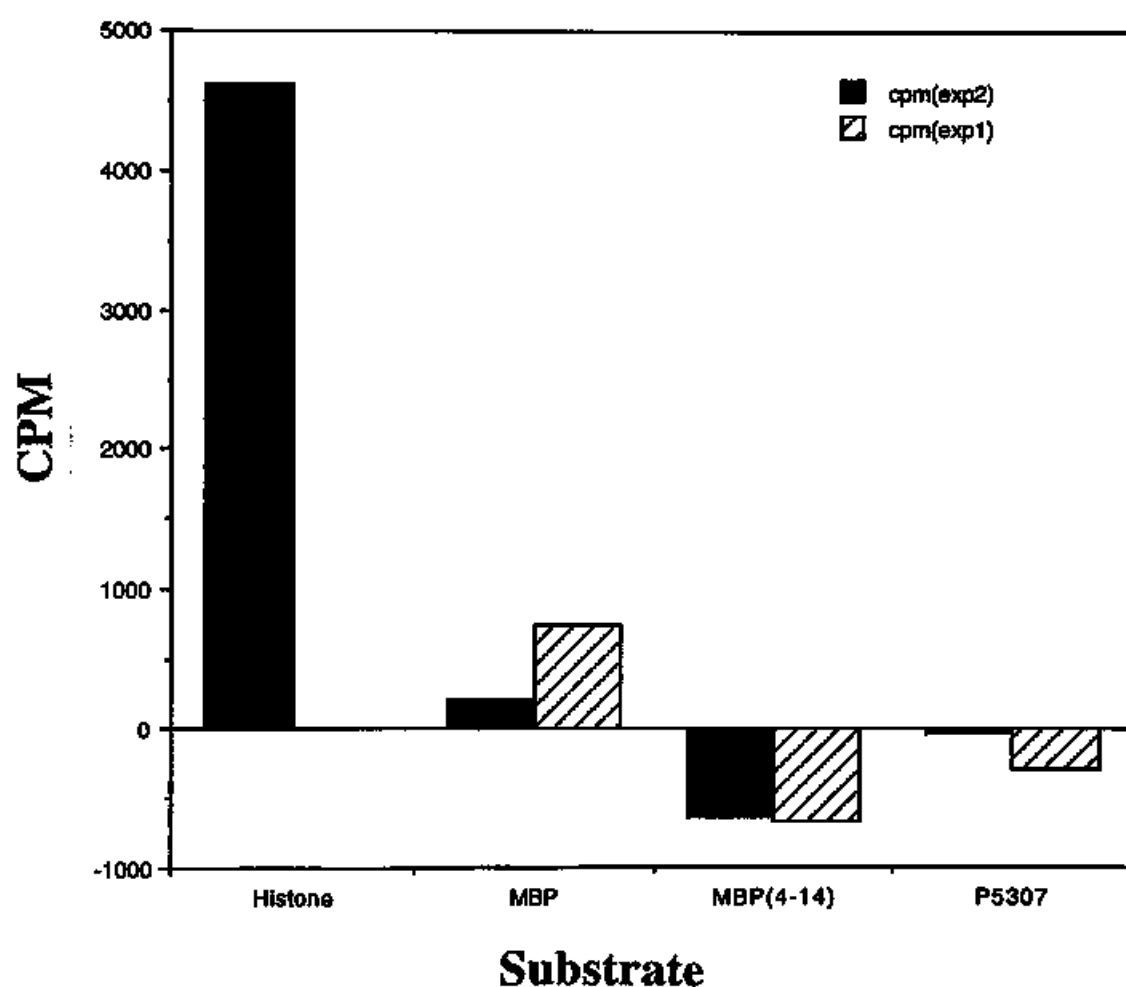


Fig. 5. Substrate specificity of 34 kDa kinase

Partially-purified p34 kinase fraction by Mono Q was incubated with histone (6 μ g), MBP (6 μ g), MBP (4-14) (133 μ M) or P5307 (133 μ M) in the presence of 25 μ M ATP and 2.5 μ Ci of [γ - 32 P] ATP at 30°C for 20 min. Reaction was stopped by adding 10% phosphoric acid and spotted on phosphocellulose paper. Phosphocellulose paper was washed extensively, dried, and then radioactivity was counted. Results of two independent experiments are shown.

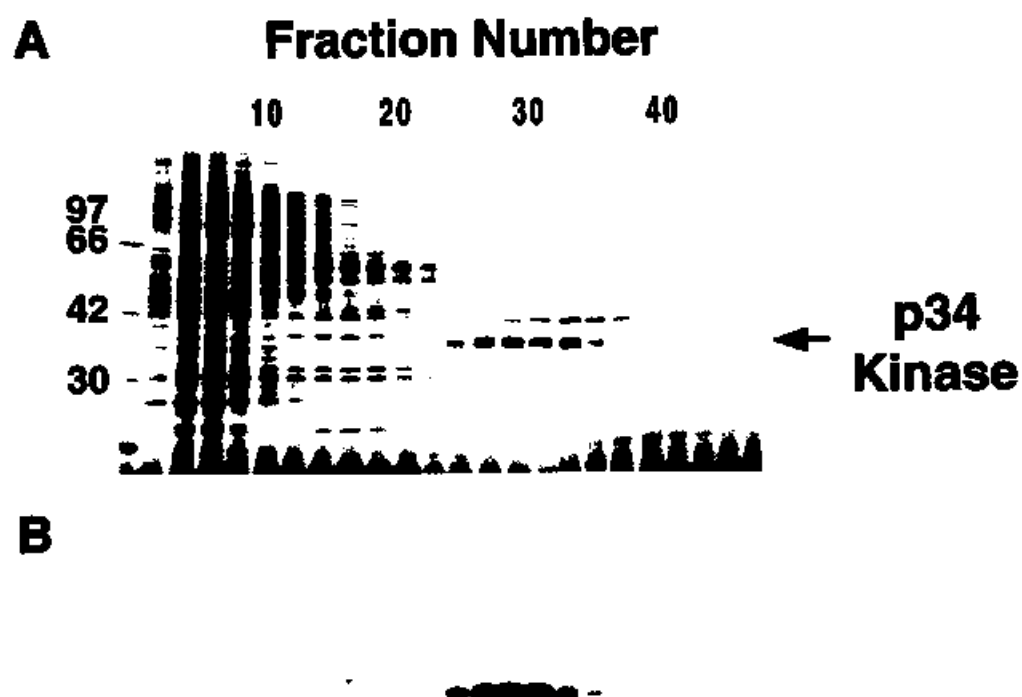


Fig. 6. Purification of the 34 kDa kinase. (A) Fractions from Superdex 75 gel filtration column chromatography were analyzed by SDS-PAGE and silver-stained. (B) The same fractions were analyzed by in-gel kinase assay using histone as substrate. Kinase-active fractions from fraction 22 to 32 were analyzed by two-dimensional electrophoresis. Gels were silver-stained (C) or subjected to in-gel kinase assay (D).

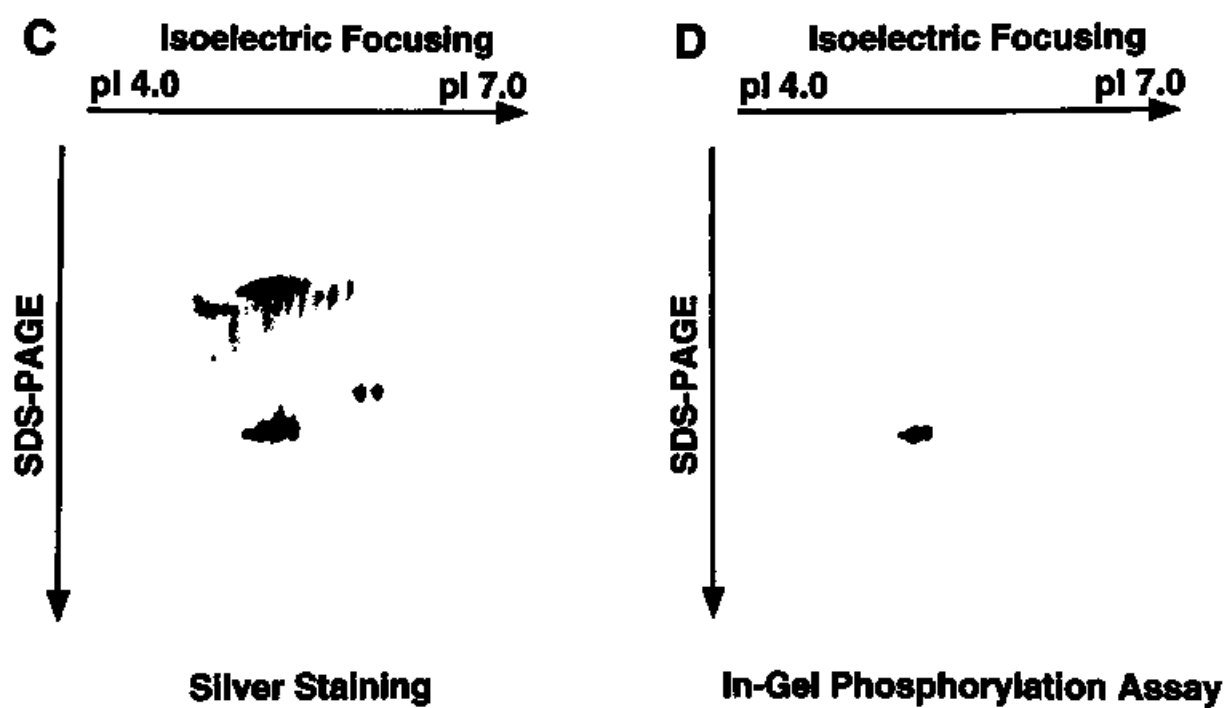


Fig. 6. continued

phosphorylation assay. Further separation was performed by two-dimensional electrophoresis and analyzed by silver staining and in-gel phosphorylation assay. In-gel phosphorylation assay showed three or four distinctive spots of kinase activity with slightly different isoelectric points (pI), ranging from pI 5.0 to pI 5.5 (Fig. 6D). Silver staining showed, however, 6 distinct protein spots with 34 kDa and the same pI range. Four of the 6 spots co-migrated with the kinase activity while the other two spots with 34 kDa were not associated with kinase activity (Fig. 6C). Fig. 6C shows the other spots of protein; two spots with 40 kDa and consecutive spots with 60-70 kDa. The protein with 40 kDa was observed also in Fig. 6A, and the consecutive spots of high molecular weight might be cytokeratins contaminated during isoelectric focusing.

The four protein spots that coincided well with the 34 kDa kinase activity were divided into halves by a slight difference of pI range. Then, the two pairs of the four protein spots, and one pair of the remaining 2 spots of the same molecular weight were separately recovered from polyacrylamide gel and digested partially with V8 protease. Resulting peptides were separated by SDS-PAGE and subjected to peptide sequencing. The peptide sequences ISIM T/Q T/Q H/C D S/Q PYVVKY and IGYNXVADI were obtained from all the 3 pairs of protein spots. A BLAST search of the sequences revealed complete identity with a protein kinase, MST/Krs, a member of the kinase family of STE20 (Fig. 7, Creasy and Chernoff, 1995a). Amino acid sequences of MST/Krs suggest that caspase-specific cleavage sites exist at the end of the kinase domain, ³²³DEMDS³²⁷ (MST1) and ³¹⁹DELDS³²³ (MST2). The calculated molecular mass of cleaved kinase domain is 35 kDa. These results show that purified 34 kDa kinase is a catalytic domain of MST/Krs, cleaved by caspases during apoptosis. Interestingly, the remaining pair of spots with 34 kDa which had no kinase activity in in-gel phosphorylation assay and

MST1	MEIVQLRNHP RRQLKKLDED SLTKQPEEVF DVLEKLGEYS YGSVKAIHK	50
MTS2	ME--Q-PPAP KSKLKKLSED SLTKQPEEVF DVLEKLGEYS YGSVKAIHK	47
MST1	ETGGIVANKO VEVESDLQEI IKEISIMQOC DSPHVVKYYG SYFKNTDLWI	100
MTS2	ESGGVATKO VEVESDLQEI IKEISIMQOC DSPHVVKYYG SYFKNTDLWI	97
MST1	VMEYCGAGSV SDIIRLRNKT LDEDEIATIL DSTLKGLEYL HFMRKIHRDI	150
MTS2	VMEYCGAGSV SDIIRLRNKT LDEDEIATIL DSTLKGLEYL HFMRKIHRDI	147
MST1	KAGNILLNTE GHAKLADGV AGQLTDIMAK RNTVIGTPEW MAPEVIOEIG	200
MTS2	KAGNILLNTE GHAKLADGV AGQLTDIMAK RNTVIGTPEW MAPEVIOEIG	197
MST1	YNGVADIWSL GITSIEMAEK KPPYADHHP RAIFMIPINP PPTFRKPELW	250
MTS2	YNGVADIWSL GITSIEMAEK KPPYADHHP RAIFMIPINP PPTFRKPELW	247
MST1	SDNFTDFVKQ CLVKSPQRA TATQLLOHPF VRSAGVSTL RDLINAEADV	300
MTS2	SDNFTDFVKK CLVKSPQRA TATQLLOHPF IKNAKEVSTL RDLINAEAEI	297
MST1	KLRROESQOR EVDQDDEENS <u>DEDEDSGTM</u> YRAVGDEMGT MRVASTIMTG	350
MTS2	KAKRHEEQOR EEEEEENS <u>DEDEDSGTM</u> VKTSVESVGT MRATSTMSEG	346
MST1	ANIMREHDET L-ESQIGTMV INAEDEEEE- GIMKRDETM OPAKPSFLEY	398
MTS2	AQIMREHDET L-ESQIGTMV INAEDEEEE- GIMKENATSP QVQPSFMDY	396
MST1	FEQKEKENDI N-SFGKSVPG FL--KN--S SIWRIPDGD YFEELKSWTVR	442
MTS2	FDKQDFKKS HENCNQNMHE PFPMKSNVFP DNWRIPDGD YFEELKNSLIE	446
MST1	ELQKRLKALD PMMERETEEI RQRYQSKROP ILDAIEAKKR ROONE	487
MTS2	ELQKRLKALD PMMERETEEI RQRYTAKROP ILDAMDAAKR ROONE	491

Fig. 7. Primary structure of MST1 and MST2.

MST1 and MST2 are aligned using GeneWorks (Intelligenetics). Conserved amino acids are boxed and shaded. N-terminal kinase domain is boxed. Peptide sequences obtained from microsequencing are underlined. Caspase recognition site is marked by asterisk and cleavage site is indicated by arrow head.

were slightly different in pI from the kinase-active spots, were also identified as MST/Krs.

4.3. Proteolytic activation of MST/Krs by caspase during apoptosis

To examine the proteolytic cleavage of MST/Krs, KB cells were transfected with FLAG-tagged MST1 and MST2 and then apoptosis was induced by the treatment with 0.5 μ g/ml of CH-11 together with 25 μ g/ml CHX. In this experiment HPB-ALL cells could not be used because of the low transfection efficiency. The immunoprecipitates from the cell lysate with anti-FLAG antibody were analyzed by immunoblotting and in-gel phosphorylation assay. As expected, FLAG-tagged MST1 and MST2 were cleaved to kinase-active 34 kDa fragment by the treatment with CH-11 time-dependently (Fig. 8). FLAG-tagged mutants of MST1 (D326N) and MST2 (D322N) in which aspartic acid, a conserved recognition amino acid of caspases, was mutated to asparagine were prepared. These mutated MST1 and MST2 were also introduced into KB cells by electroporation. Mutant MST1 (D326N) and MST2 (D322N) were never cleaved up to 5 hr in KB cells, showing that Asp³²⁶ (MST1) and Asp³²² (MST2) are cleavage sites (Fig. 8A). The same result was obtained in Jurkat cells treated with 0.5 μ g/ml of CH-11 (Fig. 9A, right panel)

The cleavage of FLAG-tagged MST was significantly inhibited by the pretreatment of Z-VAD-FK in both KB and Jurkat cells, indicating that caspases are involved in the cleavage of MST (Fig. 9A). However, Ac-DEVD-CHO, peptide inhibitor of caspase-3-like protease, inhibited very weakly while Ac-YVAD-CHO, peptide inhibitor of caspase-1-like protease, did not inhibit the proteolytic activation of MST in KB cells (Fig. 9A, left panel). In contrast, Ac-DEVD-CHO inhibited considerably

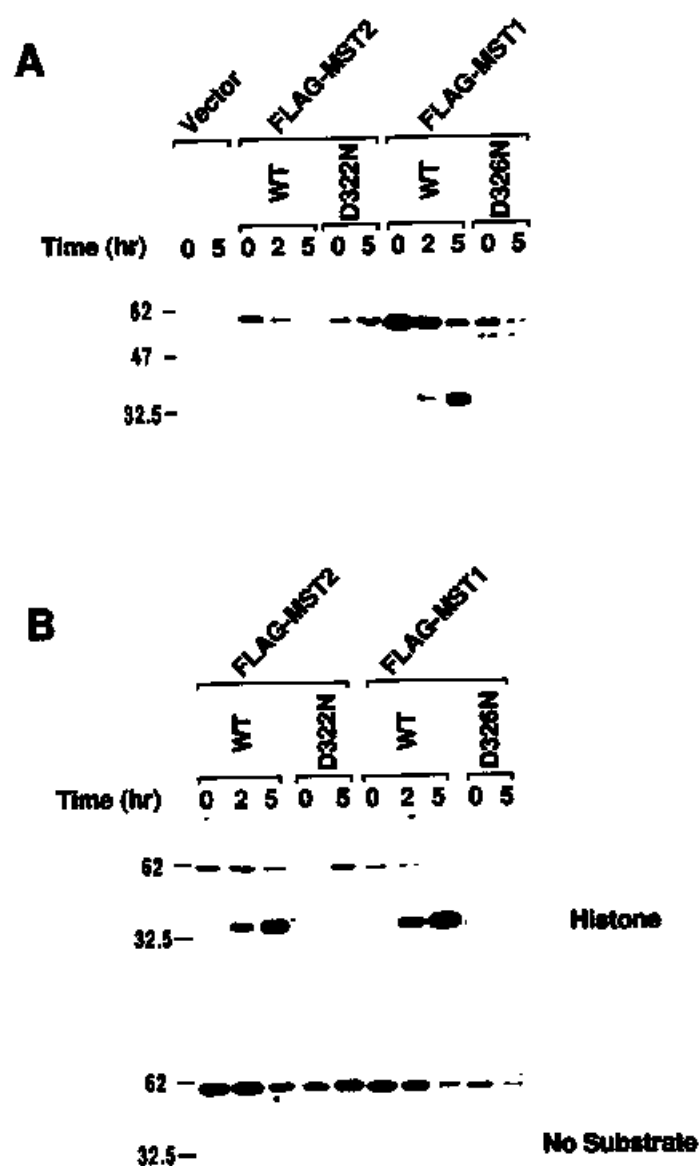


Fig. 8. Proteolytic activation of MST. KB cells were transfected with empty vector (vector alone), FLAG-tagged wild type (WT) of MST, mutant MST1 (D326N), or mutant MST2 (D322N). After 48 hr of transfection, cells were treated with CH-11 (0.5 μ g/ml) and CHX (25 μ g/ml) for the indicated periods. FLAG-tagged MST was immunoprecipitated with anti-FLAG M2 antibody and analyzed by immunoblotting with anti-FLAG M2 antibody (A) or subjected to in-gel phosphorylation assay using polyacrylamide gels containing histone (B, upper panel) or no substrate (B, lower panel).

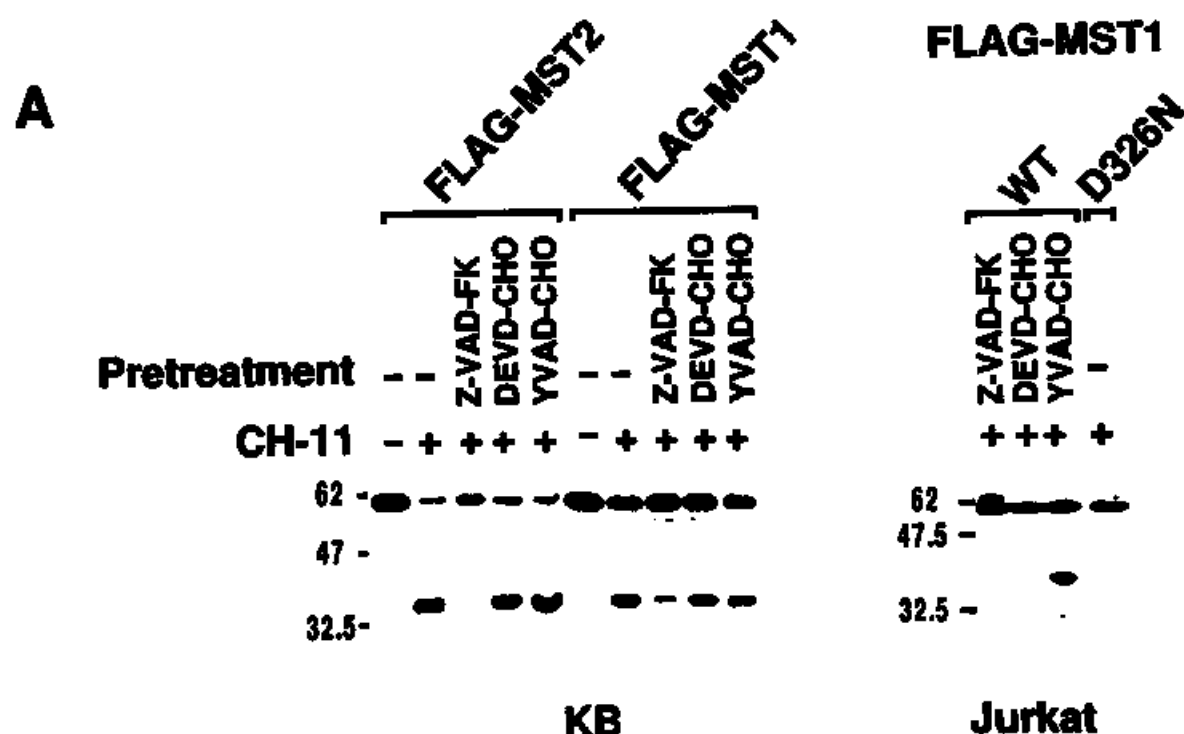


Fig. 9. Proteolytic activation of MST is induced by various apoptotic stimulations and inhibited by caspase inhibitors. (A, left panel), KB cells were transfected with FLAG-tagged MST and cultured for 48 hr. Then, cells were preincubated for 1 hr with 1% DMSO (-) or caspase inhibitors, Z-VAD-FK (25 μ M), Ac-DEVD-CHO (200 μ M), Ac-YVAD-CHO (200 μ M), and cells were treated with CH-11 (0.5 μ g/ml) + CHX (25 μ g/ml) for 5 hr at 37°C. FLAG-tagged MST was immunoprecipitated and analyzed by immunoblotting as described in the legend to Fig. 4 (A, right panel), FLAG-tagged wild type (WT) or mutant MST1 (D326N) was transfected into Jurkat cells. Transfectants were preincubated with caspase inhibitors and treated with CH-11 for 3 hr. Cell lysate was resolved on 10% SDS-PAGE and immunoblotted with anti-FLAG M2 antibody. (B), Jurkat cells (left panel) or KB cells (right panel) were transfected with FLAG-MST1, and stimulated with TNF α (20 ng/ml), staurosporine (1 μ M), C-2 ceramide (50 μ M), etoposide (100 μ M), or UV (250 J/m², 254 nm, 15 min). Cells were incubated for indicated periods at 37°C and total cell lysates were analyzed by immunoblotting.

B

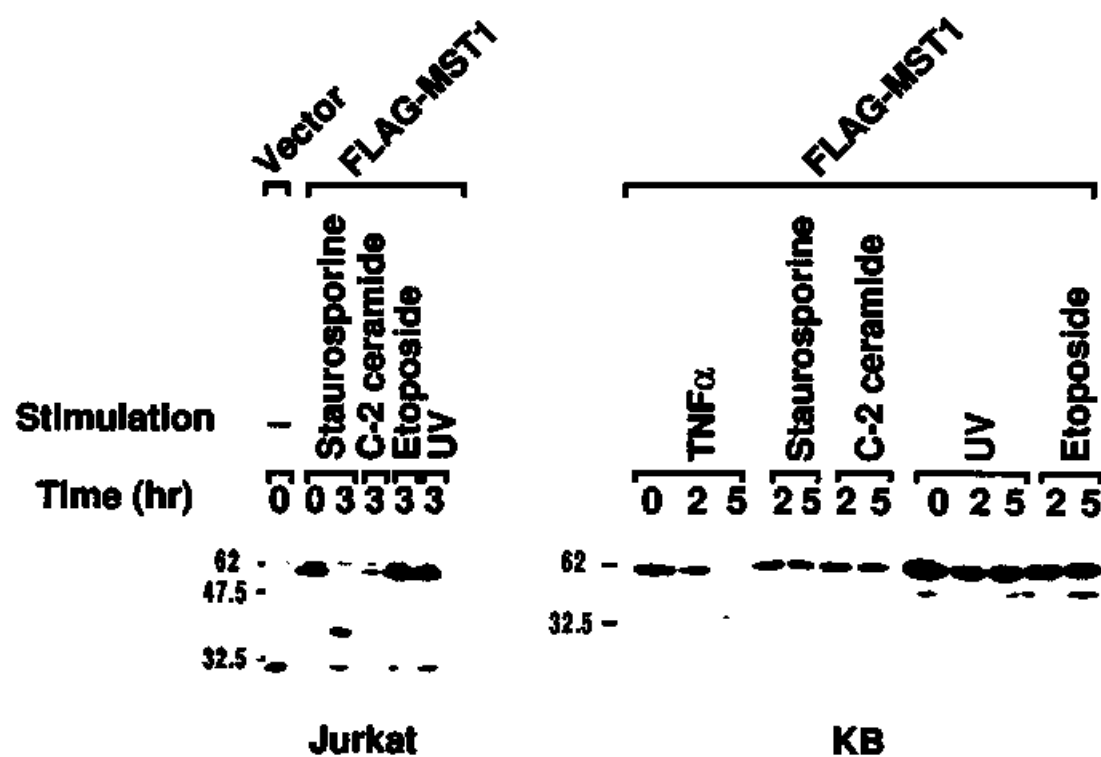


Fig. 9 continued

the proteolytic activation of MST and Ac-YVAD-CHO had no effect in Jurkat (Fig. 9A, right panel). These results suggest that different sets of caspases may be activated in Jurkat and KB cells after the stimulation with CH-11.

The proteolytic cleavage of MST coincided well with the induction of apoptosis. To examine the proteolytic cleavage of MST is general event in apoptosis, the effect of various apoptotic stimuli was studied. MST was cleaved by the treatment with TNF α in combination with CHX but MST was not cleaved to 34 kDa after stimulation with UV, etoposide, staurosporine or C-2 ceramide in KB cells (Fig 9B). MST in Jurkat cells, however, was cleaved by the treatment with staurosporine but not by UV, etoposide and C-2 ceramide (Fig 9B). These results might mean that activation and/or expression levels of various caspases are different in KB and Jurkat cells even by the same apoptotic stimulus. Taken together, proteolytic cleavage of MST may be a common event in apoptosis but the extent of cleavage may be different from cells to cells even by the same apoptotic stimulus.

The proteolytic fragment of MST1 and MST2 phosphorylated histone (Fig. 8B, upper panel) in in-gel phosphorylation assay but autophosphorylation activity of the 34 kDa fragment was not detected (Fig. 8B, lower panel). In contrast, full length-MST1 and MST2 had weak kinase activity when histone was used as substrate (Fig. 8B, upper panel) but this kinase activity was also detected when substrate was not used indicating the kinase activity of full-length MST1 and MST2 are autophosphorylation activity. Autophosphorylation activity of full-length MST1 and MST2 was not significantly activated by the treatment with CH-11 (Fig. 8B). This finding suggests that proteolytic cleavage of MST may not only increase kinase activity but also change substrate specificity.

To determine if both MST1 and MST2 are direct substrate of caspase-3 *in vitro*, purified recombinant His-MST1 and His-MST2 were incubated with purified active recombinant caspase-3 in the presence or absence of Ac-DEVD-CHO. In this study, kinase-dead mutant form of His-MST with the replacement of Lys→ Arg at the ATP binding region of kinase domain, was used because wild type MST could not be effectively expressed in *E.coli*. His-MST1 and His-MST2 were processed to 34 kDa N-terminal catalytic domain, whereas His-MST1 and His-MST2 were not cleaved when caspase-3-like protease inhibitor, Ac-DEVD-CHO was added (Fig. 10). Therefore it is concluded that caspase-3-like protease cleaves MST1 after Asp³²⁶ and MST2 after Asp³²² *in vivo* and *in vitro*.

Previously it was reported that JNK was activated in Fas-mediated apoptosis with unknown mechanism (Cahill *et al.*, 1996). Full-length MST does not reportedly activate ERK1/2 (Creasy and Chernoff, 1995b) but its involvement on the activation of JNK is unknown. To investigate whether MST activate JNK, various constructs of MST was transfected into Jurkat cells. After the stimulation with CH-11, JNK was immunoprecipitated with anti-JNK antibody and subjected to immune-complex kinase assay using GST-cJun as substrate. As shown in Fig. 11, JNK activity was slightly increased by Fas-ligation, but overexpression of kinase-negative or caspase-resistant MST1 did not inhibit the activation of JNK suggesting that JNK activation by Fas-ligation is not downstream event of MST cleavage.

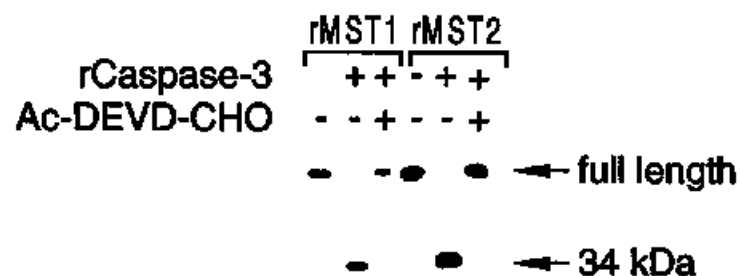


Fig. 10. MST is a substrate of caspase-3 *in vitro*. His-tagged MST1 and MST2 (10 μ g each) were incubated with recombinant caspase-3 (0.1 μ g) in the presence of DMSO(-) or 100 μ M of Ac-DEVD-CHO (+). Reaction was stopped by adding Laemmli's sample buffer and analyzed by SDS-PAGE. N-terminal 34 kDa fragment was detected by Western blotting using anti-MST monoclonal antibody (G2B).

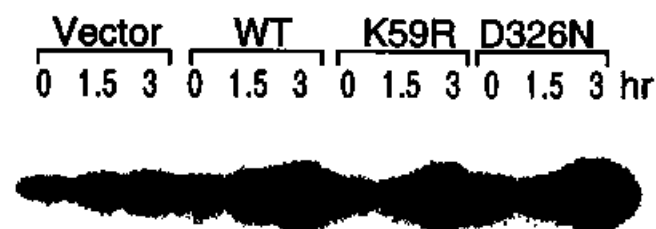


Fig. 11. MST does not activate JNK1 in Fas-mediated apoptosis. Jurkat cells were transfected with 40 μ g of pME18S (Vector), MST1 (WT), kinase-negative MST1 (K59R), or caspase-resistant MST1 (D326N). After 48 hr, transfectants were stimulated with CH-11, and incubated for indicated hours. JNK was immunoprecipitated with anti-JNK1 polyclonal antibody and subjected to immune-complex kinase assay using GST-cJun (10 μ g) as substrate.

4.4. Analysis of MST expression

Several monoclonal antibodies that specifically recognize MST were prepared. The expression of endogenous MST was examined in various cells by Western blot analysis using monoclonal antibody (Fig. 12). A monoclonal anti-MST antibody (G2B) recognized a protein of about 60 kDa in human (HPB-ALL, Jurkat, HL-60, 293T, KB, HT29), mouse (WR19L, NIH3T3, Balbc3T3), and monkey cells (COS-7). Expression of MST was confirmed in all cells examined confirming ubiquitous expression of MST (Creasy and Chernoff, 1995a). Monoclonal anti-MST antibodies also immunoprecipitated a single protein of same size from 293T cell lysate that was absent from the immunoprecipitates with control mouse IgG instead of anti-MST antibody.

4.5. Proteolytic cleavage of endogenous MST by Fas-stimulation

Proteolytic cleavage of MST was examined in HPB-ALL cells that were used in the purification of MST. HPB-ALL cells were stimulated with CH-11 for various times and endogenous MST was detected with anti-MST monoclonal antibody, G2B. 34 kDa fragment of MST was detected at 2 hr and nearly all of the MST was cleaved at 3.5 hr (Fig. 13).

Proteolytic cleavage was considerably inhibited with the treatment of Ac-DEVD-CHO confirming again that MST is cleaved by caspase-3-like protease Fas-dependently. Taken together, not only overexpressed but also endogenous MST is cleaved to catalytic 34 kDa fragment indicating that MST is a physiological substrate of caspase-3-like protease.

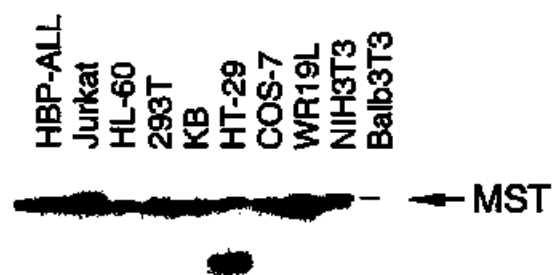


Fig. 12. Expression of MST on various cell lines. Endogenous MST was detected from total cell lysate of various cells by Western blotting using anti-MST monoclonal antibody (G2B). Lysate were normalized for protein amounts.

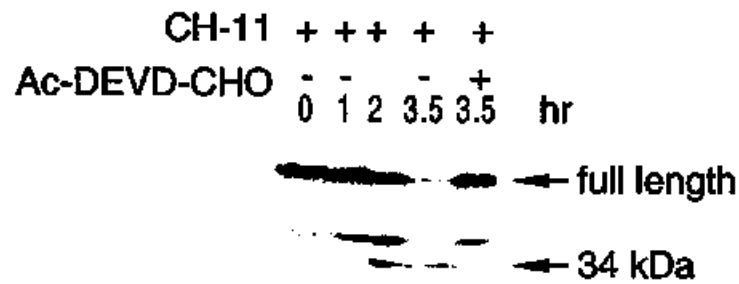


Fig. 13. Proteolytic cleavage of endogenous MST in HPB-ALL cells. HPB-ALL cells were stimulated with CH-11 for indicated hours. Ac-DEVD-CHO was pretreated for 1 hr before adding CH-11 when used. Total cell lysate was separated by SDS-PAGE and analyzed by immunoblotting as described in Fig. 12

4.6. Proteolytic activation of MST by staurosporine

Staurosporine-induced apoptosis of some cells are reported (Fig. 4A, Jacobsen *et al.*, 1996; Mehlen *et al.*, 1996) and MST was cleaved to 34 kDa fragment by staurosporine in Jurkat cells overexpressing MST (Fig. 9B). Proteolytic activation of MST by staurosporine was further analyzed to investigate the activation mechanism of MST. FLAG-tagged MST was expressed in Jurkat cells and stimulated with staurosporine or CH-11. After immunoprecipitation with anti-FLAG antibody, immunoprecipitates were analyzed by immunoblotting and in-gel phosphorylation assay. MST1 and MST2 were cleaved to about 34 kDa fragment by the treatment with staurosporine (Fig. 14A, lane 4 and lane 16). However, 34 kDa fragment generated by staurosporine was slightly slower in mobility than the proteolytic fragment of MST cleaved by CH-11 (Fig. 14A, lane 4, 6, 16, 18). The mobility change of 34 kDa fragment by the treatment with staurosporine may be caused from the modification such as phosphorylation or dephosphorylation. Furthermore, the mutant MST1 and MST2 with Asp → Asn replacement at a putative caspase cleavage site also showed the proteolytic cleavage generating about 40 kDa N-terminal fragment after the stimulation with staurosporine and CH-11 (Fig. 14A, lane 10, 12, 22, 24). The 40 kDa fragment of MST was not detected in KB cells stimulated with CH-11 or staurosporine (Fig. 8) suggesting that such activities are not activated or very weak in KB cells.

Then, the kinase activity of cleaved MST was analyzed by in-gel kinase assay. The activity of full-length MST1 was greatly stimulated by staurosporine at 0.5 hr and this activity may be autophosphorylation activity because the increase of kinase activity was still observed even when substrates were not used (Fig. 14B). In contrast, full-length MST was not activated by CH-11 confirming previous observation (Fig. 8). Great increase of autophosphorylation activity with 40 kDa was also

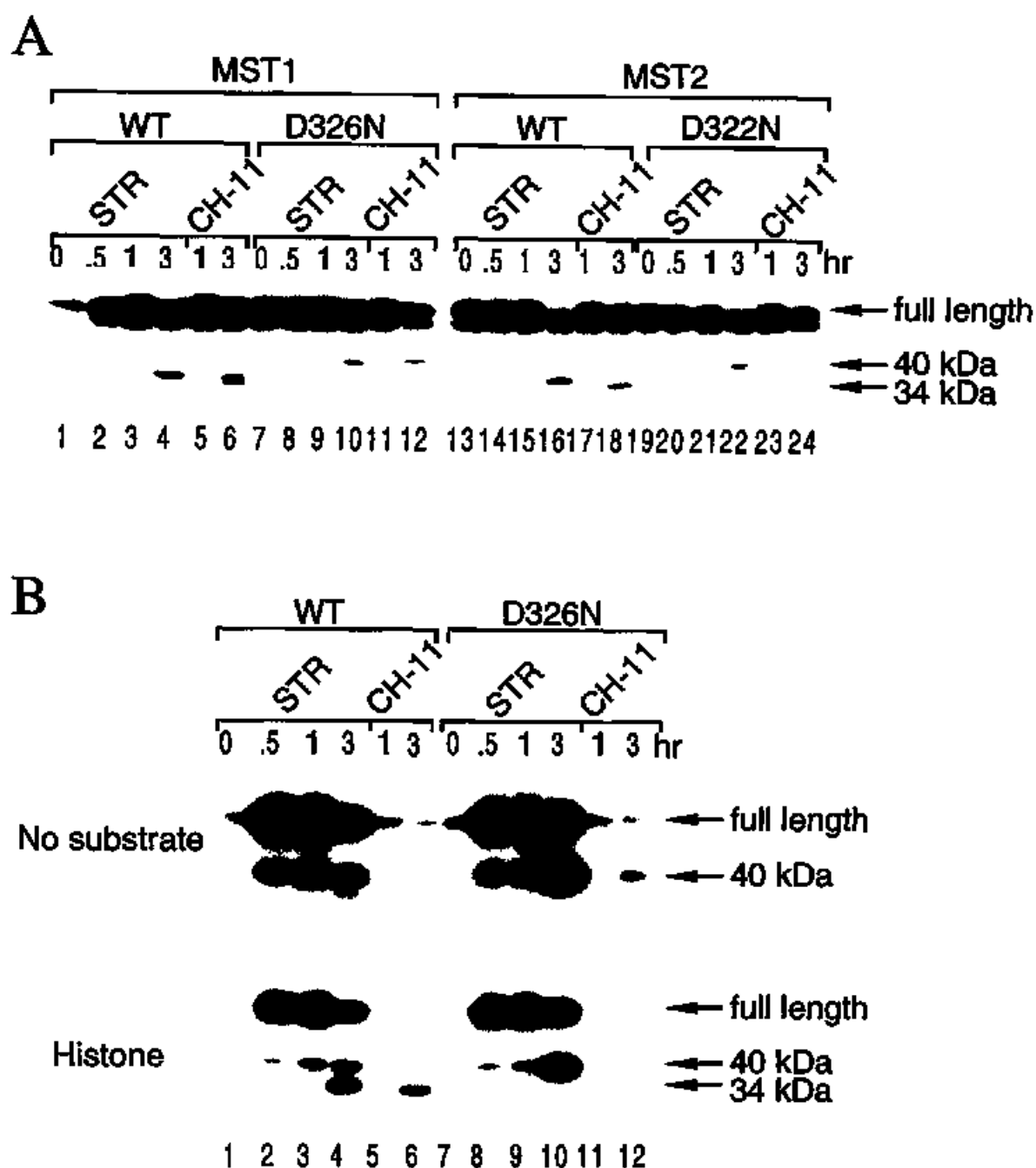


Fig. 14. Proteolytic activation of MST by staurosporine.

Jurkat cells were transfected with 40 μ g of vectors encoding FLAG-tagged wild type MST (WT) or mutant MST (D326N for MST1 or D322N for MST2). After 48 hr, transfectants were stimulated with staurosporine (STR) or CH-11 as indicated times. MST was immunoprecipitate with α -FLAG antibody and analyzed by immunoblot (A) or subjected to in-gel phosphorylation assay using no substrate (B, upper panel) or histone (B, lower panel)

detected at 0.5 -1 hr by stimulation with staurosporine (Fig. 14B, upper panel), and the activity was sustained more than 2 hr. Appearance of 34 kDa fragment with histone kinase activity was detected at 3 hr and this 34 kDa histone kinase activity was also detected by the stimulation with CH-11. Mutant full-length MST1 (D326N) was activated with the same time course as wild type MST indicating the introduction of mutation does not effect on activation of MST by staurosporine. MST1 (D326N) was cleaved to 40 kDa fragment with autophosphorylation activity by stimulation with staurosporine in the same manner as wild type MST1. The 40 kDa fragment of MST1 (D326N) generated by stimulation with CH-11 had lower kinase activity than that stimulated with staurosporine suggesting not only proteolysis but also modification may contribute to the activation of MST by stimulation with staurosporine (Fig. 14 A and B, lane 10 and 12). Autophosphorylation activity of 40 kDa of MST1 (D326N) was detected at 0.5 hr and this activity was accumulated time-dependently to 3 hr (Fig. 14B, lane 10 and 12). Accumulation of 40 kDa kinase activity was not observed in wild type MST1 and rather slight decrease was observed (Fig. 14B, lane 2, 3,4). Similar result was observed in the case of MST2 and MST2 (D322N).

These findings strongly suggest that MST is cleaved by two-step process, resulting in 34 kDa fragment followed by the generation of 40 kDa fragment by the stimulation with staurosporine and CH-11. This possibility is further supported by the fact that another putative caspase-cleavage site may exist in MST1 (³⁴⁶TMTDG³⁵⁰), an optimal sequence to caspase-6/Mch2. Actually this site was shown to be cleaved by peptide sequencing of the 40 kDa fragment of MST1 (D326N) (data not shown). However, this sequence is not conserved in MST2 suggesting another possibility that other sequences conserved in MST1 and MST2 may be cleaved by caspase or other protease.

4.7. Effect of MST in Fas-mediated apoptosis

The role of MST in Fas-mediated apoptosis was examined. Jurkat cells were co-transfected with various constructs of MST and truncated Aic2A (β -chain of IL3 receptor) and transfected Jurkat cells was selectively collected by panning using anti-Aic2A monoclonal antibody. Recovered transfectants were treated with CH-11 and effect on apoptosis was investigated. Overexpression of wild type, kinase-dead (K59R), caspase-resistant (D326N), or kinase-negative and caspase-resistant (K59R, D326N) MST did not effect on the time course of the appearance of change in morphological apoptosis in Jurkat cells (Fig. 15). Cell viability of transfectants overexpressing mutant MSTs were examined by MTS assay but no significant change of cell viability was observed (Fig. 16). Externalization of phosphatidylserine, nuclear fragmentation and formation of DNA ladder were examined but no significant change was detected, suggesting that proteolytic activation of overexpressed MST alone is not sufficient to induce apoptosis in Jurkat cells.

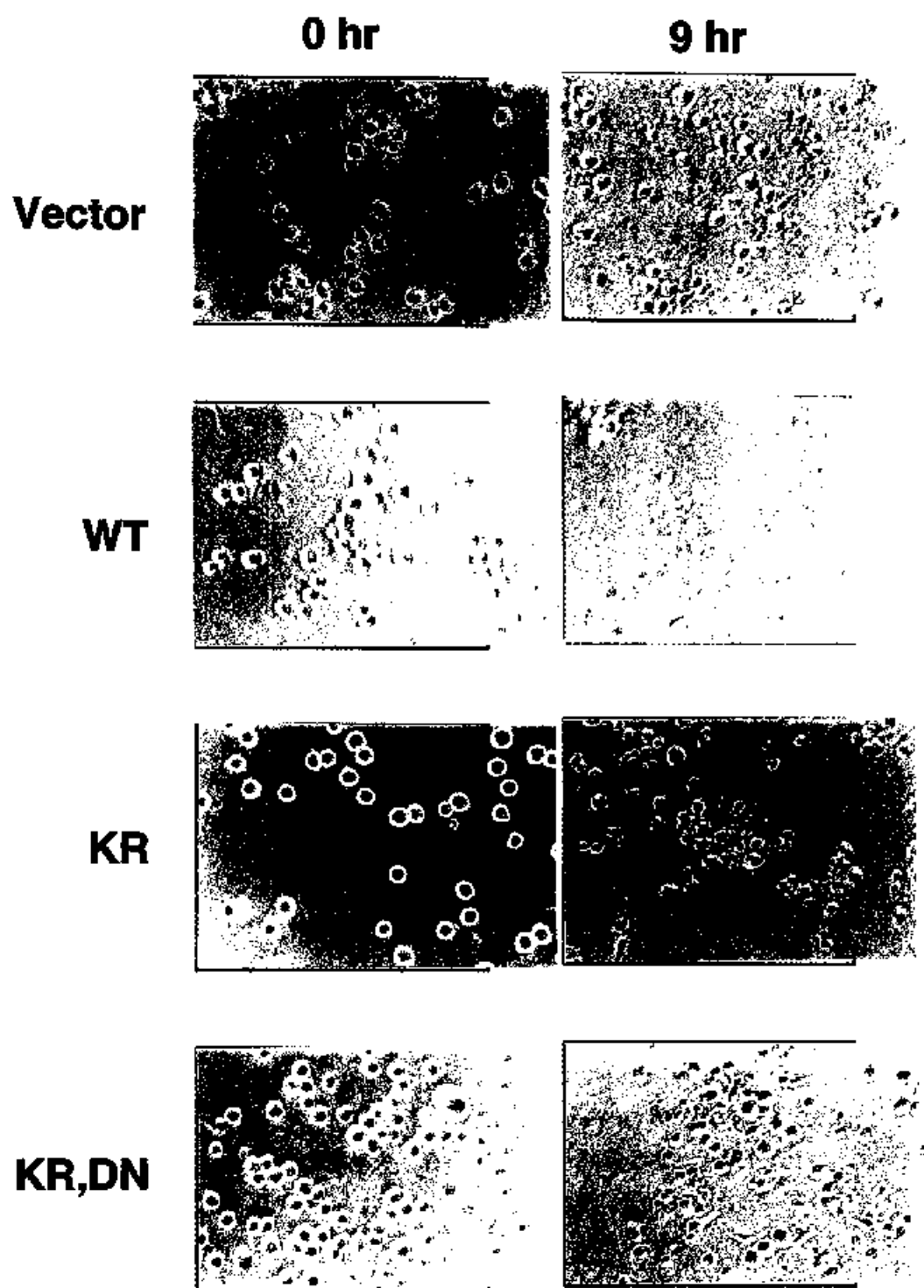


Fig. 15. Overexpression of MST does not effect on apoptotic morphology.

Vectors (40 μ g) encoding wild type MST (WT), kinase-negative (KR), kinase-negative or caspase-resistant MST (KR, DN) were co-transfected into Jurkat cells with 20 μ g of vector encoding truncated IL3 receptor (tAic2A). Transfectants were collected by panning as described in "Materials and methods" and treated with CH-11 for indicated hour and cells were photographed.

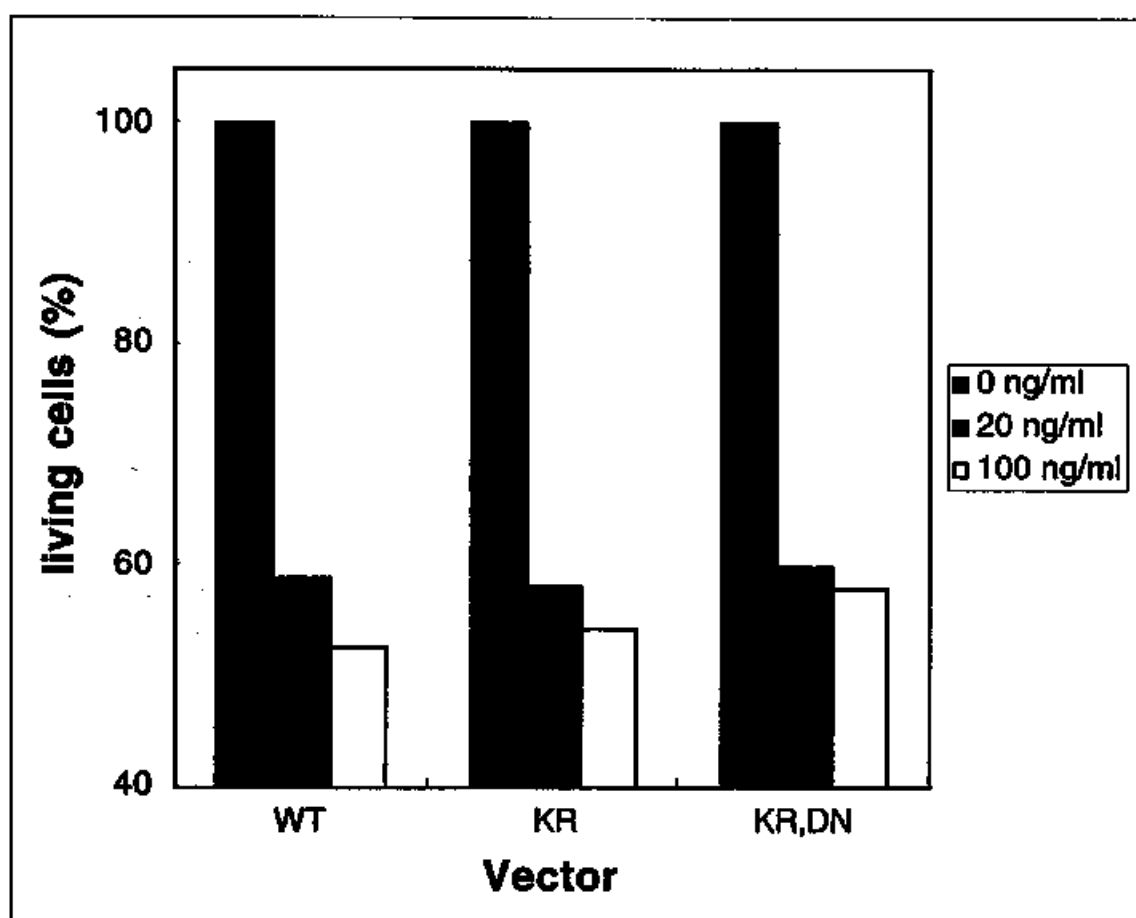


Fig. 16. Overexpression of MST does not effect on cell viability in Fas-mediated apoptosis.

2×10^4 transfectants prepared as in the legend of Fig. 15, were treated with CH-11 for 13 hr. 10 μ l of MST solution was added and further incubated at 37°C for 4 hr. Absorbance at 490 nm was measured. Cell viability represent mean values $\pm$ S.D. of three independent experiments.

4.8. Effect of PAK2 in Fas-mediated apoptosis

Recently it was reported that PAK2, another member of the STE20 kinase family, is proteolytically activated by caspase, and Jurkat cells that express a dominant-negative PAK2 mutant are resistant to the Fas-induced formation of apoptotic bodies but cause an enhanced externalization of phosphatidylserine (Rudel and Bokoch, 1997). To investigate the role of PAK2 in Fas-mediated apoptosis, pME18S vectors expressing PAK2 and mutant PAK2 (H82L, H85L, K299R) were prepared. PAK2 (H82L, H85L, K299R) have mutations at two histidine (His⁸² and His⁸⁵) residues which are necessary for the binding with Rac/cdc42 and one lysine (Lys²⁹⁹) that is essential for kinase activity, respectively. This mutant was reported to function as dominant negative form of PAK (Sells et al., 1997; Hanks et al., 1988). 293T cells were transfected with PAK2 and PAK2 (H82L, H85L, K299R) and expression was examined using polyclonal anti-PAK2 antibody. As shown Fig. 16, wild type and mutant form of PAK2 were expressed as 60 kDa form. Endogenous PAK2 was not detected in 293T cells by using polyclonal anti-PAK2 antibody. Slight change of mobility in SDS-PAGE was detected between wild type and mutant PAK2, which may be caused by displacement of amino acids.

To confirm the involvement of PAK2 in Fas-mediated apoptosis, Jurkat cells were co-transfected with tAic2A and wild type PAK2 (WT) or mutant form of PAK2 (H82L, H85L, K299R). Transfectants were selectively collected by panning and the recovered cells were analyzed by flow cytometry. As shown in Fig. 17, co-transfected cells expressing tAic2A were effectively recovered by panning procedure proving that the panning method with anti-Aic2A is powerful to recover transfected cells. Several folds of increase in expression level of PAK2 were observed in the panned cells (Fig. 18). Then, the panned transfectants were treated with CH-11 and proteolytic cleavage of PAK2 was

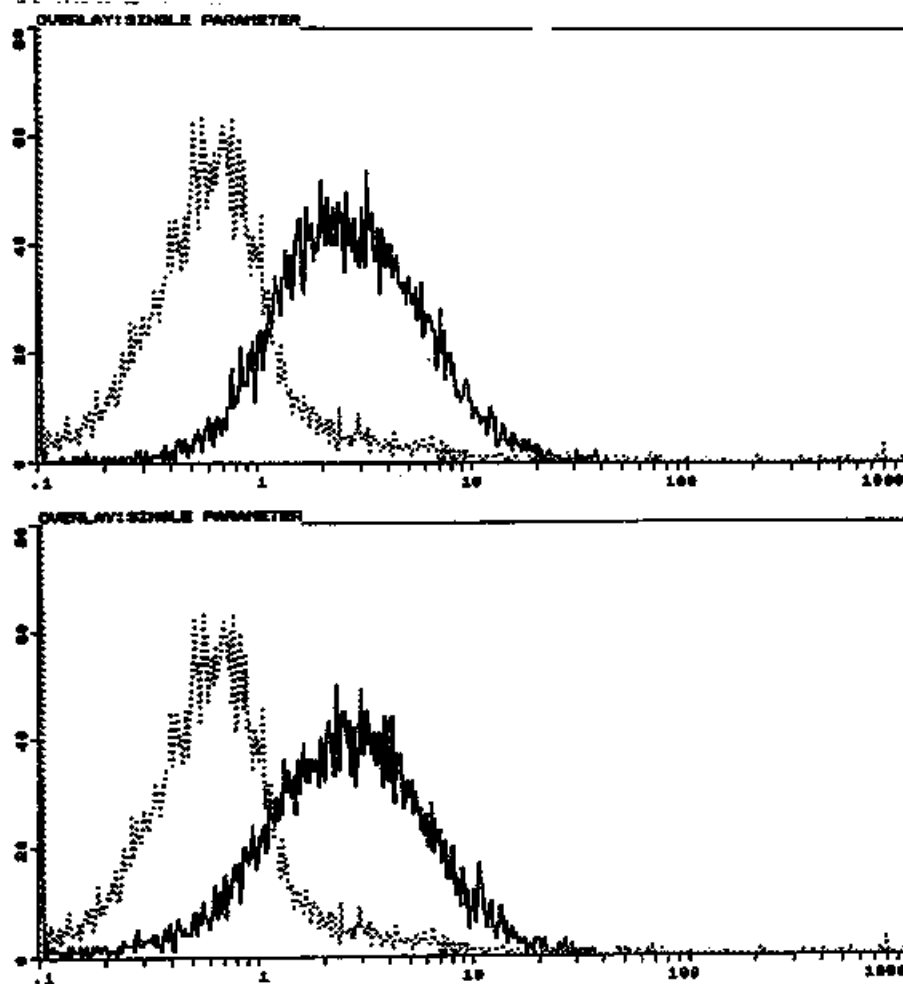


Fig. 17. Panning of transfectants overexpressing PAK2. 40 μ g of vector encoding PAK2 (upper panel) or PAK2 (H82L, H85L, K299R) (lower panel) were co-transfected with 20 μ g of tAic2A into Jurkat cells and panned as described in "Materials and methods". Panned cells were stained with FITC-anti-Aic2A monoclonal antibody and analyzed by flow cytometry.

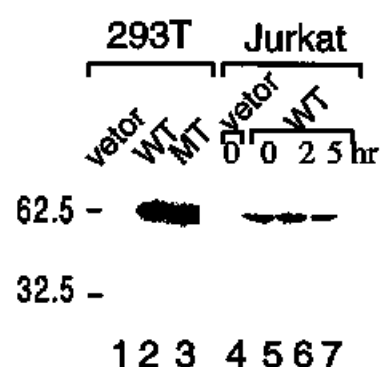


Fig. 18. Expression of overexpressed PAK2. PAK2 (WT) and PAK2 (H82L, H85L, K299R) (MT) were transfected into 293T or Jurkat cells and expression was examined by Western blotting with anti-PAK2 polyclonal antibody. Panned Jurkat transfectants overexpressing PAK2 were stimulated with CH-11 as indicated times. Total cell lysate was used to immunoblotting with anti-PAK2 polyclonal antibody.

examined. Proteolytic fragment of PAK2 could not be detected to 5 hr using polyclonal anti-PAK2 antibody (Santa Cruz) but significant decrease of full-length PAK2 was observed (Fig. 18).

The panned transfectants were stimulated with CH-11 and the effect of PAK2 on apoptosis was investigated. No morphological change was observed to 5 hr between PAK2 (WT) and PAK2 (H82L, H85L, K299R) (Fig. 19). Then, the early apoptotic change was studied by staining of cells with annexin V-FITC. Phosphatidylserine is reportedly translocated from the inner side of the plasma membrane to the outer layer in the early stage of apoptosis, exposing phosphatidylserine on the external cell surface and this process is detected by staining with annexin V, a 38 kDa protein with a strong affinity for phosphatidylserine (Fig. 20). However, no change in the externalization of phosphatidylserine was observed between PAK2 (WT) and PAK2 (H82L, H85L, K299R). Taken together, involvement of PAK2 in Fas-mediated apoptosis was not confirmed in this study. The discrepancy between this experimental result and previous one might derive from the different experimental systems used. In the previous report suggesting the active role of PAK2 in apoptosis, they used Jurkat cells stably expressing the PAK2 under IPTG inducible promoter. On the contrary, in this study, Jurkat cells transiently expressing the PAK2 were used. The role of PAK2 in Fas-mediated apoptosis should be further analyzed with more detailed experiments.

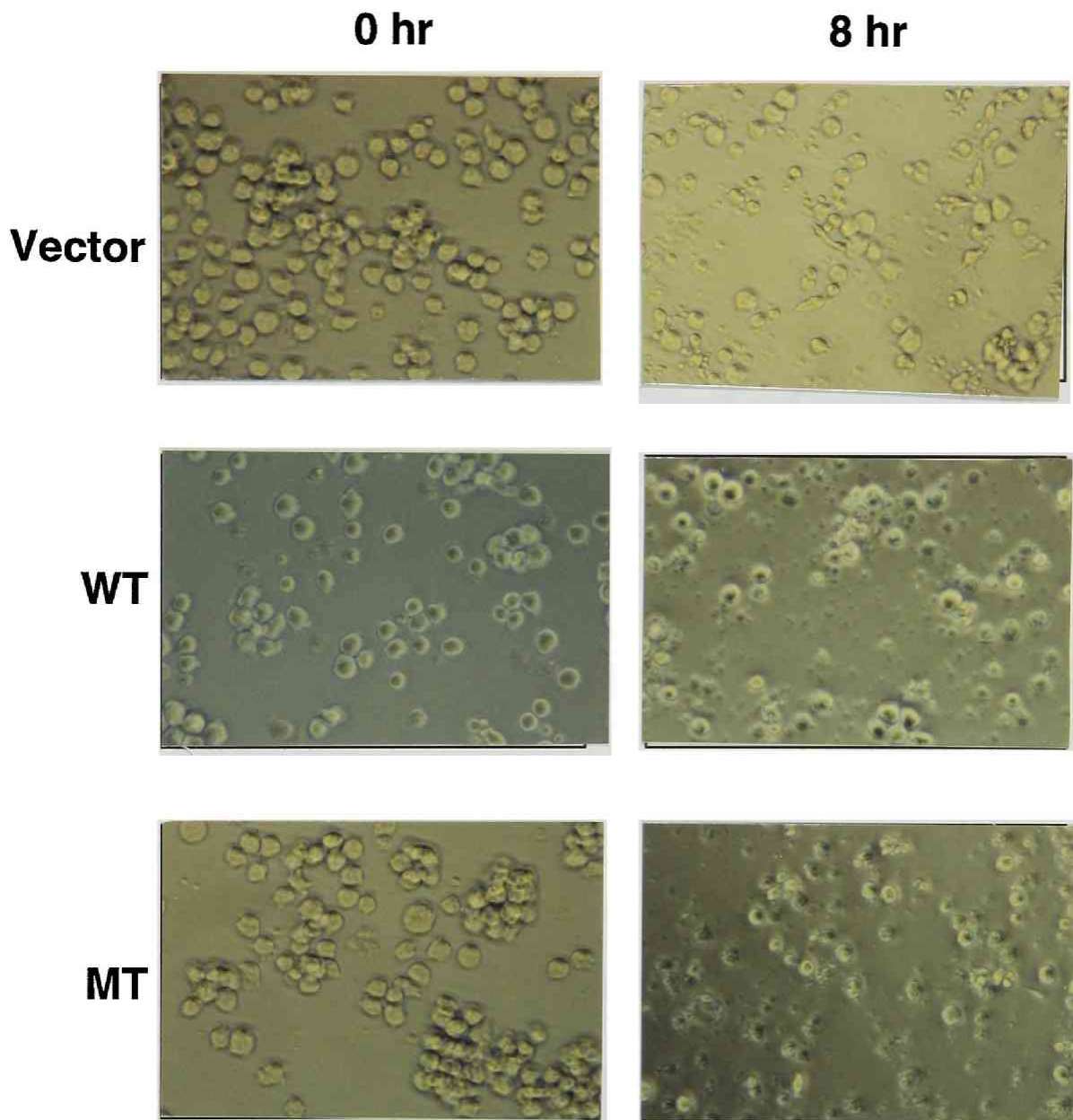


Fig. 19. Overexpression of PAK2 does not effect on apoptotic morphology

The panned transfectants of PAK2 (WT) and PAK2 (H82L, H85L, K299R) (MT) were treated with 0.5 $\mu\text{g/ml}$ of CH-11 and morphological changes was recorded by photography.

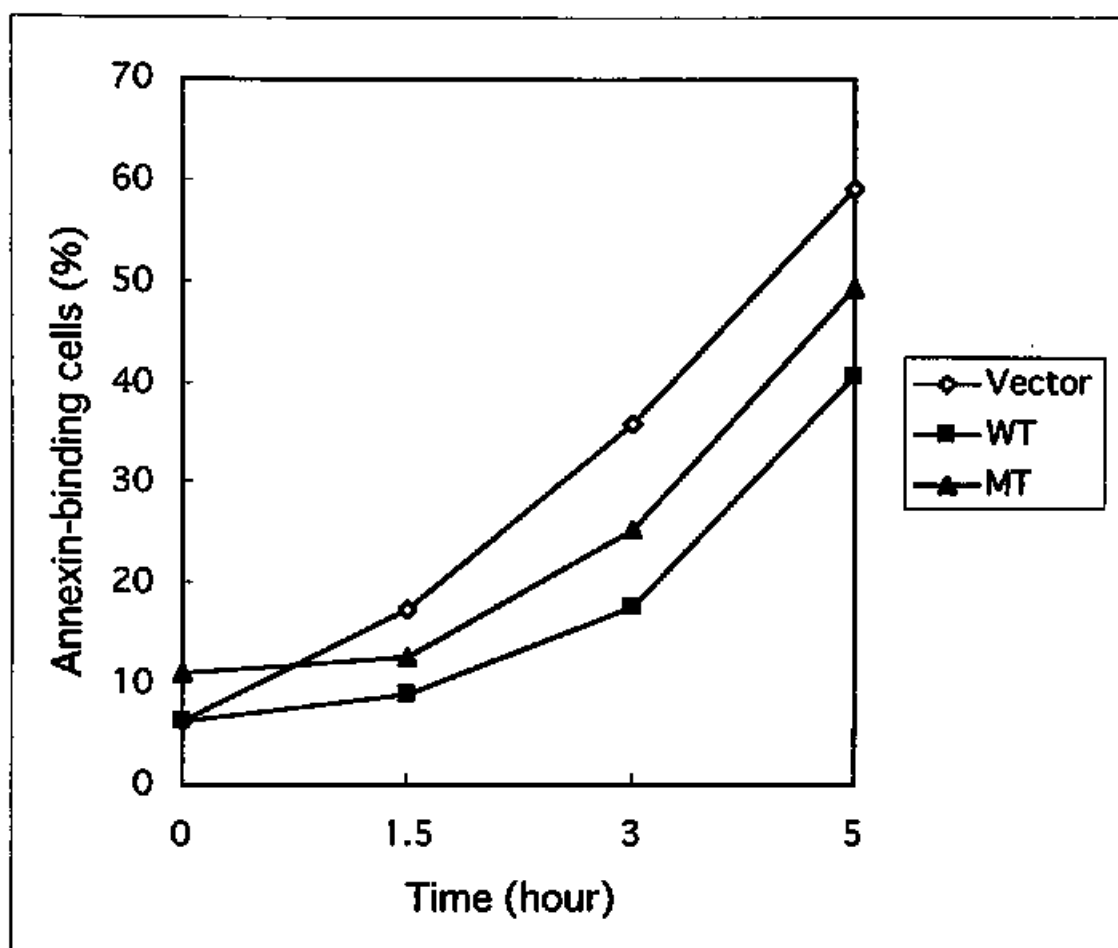


Fig. 20. Overexpression of PAK2 does not effect on the externalization of phosphatidylserine.

The panned Jurkat cells (1×10^5) were treated with CH-11 as in the legend of Fig. 19. Cells were incubated with 1 $\mu\text{g/ml}$ of FITC-annexin V for 10 min and analyzed by flow-cytometry.

5. Discussion

5.1. MST is physiological substrate of caspase

In this study, I have identified and purified a protein kinase with molecular weight of 34 kDa that is activated by the stimulation with CH-11, and the protein kinase was revealed by peptide sequencing to be a cleaved catalytic domain of MST/Krs, a member of the growing family of STE20-related protein kinase. MST was a physiological substrate of caspases and proteolytically activated during the execution of apoptosis. Some previous reports indicated that a kinase with 34 kDa is activated during apoptosis; UV irradiation-induced apoptosis leads to activation of a 36 kDa MBP kinase in HL-60 cells (Lu *et al.*, 1996) and triggering of Fas results in the induction of several kinases including 35 kDa form that phosphorylate c-Jun and histone (Cahill *et al.*, 1996). These reported kinase may be cleaved MST1 and MST2 since both MBP and histone are good substrates for the cleaved MST.

The proteolytic cleavage site of MST1/MST2 (³²³DEMDS³²⁷ and ³¹⁹DELDS³²³) is optimal to caspase-3 and MST1/MST2 is cleaved *in vitro* by recombinant caspase-3, indicating that MST may be a good substrate of caspase-3. MST expressed as FLAG-tagged forms in Jurkat or KB cells were cleaved to kinase-active 34 kDa fragment and the cleavage was inhibited by caspase-3-like-protease inhibitor. Proteolytic cleavage of endogenous MST was also confirmed using monoclonal anti-MST antibody and the cleavage was also blocked by caspase inhibitor in Jurkat cells. Mutant MSTs with the replacement at a putative caspase cleavage site were resistant to the cleavage by apoptotic stimulation. From these results, it is concluded that MST is a physiological substrate of caspase-3-like protease *in vivo* and *in vitro*. Cleavage of MST to 34 kDa fragment is blocked by Ac-DEVD-CHO in Jurkat cells but not inhibited by KB cells. Staurosporine induces the proteolytic cleavage of MST in

Jurkat cells but not in KB cells. These findings suggest that activation and/or expression levels of various caspases may be different from cells to cells even in the same apoptotic stimulus.

MST is cleaved at least by two-step procedure resulting in a kinase-active 34 kDa fragment followed by the generation of 40 kDa. This two-step cleavage might mean that at least two kinds of caspases are involved in the proteolysis of MST. The possibility is not excluded that two-step cleavage results from the different accessibility of caspase to full-length MST and 40 kDa fragment, cleaving to 40 kDa might be a more liable reaction than cleaving to 34 kDa.

5.2 Activation mechanism of MST

MST is an STE20-related kinase and classified in the germinal center kinase (GCK) subfamily (Creasy and Chernoff, 1995a, 1995b). MST and other members of the GCK subfamily have an N-terminal kinase domain and C-terminal regulatory domain. The C-terminal region of MST is reportedly necessary for dimerization and regulates the activity of the N-terminal kinase domain (Creasy *et al.*, 1996). The C-terminal domain of MST may be a negative regulatory domain since its proteolytic removal increases kinase activity markedly (Fig. 8). Recombinant C-terminal deletion mutants of MST1 and MST2 also have higher kinase activity than full-length MST (data not shown). Wild type MST does not phosphorylate histone or MBP in in-gel kinase assay although the cleaved catalytic kinase domain of MST strongly phosphorylates histone and MBP. The catalytic domain of MST (cleaved MST) has undetectable autophosphorylation activity in in-gel kinase assay though full length MST has autophosphorylation activity. Thus, removal of the C-terminal domain increases kinase activity and possibly changes the substrate specificity that may be important in apoptosis. Moreover, removal of the C-terminal

region may deregulate subcellular localization of MST, leading to the phosphorylation of various target proteins.

The protein kinase activity of MST may be regulated by not only proteolysis but also other modifications, since an inactive 34 kDa form of cleaved MST was detected by two-dimensional electrophoresis (Fig. 6C and D). This result indicates that modifications such as phosphorylation or dephosphorylation *in vivo* are critical to the activation of the cleaved form of MST. This possibility is further supported by the fact that MST is greatly activated by the treatment with staurosporine. Staurosporine induces not only proteolysis but also activation of the full-length MST which is not observed in Fas-induced apoptosis

The role of staurosporine in the activation of MST should be further studied. MST was originally purified as staurosporine-activated protein kinase which activity was detected in in-gel kinase assay. (Talyor *et al.*, 1996). However, activity in in-gel kinase assay requiring denaturation and renaturation of kinase may not reflect on the native activity in cells. The possibility that MST might be directly inhibited in cells by staurosporine, a general inhibitor of serine/threonine kinase, could not be excluded. Staurosporine may indirectly regulate activity of MST acting on the upstream activator or regulator of MST.

Another possible activation mechanism of MST is autophosphorylation. Some members of the STE20 family are known to be activated by autophosphorylation although the role of autophosphorylation in the activation of MST is not known. In this study, autophosphorylation activity of MST was shown to greatly increase by the treatment with staurosporine, suggesting the possibility that autophosphorylation is one activation mechanism of MST. MST has high kinase activity when overexpressed in cells and is known to dimerize through the C-terminal domain. These results also may support the

activation of MST by autophosphorylation followed by dimerization. However, optimal activity of MST which might not be obtained by autophosphorylation may require other stimulation as in the case of SOK1, another family of STE20. Taken together, change of phosphorylation state is one possible activation mechanism of MST and function of autophosphorylation should be further analyzed.

The physiological events leading to activation of MST and the biochemical role of MST itself are not known. MST does not reportedly respond to UV irradiation or hypertonic stress but becomes activated by heat-shock of 55°C, and high concentration of sodium arsenite, okadaic acid and staurosporine (Taylor *et al.*, 1996). Some GCK family kinases are reportedly activated by stress; GCK is activated by inflammatory cytokines such as TNF α (Pombo *et al.*, 1995), SOK1 is activated by oxidative stress such as H₂O₂ (Pombo *et al.*, 1996), and the expression level of Sps1p, a member of GCK family in *Schizosaccharomyces pombe*, increases markedly during sporulation, a process that is initiated by nutrient deprivation (Friesen *et al.*, 1994). Thus, GCK family kinases, including MST may be involved in cellular responses to various forms of stress. Therefore, it could not be ruled out that proteolytic activation of MST is necessary for the response to inflammatory stress.

5.3. The role of MST and PAK2 in apoptosis

The role of the cleaved form of MST in apoptosis is still unknown. I transfected various mutants of MST1 and MST2 into Jurkat cells; kinase-negative mutants, caspase-resistant mutants, N-terminal kinase domains and C-terminal regulatory domains, and then the transfected cells were treated with CH-11. However, no detectable changes were observed in apoptotic morphology, cell survival, fragmentation of DNA and nucleus, or the export of phosphatidylserine

Very recently it was reported that PAK2, another member of the STE20 kinase family, is proteolytically activated by caspase, and Jurkat cells that express a dominant-negative PAK2 mutant are resistant to the Fas-induced formation of apoptotic bodies but cause the enhanced externalization of phosphatidylserine (Rudel and Bokoch, 1997). This suggested that caspase-mediated activation of STE20 family kinases regulate some aspects of apoptosis although the role of the cleaved MST in apoptosis is unclear. The role of PAK2 in Fas-mediated apoptosis was not confirmed in this study, using different experimental systems. It is not clear whether the discrepancy in experimental result might derive from the different experimental systems used. More detailed experiment with well-designed system should be further performed to manifest the role of PAK2 in Fas-mediated apoptosis.

PAK has a long N-terminal extension containing p21-binding domain that is different from the C-terminal tail of MST, although the C-terminal kinase domain of PAK is very similar to the N-terminal kinase domain of MST. The differences in fine structure especially in the non-catalytic domain might explain the lack of apparent morphological changes in cells transfected various mutants of MST. There remains the possibility, however, that the cleaved MST plus other intracellular events are involved in the execution of apoptosis downstream of the caspase cascade, and the clarification of the biological and biochemical function of MST may be necessary for the understanding of the role of the cleaved MST in apoptosis.

6. Conclusion

Identification of the substrate protein of caspases and clarification of the biochemical function of the substrates are essential in understanding the molecular mechanism of apoptosis. Here I have identified and purified a protein kinase with molecular weight of 34 kDa that is activated by the stimulation with CH-11, and the protein kinase was revealed by peptide sequencing to be a cleaved catalytic domain of MST/Krs, a member of the growing family of STE20-related protein kinase. MST is added as a new member of the substrate to caspase, growing in number. The function of MST in cells should be clarified to understand the role in apoptosis and this may lead to understand the cellular function of growing kinases of STE20 family.

6. References

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Acknowledgment

I am deeply appreciated to Prof. Yonehara for his continuous guidance, constant encouragement and critical advice throughout this research. The author also wishes to thank to Dr. Murakami and Dr. Sakamaki for general support and advice. I would like to express gratitude to the all the staff and students of the laboratory, especially to Mr. Murakawa for co-working throughout this research. I thank to Dr. Kawashima and Dr. Tsubuki for peptide sequencing. I wish to thank my wife and two sons for their spiritual support, generous understanding and endurance of the unfamiliar foreign life in Japan. I also thank my parent and grandmother for their endless encouragement. The doctoral course of the author was supported by Monbusho scholarship of Japan.

Proteolytic Activation of MST/Krs, STE20-related Protein Kinase, by Caspase during Apoptosis

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Running Title: Proteolytic activation of MST during apoptosis

Key words: Fas (Apo-1/CD95); apoptosis; caspase; protein kinase; signal transduction

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Abstract

The Fas system has been extensively investigated as a model of apoptosis and the caspase cascade has been shown to be a characteristic mechanism of signaling of apoptosis. We have identified and purified a kinase that was activated after the stimulation of Fas on human thymoma-derived HPB-ALL cells. Partial amino acid sequencing of the purified kinase revealed it to be MST/Krs, member of the yeast STE20 family of protein kinases. MST/Krs was activated by proteolytic cleavage and proteolytic activation was blocked by the caspase inhibitor, Z-VAD-FK. A mutant MST with Asp → Asn replacement at a putative caspase cleavage site was resistant to either the proteolytic cleavage or the activation of the kinase activity. These findings suggest that proteolytic activation is one activation mechanism of MST and plays a role in apoptosis.

Introduction

Fas (Fas antigen/CD95/Apo-1) is a type I-membrane protein belonging to the TNF receptor family (Itoh *et al.*, 1991), which includes TNF receptor 1 and 2 (TNFR1 and TNFR2), low affinity nerve growth factor receptor, CD27, CD30, CD40 and OX40 (Nagata and Golstein, 1995). Fas ligand (FasL) also belongs to the TNF family and is synthesized as a type-II membrane protein (Suda *et al.*, 1993). Ligation of Fas with FasL or with agonistic anti-Fas monoclonal antibody induce apoptotic cell death in various cells (Suda *et al.*, 1993; Yonehara *et al.*, 1989; Trauth *et al.*, 1989). Fas-mediated apoptosis plays pivotal roles in T and B cell homeostasis (Nagata and Golstein, 1995; Abbas, 1996; Nagata, 1997); activation-induced death of T cell, maintenance of immune privilege, and deletion of activated or autoreactive B cell. The intracellular domain of Fas contains the death domain required for induction of apoptosis (Nagata and Golstein, 1995). The death domain consists of approximately 70 amino acids and is conserved among several proteins including TNFR, FADD/MORT1, and TRADD (Yuan, 1997). The downstream signals of Fas/FasL have been studied extensively. Triggering of Fas recruits FADD/MORT1 to Fas via interaction between the death domains of Fas and FADD (Boldin *et al.*, 1995; Chinnaiyan *et al.*, 1995). Pro-caspase-8/MACH/FLICE is also recruited to Fas through interaction between death effector domains of FADD and pro-caspase-8, after which several caspases are activated (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Activation of the caspase cascade is a unique biochemical mechanism of apoptosis (Nagata, 1997). Caspases cause apoptosis when overexpressed in cells. Caspase inhibitors such as Z-VAD-FK and Ac-DEVD-CHO can block apoptosis, suggesting that the caspase cascade is an essential mechanism of apoptosis. Known molecular targets of caspases are growing in number; DFF (a heterodimeric protein, triggers DNA fragmentation in cell-free systems after proteolytic activation by caspase-3) (Liu *et al.*, 1997), Lamin (Takahashi

et al., 1996), poly(ADP-ribose) polymerase (PARP), sterol-regulatory element-binding protein (SREBP) (Wang *et al.*, 1996), 70 kDa peptide of U1 snRNP (Casciola *et al.*, 1994), protein kinase C δ (PKC δ) (Emoto *et al.*, 1995), and DNA-dependent protein kinase (DNA-PK) (Casciola *et al.*, 1995) are also cleaved by caspases. It is not known, however, whether the cleavage of these substrates plays a role in apoptosis.

Protein kinases and (or) phosphatases have been suggested to play a role in apoptosis although the caspase cascade is essential to apoptosis. FAP-1 (Sato *et al.*, 1995), a Fas-associated phosphatase, negatively regulates Fas-mediated apoptosis in some cells. PKC δ is proteolytically activated by various apoptotic stimuli. Staurosporine, a serine/threonine kinase inhibitor, induces apoptosis (Jacobsen *et al.*, 1996; Mehlen *et al.*, 1996). Butyrolactone I, a CDK-specific inhibitor, inhibits Fas-mediated apoptosis (Furukawa *et al.*, 1996).

SAPK/JNK or p38 kinase activity increases during apoptosis (Cahill *et al.*, 1996) and activation of SAPK is reportedly necessary for the neuronal apoptosis (Xia *et al.*, 1995). A specific 34 kDa kinase becomes activated by various apoptotic stimuli (Cahill *et al.*, 1996; Lu *et al.*, 1996). Recently, PAK2, a serine threonine kinase whose activity is regulated by Rac and Cdc42, was reported to be activated by caspase-dependent cleavage and suggested to be involved in apoptosis (Rudel and Bokoch, 1997). But the biochemical role of kinase/phosphatase in apoptosis is still unknown.

To investigate the involvement of kinase/phosphatase in Fas-mediated apoptosis, we undertook studies to identify the kinase that was activated during apoptosis. We identified the activity of 34 kDa protein kinase by in-gel phosphorylation assay using histone as substrate. Here, we report that 1) this protein kinase is identical to catalytic domain of MST1/Krs2 and MST2/Krs1, STE20 family kinases, 2) MST1/MST2 is proteolytically activated by apoptotic stimuli and 3) activation is blocked by caspase inhibitor, suggesting proteolytic activation of MST plays a role in generating apoptosis-

inducing signals downstream of caspases.

Results

Identification of protein kinase activity induced during the Fas-mediated apoptosis

Human HPB-ALL thymoma cells were treated with anti-Fas mAb and incubated for varying intervals. Then, we studied the changes of kinase activity using in-gel kinase assay. We detected activity of a histone kinase of 34 kDa after 1 hr incubation with anti-Fas mAb which maximized after 2-3 hr (Fig. 1) The kinetics of activation of the kinase correlated well with DNA ladder formation (Fig. 1A) and the onset of morphologic apoptosis (data not shown). Longer exposure to anti-Fas mAb showed decreased kinase activity, suggesting the loss of cell components by apoptosis. Myelin basic protein (MBP) and c-Jun were weakly phosphorylated by this kinase although casein and MAP2 were not significantly phosphorylated. Autophosphorylation activity of this kinase was not detected in in-gel kinase assay in the absence of substrates (data not shown). The kinase activity was induced with anti-Fas mAb dose-dependently (Fig. 1C). Cytoplasmic extracts of HPB-ALL cells were analyzed by Q-Sepharose chromatography and the kinase activity was eluted at 0.3 M NaCl from the anti-Fas mAb-stimulated but not from unstimulated cell extract. We observed neither increase nor decrease of other histone kinase activities after the stimulation with anti-Fas mAb (data not shown).

The activity of the 34 kDa protein kinase was also induced in HPB-ALL cells by the stimulation of staurosporine or C-2 ceramide in combination with cycloheximide (CHX) with similar time course as anti-Fas mAb, though C-2 ceramide or CHX alone did not induce the activity (Fig. 2B). The activation of the kinase coincided well with the induction of apoptosis, since staurosporine or C-2 ceramide plus CHX induced apoptosis while C-2 ceramide or CHX alone did not, in HPB-ALL cells (Fig. 2A and B). A 60 kDa kinase activity was also induced by the treatment with staurosporine (Fig.

2B). This activity was not inhibited by a caspase inhibitor, Z-VAD-FK, although Z-VAD-FK markedly inhibited the activation of the 34 kDa kinase by the treatment with anti-Fas mAb, C-2 ceramide or staurosporine (data not shown). Ac-DEVD-CHO, an inhibitor for caspase-3-like protease inhibited the activation of the 34 kDa kinase to a lesser extent than Z-VAD-FK, and the inhibition profile correlated well with DNA ladder formation (data not shown), implying that the protein kinase was activated at downstream of Ac-DEVD-CHO-sensitive caspase-3-like protease. In contrast, Ac-YVAD-CHO, an inhibitor for caspase-1-like protease, did not inhibit the activation of the kinase (data not shown) suggesting that Ac-YVAD-CHO-sensitive caspase-1-like protease is not involved in Fas-mediated apoptosis in HPB-ALL cells.

Purification of the 34 kDa protein kinase

The protein kinase activity was purified with stepwise column chromatography procedure; SP Sepharose, Q-Sepharose, butyl Sepharose, Mono Q and Superdex 75 gel filtration column chromatography. Partially purified kinase did not react to anti-PKC δ antibody and the protein kinase activity was not inhibited by PKC specific inhibitors. The 34 kDa protein kinase did not efficiently phosphorylate MBP (amino acid 4-14), a good substrate for PKC δ (data not shown). These results suggest that the protein kinase with 34 kDa is not a cleaved form of the PKC δ with 40 kDa (Emoto *et al.*, 1995).

Active fractions from the final Superdex 75 column chromatography were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and distribution of kinase activity and protein profile were observed by in-gel kinase assay and silver staining, respectively (Fig. 3). No histone kinase activity of higher molecular weight was detected. Further separation was performed by two-dimensional electrophoresis. In-gel phosphorylation assay showed three or four distinctive spots of kinase activity with slightly different isoelectric

points (pI), ranging from pI 5.0 to pI 5.5 (Fig. 3D). Silver staining showed, however, 6 distinct protein spots with 34 kDa and the same pI range. Four of the 6 spots co-migrated with the kinase activity while the other two spots with 34 kDa were not associated with kinase activity (Fig. 3C). Figure 3C shows the other spots of protein; two spots with 40 kDa and consecutive spots with 60-70 kDa. The protein with 40 kDa was observed also in Figure 3A, and the consecutive spots of high molecular weight might be cytokeratins contaminated during isoelectric focusing.

The four protein spots that coincided well with the kinase activity were divided into halves by a slight difference of pI range. Then, the two pairs of the four protein spots, and one pair of the remaining 2 spots of the same molecular weight were separately recovered from polyacrylamide gel and digested partially with V8 protease. Resulting peptides were separated by SDS-PAGE and subjected to peptide sequencing. The peptide sequences ISIM T/Q T/Q H/C D S/Q PYVVKY and IGYNXVADI were obtained from all the 3 pairs of protein spots. A BLAST search of the sequences revealed complete identity with the well-conserved catalytic domain of protein kinases, MST1/Krs2 and MST2/Krs1, closely related members of the kinase family of STE20 (Fig. 4A), suggesting that the 6 distinct protein spots with 34 kDa and the same pI range may be composed of MST1 and MST2. Amino acid sequences of MST/Krs (Fig. 4A) suggest that caspase-specific cleavage sites exist at the end of the kinase domain, ³²³DEMDS³²⁷ (MST1) and ³¹⁹DELDS³²³ (MST2) (Creasy and Chernoff, 1995a). The calculated molecular mass of cleaved kinase domain is 35 kDa. These results show that our purified protein kinase is the catalytic domain of MST/Krs, cleaved by caspases. Interestingly, the remaining pair of spots with 34 kDa which had no kinase activity in in-gel phosphorylation assay and were slightly different in pI from the kinase-active spots, were also identified as MST/Krs.

Proteolytic activation of MST/Krs by caspase during apoptosis

To examine the proteolytic cleavage of MST/Krs, KB cells were transfected with FLAG-tagged MST1 and MST2 and then apoptosis was induced by the treatment with 0.5 $\mu\text{g/ml}$ anti-Fas mAb (CH-11) together with 25 $\mu\text{g/ml}$ CHX. The immunoprecipitates from the cell lysate with anti-FLAG antibody were analyzed by immunoblotting and in-gel phosphorylation assay. As expected, FLAG-tagged MST1/MST2 were cleaved to kinase-active 34 kDa fragment by the treatment with anti-Fas mAb time-dependently (Fig. 4). We prepared FLAG-tagged mutants of MST1 (D326N) and MST2 (D322N) in which aspartic acid, a conserved recognition amino acid of caspases was mutated to asparagine. These mutated MST1 and MST2 were also introduced into KB cells by electroporation. Mutant MST1 (D326N) and MST2 (D322N) were never cleaved up to 5 hr in KB cells, showing that Asp³²⁶ (MST1) and Asp³²² (MST2) are cleavage sites (Fig. 4B). The same result was obtained in Jurkat cells treated with 0.5 $\mu\text{g/ml}$ anti-Fas mAb (Fig. 5A, right panel and unpublished data).

The proteolytic fragment of MST1/MST2 phosphorylated histone (Fig. 4C, upper panel) and MBP in in-gel kinase assay (data not shown) but we did not observe autophosphorylation activity of the 34 kDa fragment (Fig. 4C, lower panel). In contrast, full length-MST1/MST2 had weak autophosphorylation activity but did not phosphorylate histone or MBP (Fig. 4B and C). Autophosphorylation activity of full-length MST1/MST2 was not significantly activated by the treatment with anti-Fas mAb (Fig. 4C). We analyzed the activation of JNK and p38 protein kinase activity in KB and Jurkat cells transfected with MST1/MST2 and the 34 kDa form of MST, showing that JNK and p38 activity was not elevated by the proteolytic activation of MST (data not shown).

The cleavage of FLAG-tagged MST was significantly inhibited by Z-VAD-FK in both KB and Jurkat cells, indicating that caspases cleave MST (Fig. 5A).

Ac-DEVD-CHO, however, inhibited very weakly while Ac-YVAD-CHO did not inhibit at all, the proteolytic activation of MST in KB cells (Fig. 5A, left panel). In contrast, Ac-DEVD-CHO inhibited considerably the proteolytic activation of MST in Jurkat (Fig. 5A, right panel). These results suggest that different sets of caspases may be activated in Jurkat and KB cells after the stimulation with anti-Fas mAb. We analyzed the *in vitro* effect of the recombinant caspase-3 to FLAG-tagged MST. Both MST1 and MST2 were directly cleaved to 34 kDa fragments with kinase activity (data not shown), suggesting that MST is a physiological substrate of caspases.

The proteolytic cleavage of MST coincided well with the induction of apoptosis. Overexpressed recombinant MST was cleaved by the treatment with TNF α in combination with CHX but we could not detect the cleavage form of the recombinant MST after stimulation with UV, etoposide, staurosporine or C-2 ceramide in KB cells (Fig 5B). Overexpressed recombinant MST in Jurkat cells, however, was cleaved by the treatment with staurosporine but not by UV, etoposide and C-2 ceramide (Fig 5B). These results might mean that activation and/or expression levels of various caspases are different in KB and Jurkat cells.

Discussion

We show, here, that a protein kinase with Mr 34 kDa is activated by the stimulation with anti-Fas mAb, and the protein kinase is a cleaved catalytic domain of MST/Krs. MST is a physiological substrate of caspases and proteolytically activated during the execution of apoptosis. Some previous reports indicated that a kinase with 34 kDa is activated during apoptosis; UV irradiation-induced apoptosis leads to activation of a 36 kDa MBP kinase in HL-60 cells (Lu *et al.*, 1996) and triggering of Fas results in the induction of several kinases including 35 kDa form that phosphorylate c-Jun and histone (Cahill *et al.*, 1996). These reported kinase may be cleaved MST1/MST2 since both MBP and histone are good substrates for the cleaved MST. PAK2, another member of the STE20 kinase family is also reportedly cleaved to active 34 kDa fragment by caspase in Fas-mediated apoptosis (Rudel and Bokoch, 1997). The fragment of PAK2, however, was not detected in our purified fraction suggesting that the intracellular content of MST may be much higher than PAK-2 or MST may be more favorable substrate of caspase, or catalytic domain of MST and PAK2 may be separable by purification procedures.

The proteolytic cleavage site of MST1/MST2 (³²³DEMDS³²⁷ and ³¹⁹DELDS³²³) is optimal to caspase-3 and MST1/MST2 were cleaved by recombinant caspase-3, indicating that MST may be a good substrate of caspase-3. Identification of the substrate protein of caspases and clarification of the biochemical function of the substrates are essential in understanding the molecular mechanism of apoptosis. In this points, biological and biochemical functions of cleaved MST must be explained.

MST is an STE20-related kinase and classified in the germinal center kinase (GCK) subfamily (Creasy and Chernoff, 1995a, 1995b). MST and other members of the GCK subfamily have an N-terminal kinase domain and C-terminal regulatory domain. The C-terminal region of MST is reportedly

needed for dimerization and regulates the activity of the N-terminal kinase domain (Creasy *et al.*, 1996). The C-terminal domain of MST may be a negative regulatory domain since its proteolytic removal increases kinase activity markedly (Fig. 4). Recombinant C-terminal deletion mutants of MST1 and MST2 also have higher kinase activity than full-length MST (data not shown). Wild type MST does not phosphorylate histone or MBP in in-gel kinase assay although the cleaved catalytic kinase domain of MST strongly phosphorylates histone and MBP. The catalytic domain of MST (cleaved MST) has undetectable autophosphorylation activity in in-gel kinase assay though full length MST has autophosphorylation activity. Thus, removal of the C-terminal domain increases kinase activity and changes the substrate specificity which may be important in apoptosis. Moreover, removal of the C-terminal region may deregulate subcellular localization of MST, leading to the phosphorylation of various target proteins.

The protein kinase activity of MST may be regulated by not only proteolysis but also other modifications, since we detected an inactive 34 kDa form of cleaved MST by two-dimensional electrophoresis (Fig. 3C and D). This result indicates that modifications such as phosphorylation or dephosphorylation *in vivo* are critical to the activation of the cleaved form of MST. The physiological stimuli leading to activation of MST and the biochemical role of MST itself are not known. MST does not reportedly respond to UV irradiation or hypertonic stress but becomes activated by heat-shock of 55°C, and high concentration of sodium arsenite, okadaic acid and staurosporine (Taylor *et al.*, 1996). Some GCK family kinases are reportedly activated by stress; GCK is activated by inflammatory cytokines such as TNF α (Pombo *et al.*, 1995), SOK1 is activated by oxidative stress such as H₂O₂ (Pombo *et al.*, 1996), and the expression level of Sps1p, a member of GCK family in *Schizosaccharomyces pombe*, increases markedly during sporulation, a process that is initiated by nutrient deprivation (Friesen

et al., 1994). Thus, GCK family kinases, including MST may be involved in cellular responses to various forms of stress. Therefore, it could not be ruled out that proteolytic activation of MST is necessary for the response to inflammatory stress.

Recently it was reported that PAK2, another member of the STE20 kinase family, is proteolytically activated by caspase, and Jurkat cells that express a dominant-negative PAK2 mutant are resistant to the Fas-induced formation of apoptotic bodies but cause the enhanced externalization of phosphatidylserine (Rudel and Bokoch, 1997). This suggests that caspase-mediated activation of STE20 kinases regulate some aspects of apoptosis although the role of the cleaved MST in apoptosis is unclear. PAK has a long N-terminal extension containing p21-binding domain which is different from the C-terminal tail of MST, although the C-terminal kinase domain of PAK is very similar to the N-terminal kinase domain of MST. The differences in fine structure especially in the non-catalytic domain might cause different biological activity of MST and PAK2. It is necessary to clarify whether the cleaved MST is involved in the execution of apoptosis downstream of the caspase cascade, and the clarification of the biological and biochemical function of MST may be necessary for the understanding of the role of the cleaved MST in apoptosis.

Materials and methods

Purification of the 34 kDa protein kinase

Human thymoma-derived cell line, HPB-ALL was cultured to 5×10^5 cell/ml in 40 liter of RPMI 1640 (Nissui, Tokyo) supplemented with 10% fetal calf serum (FCS), 20 mM HEPES (pH 7.3), 50 μ M 2-mercaptoethanol, 50 unit/ml penicillin, and 50 μ g/ml streptomycin. Cells were concentrated to a density of 2.5×10^7 cells/ml and stimulated with 1 μ g/ml anti-Fas mAb CH-11 (Yonehara *et al.*, 1989), for 2 hr. After washing once with cold phosphate-buffered saline (PBS), cells were harvested and frozen in liquid nitrogen. Cell pellets were thawed on ice and suspended with 200 ml of 20 mM Tris·HCl (pH 7.5) containing 10% glycerol, 50 mM NaF, 10 mM β -glycerol phosphate, 2 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μ g/ml aprotinin (buffer IA) and cells were homogenized in Dounce homogenizer with 10 strokes. After centrifugation for 1 hr at $100,000 \times g$ at 4°C, the supernatant was loaded onto SP Sepharose FF (40 ml). After adding NaCl to 0.15 M, 1/3 of the flow-through fraction was loaded onto HiPrep 16/10 Q Sepharose FF. After washing with buffer QA (buffer IA + 0.15 M NaCl), the column was eluted with a 200-ml linear gradient of 0.15 M to 0.5 M NaCl. The column was run three times and eluted fractions were assayed with in-gel kinase assay (see below) using histone (Sigma) as substrate. The protein kinase fractions were pooled and 1/8 the volume of saturated ammonium sulfate in 20 mM Tris·HCl (pH 7.5) containing 50 mM NaF, 10 mM β -glycerol phosphate, 2 mM EDTA, 0.1 mM DTT, 0.1 mM vanadate, 0.1 mM PMSF and 5 μ g/ml aprotinin (buffer BA) was slowly added with gentle stirring and further incubated for 30 min on ice. After centrifugation for 30 min at $10,000 \times g$ at 4°C, 1/2 the volume of the supernatant was loaded onto butyl Sepharose FF (7 ml). The column was eluted with buffer BA containing 50% ethylene glycol. The column was run twice and the kinase-active fractions were

dialyzed against buffer QA. Then, the active fractions were loaded onto a Mono Q column and eluted with a 20 ml gradient of 0.15 M to 0.5 M NaCl. Activity fractions were concentrated to 2.5 ml by vacuum evaporation and resolved on Hiload 16/60 Superdex 75. Aliquots of the kinase fraction were subjected to two-dimensional electrophoresis and analyzed by silver staining and in-gel kinase assay.

In-gel kinase assay

In-gel kinase assay was performed as described previously (Kameshita and Fujisawa, 1989). In brief, gels for SDS-PAGE were prepared with 0.5 mg/ml of substrate protein co-polymerized in the resolving gel. After electrophoresis, gel was incubated for 30 min twice with wash buffer [50 mM Tris·Cl (pH 7.4), 5 mM β -mercaptoethanol and 20% isopropanol]. The gel was denatured by incubating in two changes of 6 M guanidine·HCl in wash buffer for 30 min each, followed by renaturation by incubating the gel overnight in 0.04% Tween 40 in wash buffer at 4°C. The renatured gel was then equilibrated with kinase reaction buffer [40 mM HEPES (pH 7.4), 5 mM MgCl₂, 2 mM DTT and 0.1 mM EGTA] for 10 min at room temperature. The kinase reaction was initiated by the addition of 25 μ Ci of [γ -³²P] ATP and the gel was incubated at room temperature for 1 hr. The reaction was terminated by washing the gel extensively with 1% pyrophosphate in 5% trichloroacetic acid. The gel was dried and subjected to autoradiography.

Peptide sequencing of the protein kinase

Following the final Superdex 75 column chromatography, the kinase-active fractions were pooled and concentrated by vacuum evaporation. After trichloroacetic acid precipitation and washing with ice-cold acetone, precipitate was dissolved in 8 M urea, 65 mM DTT, 2% pharmalyte 3-10 (Pharmacia), 0.5% Triton X-100 and 0.1% SDS, and resolved by two-

dimensional electrophoresis using Multiphor II system (Pharmacia) and Investigator (Millipore). Protein spots which comigrated with protein kinase activity in the in-gel kinase assay were recovered and partially digested with V8 protease (Boehringer Mannheim). Peptides were resolved on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (ABI). Resulting peptides were subjected to amino acid sequence analysis.

Plasmid constructs

MST cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) from human HPB-ALL total RNA by using LA-PCR (Takara, Kyoto). For cloning of MST2 primers, 5' GTAGGATCCATGGAGCAGCCGCCGGCGCCT-3' and 5' GTAGAATTCGGGAATTTACCTGGGCATGTACCATTGTCA-3' were used. Primers, 5'-GTAGGATTCATGGAGACGGTACAG-3' and 5' GTAGAATTCTGGCTAACAAACATGAGGC-3' were used for cloning of MST1. PCR products were double-digested with BamHI/EcoRI and subcloned into pBluescriptII-SK and confirmed by sequencing. FLAG-tagged full-length MST and C-terminal deletion spanning amino acid 1-299 (MST1) and amino acid 1-300 (MST2) were generated by PCR. The coding region was inserted into mammalian expression vector pME18S (Sakamaki *et. al.*, 1992) as FLAG-tagged forms. Point mutants of MST were obtained by Quick-Change site-directed mutagenesis (Stratagene) and confirmed by sequencing.

Cell culture and transient transfection

Jurkat and HPB-ALL cells were cultured in RPMI 1640 with 10% FCS, 20 mM HEPES (pH 7.3), 50 μ M 2-mercaptoethanol, 50 unit/ml penicillin and 50 μ g/ml streptomycin. KB cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine fetal calf serum (FCS), and 100 μ g/ml kanamycin. Jurkat (1×10^7 cells) cells, grown to 2×10^5 cells/ml

and KB cells, grown in 10-mm dishes to 70% confluency were transfected with expression vectors encoding various forms of MST1 and MST2 by electroporation at 300 volt with a capacitance of 960 μ F using Gene Pulser (Bio-Rad). Cells were incubated for 48 hr at 37°C and then treated with various stimulants.

Analysis of DNA fragmentation by agarose gel electrophoresis

Cells (1×10^6) were lysed in 100 μ l of 10 mM Tris-HCl (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. Supernatant was mixed with 40 μ g of RNase A and incubated at 37°C for 1 hr. After incubation with 40 μ g of proteinase K at 37°C for 1 hr, the digested sample was precipitated with 20 μ l of 5 M NaCl and 120 μ l of isopropanol. The DNA was dissolved in 10 mM Tris-HCl (pH 8.0) containing 10 mM EDTA and separated in 1.5% agarose gels.

Western blot analysis

Cellular total proteins were (30 μ g) separated by SDS-PAGE and transferred to PVDF membrane (Millipore). The membranes were blocked in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20 (TBST) with 5% skim milk at room temperature for 1 hr. Antibodies were applied in TBST containing 5% skim milk at appropriate dilutions for 1 hr. The membranes were then washed with TBST and incubated for 1 h with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham) or rabbit anti-goat IgG (Organon Teknika Co.). The specific signals were detected on X-ray films using an enhanced chemiluminescence (ECL) detection system (Amersham).

MST kinase assay

Various forms of FLAG-tagged MST were immunoprecipitated from total

cell lysate with 2 μ g of anti-FLAG M2 antibody (Kodak) and protein G-Sepharose (Pharmacia) for 2 hr at 4°C, and then washed extensively. Same amounts of immunoprecipitates as analyzed by Western blot were subjected to in-gel kinase assay using 0.5 mg/ml of histone (Sigma), MBP (Sigma) or glutathione S-transferase (GST)-cJun (1-79) as substrates. For immune complex kinase assay, immunoprecipitates were incubated with 5 μ g of substrate in 20 μ l of kinase reaction buffer [40 mM HEPES (pH 7.5) with 20 mM MgCl_2 , 20 mM β -glycerol phosphate and 0.1 mM vanadate] containing 25 μ M ATP and 2.5 μ Ci of [γ - ^{32}P] ATP for 20 min at 30°C. Reactions were terminated by adding 7 ml of 4 $\times$ Laemmli's sample buffer and boiling for 5 min. A portion of the sample (15 μ l) was separated on a 12% SDS-polyacrylamide gel and autoradiographed.

Acknowledgments

This work was supported in part by a Grant-in-Aid for cancer research from the Ministry of Education, Science and Culture of Japan

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Legends to figures

Fig. 1. Activation of 34 kDa protein kinase in Fas-induced apoptosis. HPB-ALL cells were treated with 1 $\mu\text{g/ml}$ anti-Fas mAb (CH-11) and harvested at the indicated times. Cell lysate, prepared as described in "Materials and methods", was resolved on 1.5% agarose and DNA fragmentation was monitored by staining with ethidium bromide (A), or resolved on 12% polyacrylamide gel containing 0.2 mg/ml histone as substrate and analyzed by autoradiography (B). (C), HPB-ALL cells were treated with indicated amounts of anti-Fas mAb and harvested at 2 hr. Total cell lysate was subjected to in-gel kinase assay using histone as substrate and the kinase activity with 34 kDa was measured after autoradiography.

Fig. 2. The 34 kDa protein kinase is activated by various apoptotic stimuli and inhibited by caspase inhibitor. HPB-ALL cells were preincubated for 1 hr with (+) or without (-) cycloheximide (CHX, 25 $\mu\text{g/ml}$), and with 1% DMSO (-) or Z-VAD-FK (25 μM). Then anti-Fas mAb (1 $\mu\text{g/ml}$), C-2 ceramide (100 μM) or staurosporine (1 μM) was added for 2 hr. The stimulated cells were resolved on 1.5% agarose gel (A) or subjected to in-gel kinase assay (B) as described in the legend to Figure 1.

Fig. 3. Purification of the 34 kDa kinase. (A) Fractions from Superdex 75 gel filtration column chromatography were analyzed by SDS-PAGE and silver-stained. (B) The same fractions were analyzed by in-gel kinase assay using histone as substrate. Kinase-active fractions from fraction 22 to 32 were analyzed by 2-dimensional electrophoresis. Gels were silver-stained (C) or subjected to in-gel kinase assay (D).

Fig. 4. Proteolytic activation of MST. (A) Schematic representation of the primary structure of MST1 and MST2. The determined peptide sequences of purified protein kinase and amino acid sequences of MSTs around them are indicated. Amino acid sequences of MSTs around the putative cleavage sites by caspase are also shown. Putative recognition sites are boxed and cleavage sites are indicated by arrow. (B and C) KB cells were transfected with empty vector (vector alone), FLAG-tagged wild type (WT) of MST, mutant MST1 (D326N), and mutant MST2 (D322N). After 48 hr of transfection, cells were treated with anti-Fas mAb (0.5 μ g/ml) and CHX (25 μ g/ml) for the indicated periods. FLAG-tagged MST was immunoprecipitated with anti-FLAG M2 antibody and analyzed by immunoblotting with anti-FLAG M2 antibody (B) or subjected to in-gel phosphorylation assay using polyacrylamide gels containing histone (C, upper panel) or no substrate (C, lower panel).

Fig. 5. Proteolytic activation of MST is induced by various apoptotic stimulations and inhibited by caspase inhibitors. (A, left panel), KB cells were transfected with FLAG-tagged MST and 48 hr after the transfection, cells were preincubated for 1 hr with 1% DMSO (-) or caspase inhibitors, Z-VAD-FK (25 μ M), Ac-DEVD-CHO (200 μ M), Ac-YVAD-CHO (200 μ M). Then, cells were treated with anti-Fas mAb (0.5 μ g/ml) + CHX (25 μ g/ml) for 5 hr at 37°C. FLAG-tagged MST was immunoprecipitated and analyzed by immunoblotting as described in the legend to Fig. 4. (A, right panel), FLAG-tagged wild type (WT) or mutant (D326N) MST1 was transfected into Jurkat cells. Transfectants were preincubated with caspase inhibitors and treated with anti-Fas mAb for 3 hr. Cell lysate was resolved on 10% SDS-PAGE and immunoblotted with anti-FLAG M2 antibody. (B), Jurkat cells (left panel) or KB cells (right panel) were transfected with FLAG-MST1, and stimulated with TNF α (20 ng/ml), staurosporine (1 μ M),

C-2 ceramide (50 μM), etoposide (100 μM), or UV (250 J/m², 254 nm, 15 min). Cells were incubated for indicated periods at 37°C and total cell lysates were analyzed by immunoblotting.

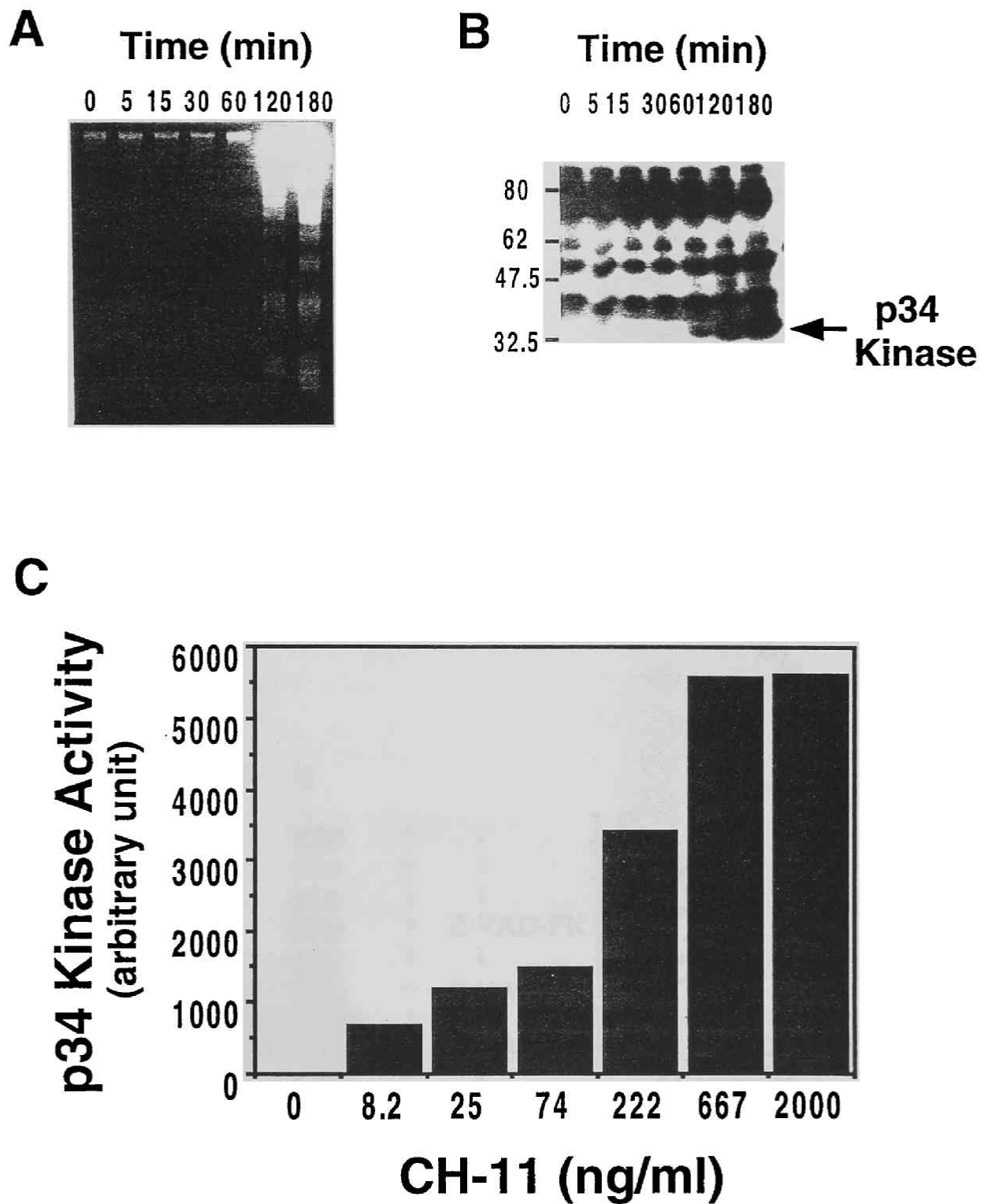
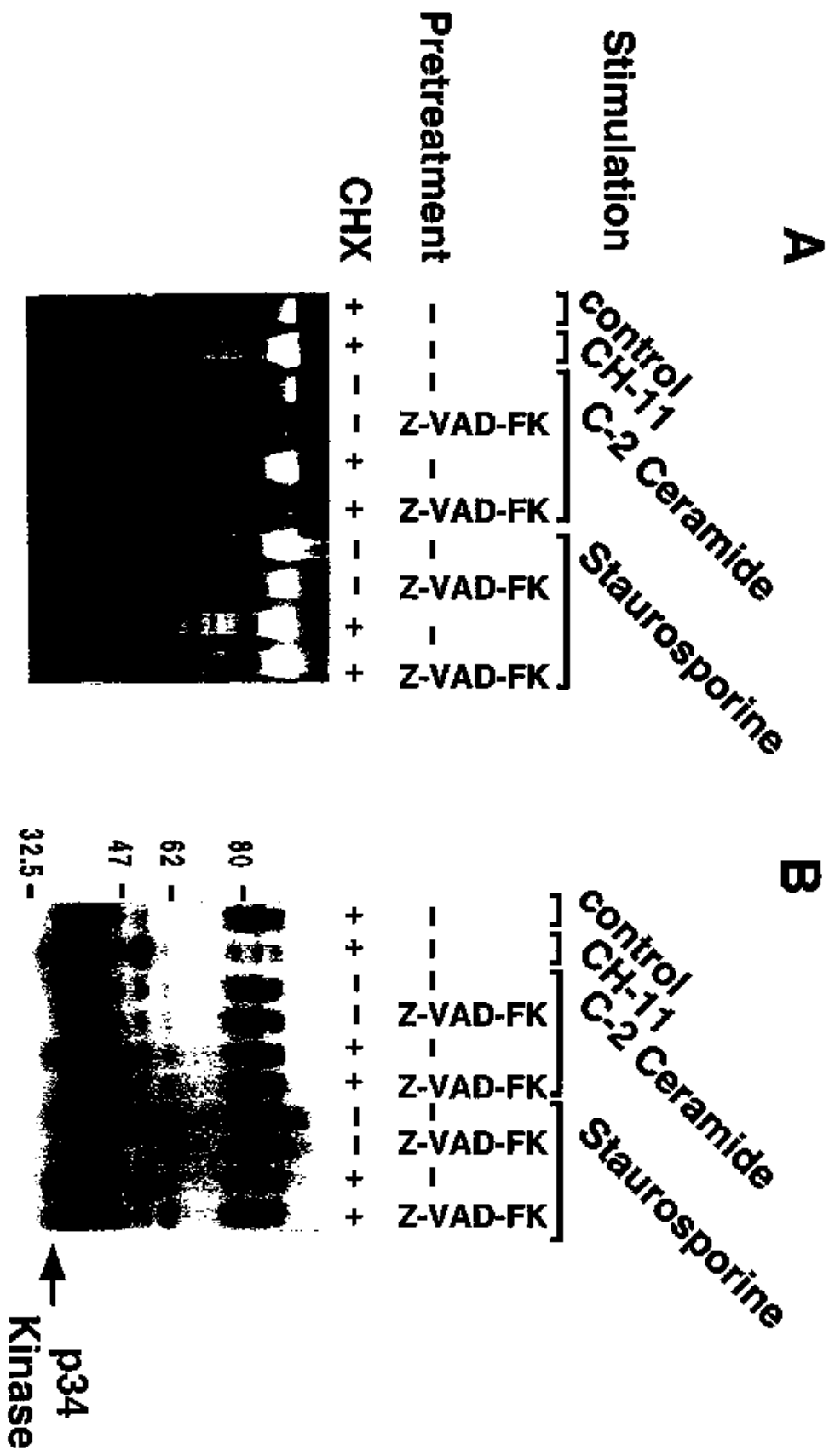
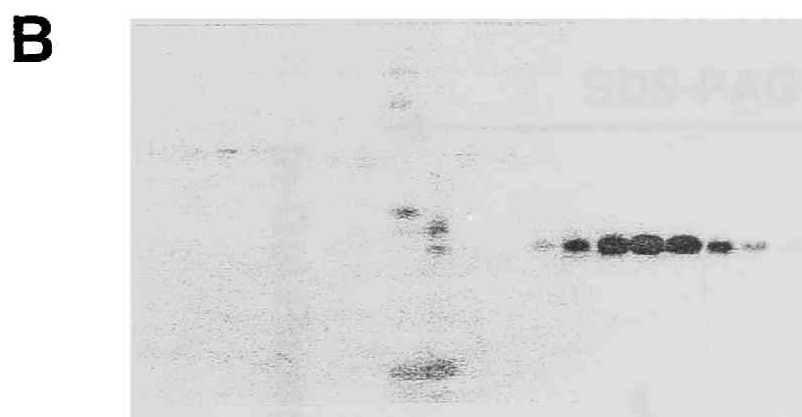
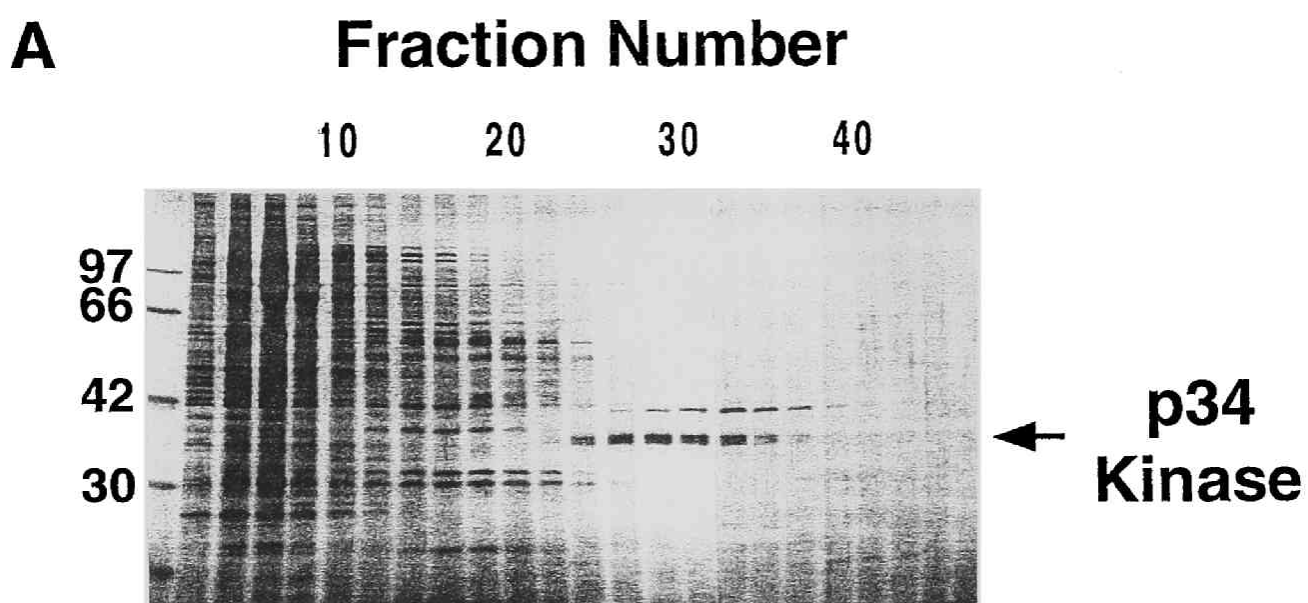
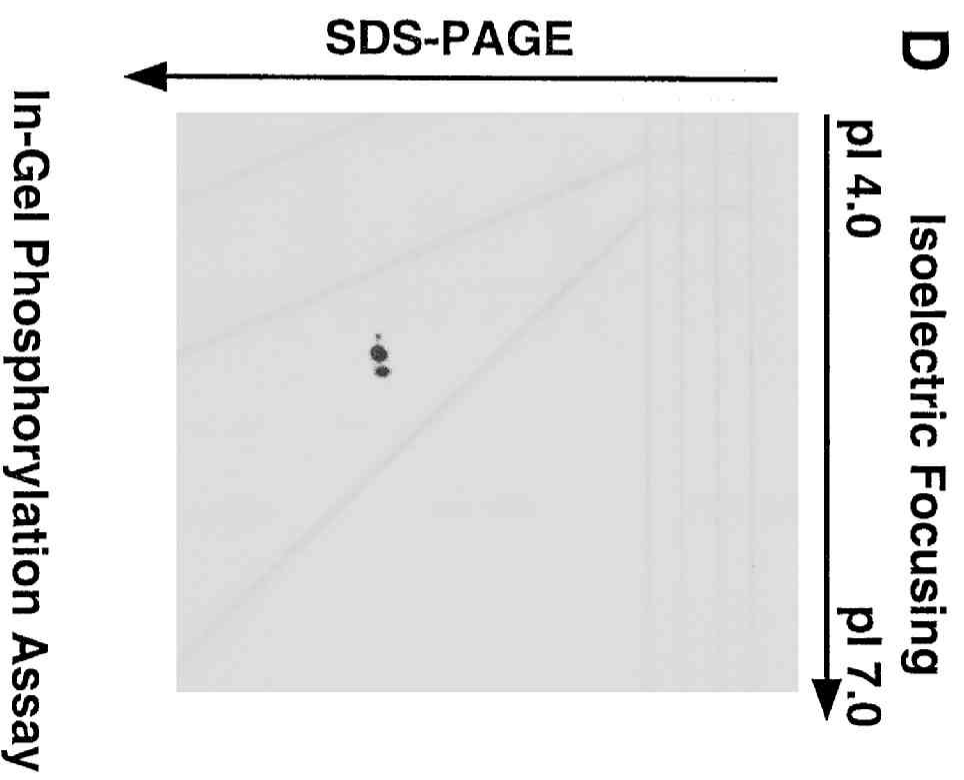
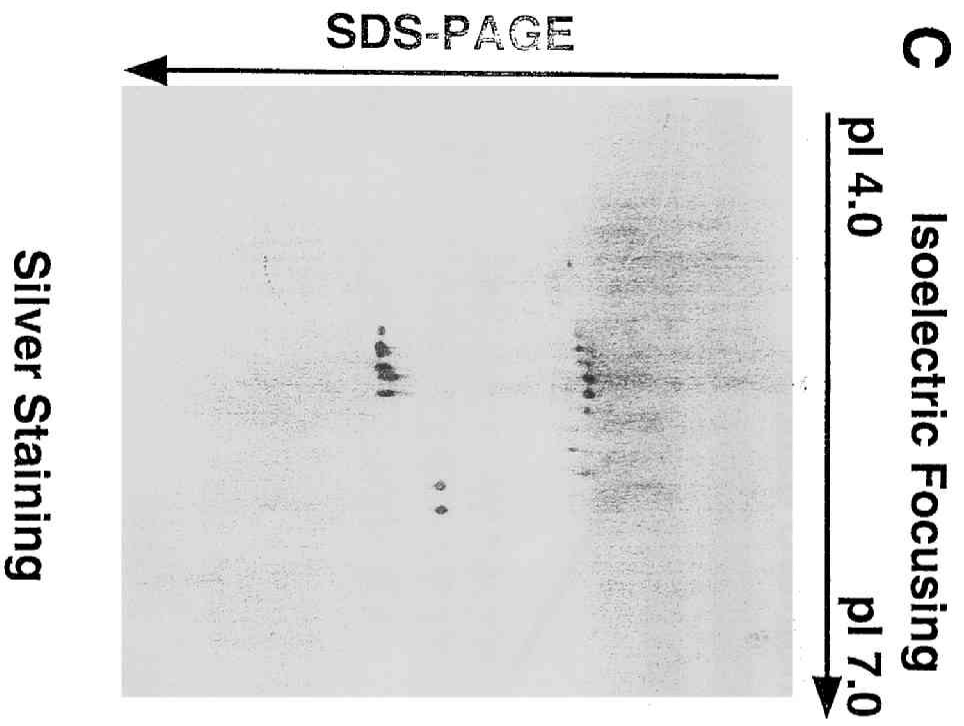
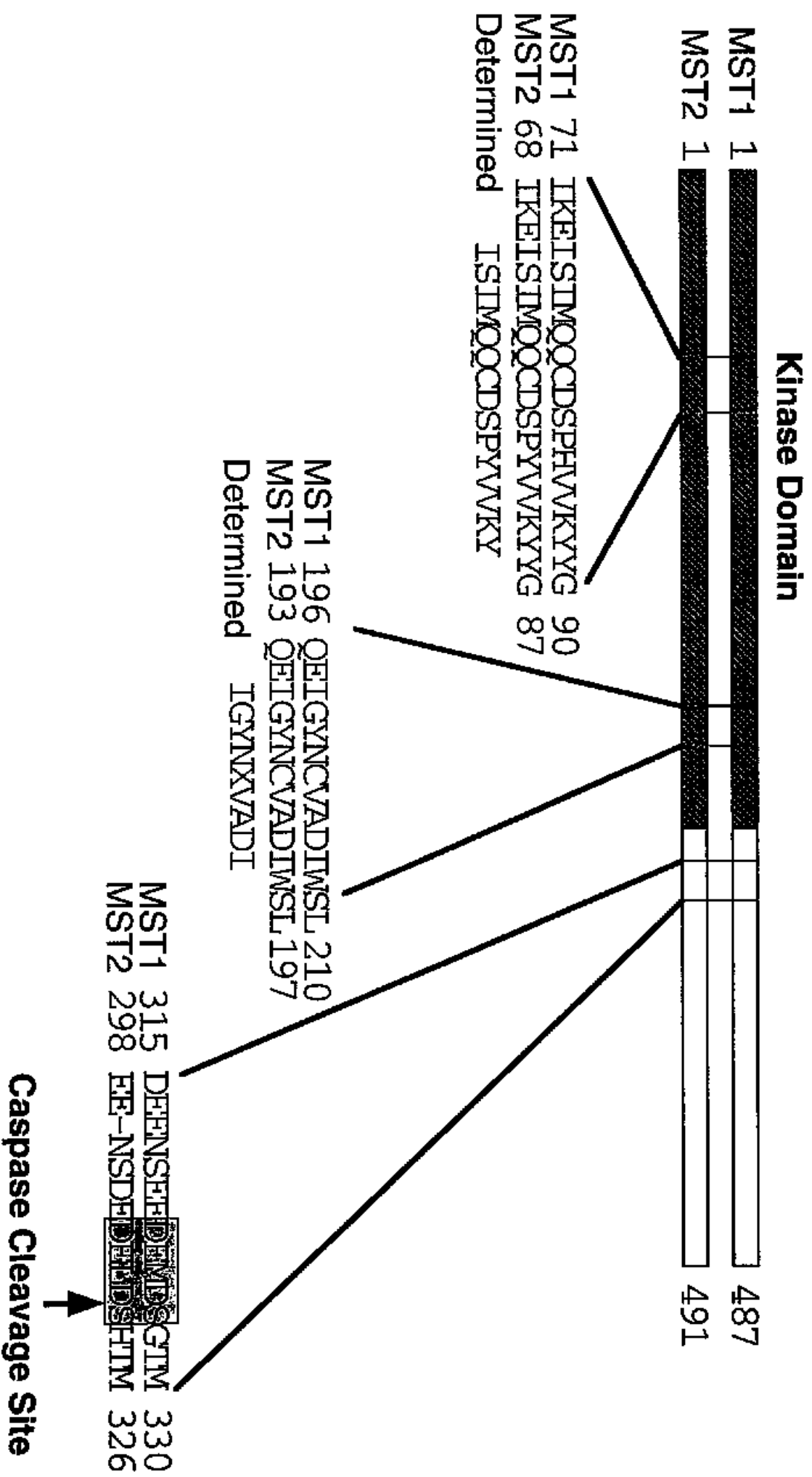


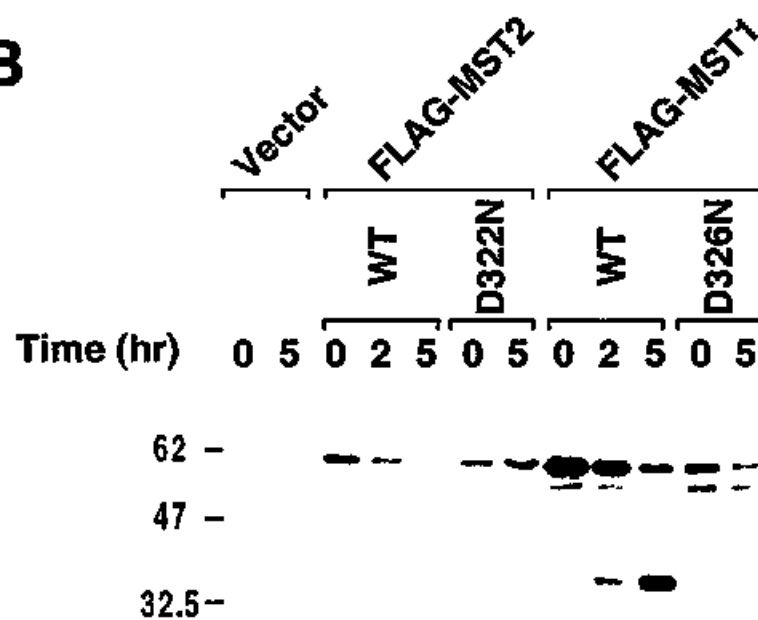
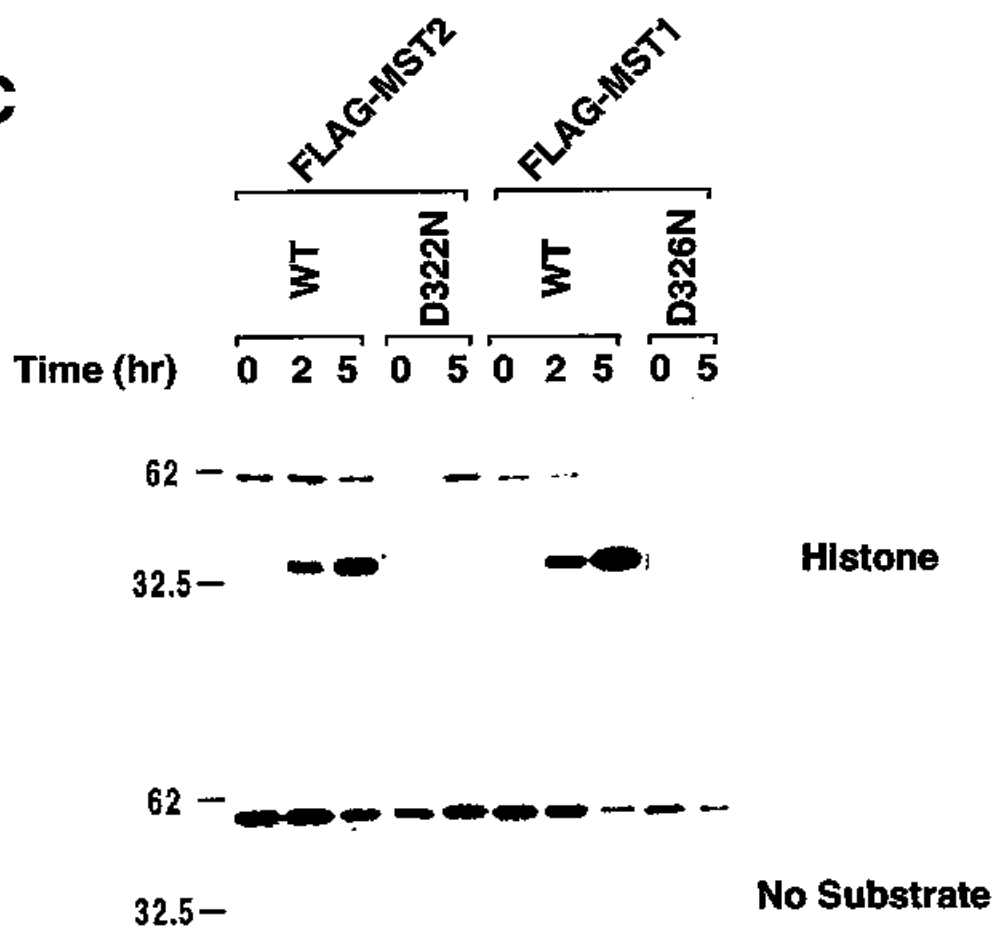
Fig 2



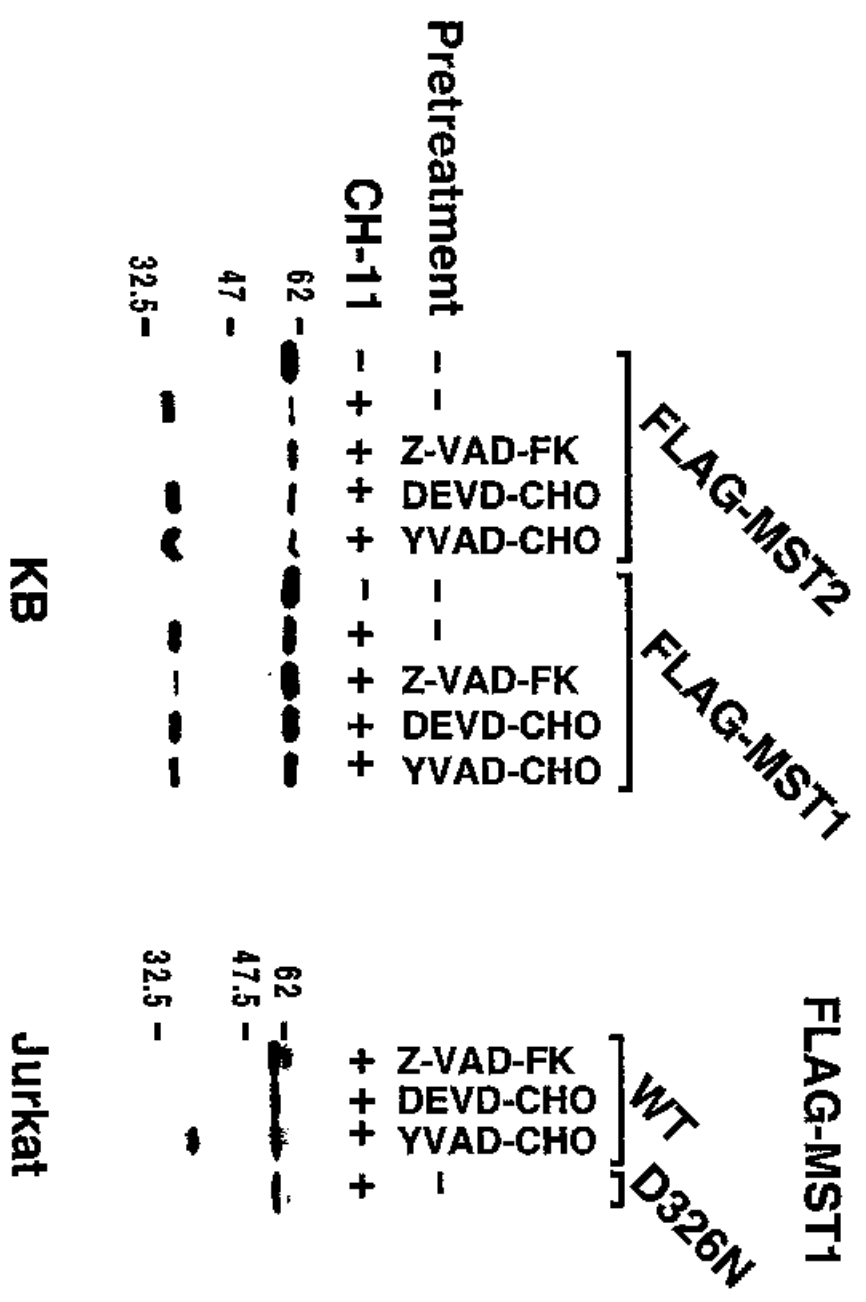




A

B**C**

A



B