MAPK Upstream Kinase (MUK)-binding Inhibitory Protein, a Negative Regulator of MUK/Dual Leucine Zipper-bearing Kinase/Leucine Zipper Protein Kinase*

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Mitogen-activated protein kinase upstream kinase/dual leucine zipper-bearing kinase/leucine-zipper protein kinase (MUK/DLK/ZPK) is a MAPKKK class protein kinase that induces JNK/SAPK activation. We report here a protein named MBIP that binds to MUK/DLK/ZPK. MUK-binding inhibitory protein (MBIP) contains two tandemly orientated leucine-zipper-like motifs with a cluster of basic amino acids located between the two motifs. MBIP interacts with one of the two leucine-zipper-like motifs of MUK/DLK/ZPK and inhibits the activity of MUK/DLK/ZPK to induce JNK/SAPK activation. Notably, no similar effect was observed with another JNK/SAPK-inducing MAPKKK, COT/Tpl-2, showing the specificity of MBIP action. Furthermore, the overexpression of MBIP partially inhibits the activation of JNK by 0.3 M sorbitol in 293T cells. Taken together, these observations indicate that MBIP can function as a regulator of MUK/DLK/ZPK, a finding that may provide a clue to understanding the molecular mechanism of JNK/SAPK activation by hyperosmotic stress.

JNK/SAPK is essential for the survival as well as the death of neural cells in developing mouse brain (1–3). In cultured mammalian cells, JNK/SAPK is well characterized as a stress-inducing protein kinase that is activated by several kinds of cellular stresses, including ultraviolet light, heat shock, protein synthesis inhibitors, and osmotic shock, as well as by different kinds of cytokines and their receptors, such as TNFα and Fas (4–7). JNK/SAPK activity is induced by mitogen-activated protein kinase kinase class protein kinases, SEK1/MKK4/JNKK1/SSK1 or SEK2/MKK7/JNKK2/SSK4, whose activity is induced by further upstream kinases. More of such upstream protein kinases, the so-called MAPKKK, are now reported and include mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-related protein kinases (8, 9), TAK1 (10, 10), Cot/Tpl-2 (11), and ASK1 (12). We and others have reported that the overexpression of mixed lineage kinases (MLKs), including MUK/DLK/ZPK, LZK, MST/MLK2, and SPRK/MLK3/PTK1, induces the activation of JNK/SAPK in mammalian cells (13–18). Using purified recombinant proteins, we have further shown that MST/MLK2 directly activates protein kinases of the mitogen-activated protein kinase kinase class, SEK1/MKK4/JNKK1/SSK1 or SEK2/MKK7/JNKK2/SSK4 (17, 19). Therefore, all members of the MLK family may act as MAPKKK in the JNK/SAPK pathway.

Members of the MLK family share a characteristic protein kinase domain that shows structural features of both tyrosine-specific and serine/threonine-specific protein kinases and two leucine-zipper-like motifs located at the C-terminal side of the kinase domain (20). Despite these common features, members of the MLK family show considerable differences in their primary structures, and they comprise two subgroups that can be distinguished by the primary structure of the kinase domain and the presence or absence of an SH3 domain located at the N-terminal side of the kinase domain. The SH3-containing group of MLK includes MLK1, MST/MLK2, and SPRK/MLK3/PTK1. They also contain a Cdc42 and Rac interactive binding domain conserved among several Cdc42- and Rac-binding proteins such as PAK and WASP (21); this domain is heavily degenerated in members of another, non-SH3-containing group of MLK including MUK/DLK/ZPK and LZK. Such structural differences between these two groups suggest their different regulatory mechanisms and physiological functions. However, neither the molecular mechanism of the regulation of MLK activity nor the physiological function of the MLK-JNK/SAPK pathway has been explored.

Nevertheless, there are some candidates for the upstream regulators of the SH3-containing group of MLK. Based on an analogy with a mitogen-activated protein kinase pathway in budding yeast, it is expected that an STE20-like protein kinase acts as an activator of MAPKKK class protein kinases including MLKs. This possibility is supported by the observations that an STE20-like kinase, HPK1, interacts with SPRK/MLK3/PTK1 through the SH3 domain and that SPRK/MLK3/PTK1 can be phosphorylated by HPK1 in vitro (22). Although the effect of HPK1 interaction on the kinase activity of SPRK/
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MLK3/PTK1 has not been explored, this STE20-like kinase is a strong candidate for an upstream regulator of SH3-containing MLKs. On the other hand, the presence of a Cdc42 and Rac interactive binding site in those MLK members suggests the regulation of kinase activity by Rac and Cdc42, known activators of the JNK/SAPK pathway. In fact, the GTP-dependent binding of these small GTP-binding proteins to MST/MK2 and SPRK/MK3/PTK1 has been shown by overlay assay and by a yeast two-hybrid system (21, 23). However, the effect of Rac/Cdc42 binding on MLK activity is obscure, and the binding could be meaningful in the sense of the targeting of MST/MK2 and SPRK/MK3/PTK1 to a certain subcellular component (23–25).

As to members of the SH3 domain noncontaining group of MLK, MUK/DLK/ZPK and LZX, information about upstream regulators is quite limited. There are some reports showing the possible involvement of MUK/DLK/ZPK in JNK/SAPK activation induced by Rac or C3G (15, 26). However, whether MUK/DLK/ZPK binds directly to and is activated by these proteins or not is obscure. On the other hand, MUK/DLK/ZPK as well as other MLKs include two leucine-zipper-like motifs, putative protein interaction domains, that could be essential for the MLK activity (13, 27). Therefore, it is conceivable that the MUK/DLK/ZPK activity is regulated by a certain protein interacting with these motifs. To identify such a regulatory protein binding directly to MUK/DLK/ZPK, we employed a yeast two-hybrid system. By using a part of MUK/DLK/ZPK protein including one of the two leucine-zipper-like motifs as a bait, we identified a MUK/DLK/ZPK-binding protein that specifically inhibits the MUK/DLK/ZPK activity to induce the activation of the JNK/SAPK pathway.

EXPERIMENTAL PROCEDURE

Library Screening—Yeast strain Y190 cells were transformed first with a bait plasmid encoding a LexA DNA binding domain fused to a part of MUK (amino acids 453–604). A selected bait-containing clone was then transformed with plasmids containing human brain cDNA expression library clones fused to DNA encoding a GAL4 activation domain. From ~2 × 10^7 transformants, we obtained 339 His^+ /LexA^+ clones that originated from six distinct gene products. To obtain a cDNA clone including the full-length MBIP coding sequence, we screened a human kidney cDNA library using one of the initial isolates (clone3–3) encoding amino acid residues 171–344 of MBIP as a probe. Hybridization was done overnight at 65° C in 0.9 M NaCl, 90 mM NaHCO3, 5 × Denhardt’s solution, 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA, and 1 × 10^6 cpm/ml of the cDNA probe. Filters were subsequently washed with 2 × SSC containing 0.1% SDS twice at room temperature for 10 min and with 0.1 × SSC containing 0.1% SDS twice at 65° C for 20 min, and they were exposed to x-ray films at ~80° C.

Northern Blot Analysis—Multiple human tissue Northern blots (CLONTECH) were hybridized to a ^32P-labeled MBIP cDNA probe at 68° C for 1 h and washed at 50° C for 40 min to a final stringency of 0.1 × SSC, 0.1% (w/v) SDS before exposure to x-ray film.

Expression Vectors—The expression vectors for MUK and Cot were constructed with a mammalian expression vector containing an EF1 promoter and a cDNA encoding rat MUK (13) or human Cot (kindly provided by Dr. Jun Miyoshi). The expression vectors for MBIP and its homologues were prepared by cloning 3′- or 5′-terminal part of MUK or anti-FLAG M2 monoclonal antibody tag (Zyto) for 60 min at 37° C, and washed three times for 15 min each with TBST. After incubation with secondary antibodies, fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Cappel) and Cy3-conjugated goat anti-mouse IgG (Amersham Pharmacia Biotech), the cells were washed three times for 15 min each with TBST. Samples were observed under an Olympus BX50, and images were captured with a Princeton Instruments digital camera.

Immunoprecipitation—The expression vectors transfected with expression vectors were collected directly in SDS-sample buffer. After SDS-polyacrylamide gel electrophoresis, the separated proteins were electrothermically transferred to a polyvinylidene difluoride membrane. The blotted membrane was soaked in PBS containing 5% skim milk overnight at 4° C. The membrane was then incubated with appropriately diluted anti-active JNK pAb (pTPpY) (Promega), phosphospecific SEK1/MEKK4 antibody (Thr223) (New England Biolabs), anti-T7 tag monoclonal antibody (Novagen), anti-FLAG M2 monoclonal antibody (Sigma), or anti-GST antibody in TBST containing 0.1% bovine serum albumin for 1 h at 37° C. After washing with TBST, the membrane was incubated with peroxidase-conjugated goat anti-rabbit or mouse IgG antibody (Amersham Pharmacia Biotech) in TBST containing 0.1% bovine serum albumin. The membrane was washed again and the specific bands were visualized with an ECL system (Amersham Pharmacia Biotech).

Stimulation of Cells—293T cells were routinely grown in Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum (10% fetal bovine serum/Dulbecco’s modified Eagle’s medium). The cells were transfected with the MBIP expression vector or empty vector together with the T7-JNK1 expression vector by calcium phosphate co-precipitation methods at a density of 2 × 10^5 cells/cm^2. After a 10-h exposure to the calcium phosphate-DNA precipitate, the medium was changed to fresh 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium and the cells were further cultured for 24 h. The cells were then stimulated with 0.3% sorbitol for 5, 15, 60, or 120 min, heat shock for 44° for 15 min, or 100 ng/ml TNFα for 15 min, and the cells were washed with PBS and lysed in SDS-polyacrylamide gel electrophoresis sample buffer.

RESULTS

Molecular Cloning of a MUK-binding Protein, MBIP—To isolate cDNA clones encoding MUK-binding proteins, we employed a yeast two-hybrid system using part of MUK including a leucine-zipper-like motif and a cluster of basic amino acids as bait (see Ref. 13 and Fig. 4). By screening ~2 × 10^7 clones of a human brain cDNA library, we identified 339 positive clones originating from six different gene products. The expression of one of these clones, clone 3–3, in 293T cells resulted in the inhibition of the overexpressed MUK activity to induce JNK activation, whereas no significant effect was observed with the other clones (data not shown). Therefore, we chose clone 3–3 for further analyses. To isolate cDNA clones encoding the entire protein, a human kidney cDNA library was screened using a 3–3 cDNA fragment as a probe. Among 16 cDNA clones isolated, the complete sequences of 2 clones containing the 5′- or 3′-parts of the long open reading frame were determined (Fig. 1, A and B). The open reading frame encodes 344 amino acids, and the nucleotides flanking the initiation codon fulfill Kozack’s consensus sequence (28). In addition...
in-frame termination codons were found in the 5′-nontranslated sequence, and a poly(A) addition signal was identified 22 base pairs upstream of the poly(A) tail in the 3′-nontranslated sequence (Fig. 1B). Importantly, the reading frame is the same as that of clone 3–3 on the yeast expression vector, which covers the C-terminal part (Fig. 1A). Taken together, these structural features indicate that this open reading frame is translated into a protein harboring a MUK binding region in the C-terminal part. We named this protein MBIP: MUK binding inhibitory protein.

MBIP shows no significant homology in its primary structure with any other proteins in the GenBank™ data base, although we could find several human expressed sequence tag clones that together covered almost the entire MBIP sequence. MBIP contains two leucine-zipper-like motifs that are predicted to form short amphipathic α-helixes (Fig. 1C). The joining region between the two leucine-zipper-like motifs is 28 amino acids long and includes a cluster of basic amino acids related to an SV40T-type nuclear localization sequence (Fig. 1B). The mouse expressed sequence tag clone AA879837 covers part of the MBIP mRNA.

**Fig. 1. Structure and expression of MBIP.** A, structure of human cDNA clones encoding MBIP. Clone 3–3 was isolated using the yeast two-hybrid system. Clones 17 and 11 were isolated from a human kidney cDNA library using clone 3–3 as a probe. The open box represents an open reading frame. B, nucleotide and amino acid sequences of MBIP. Underlining in the 5′-noncoding sequence indicates in-frame stop codons. A poly(A) addition signal found in the 3′-noncoding sequence is also underlined. Underlined amino acids are hydrophobic amino acids involved in leucine zipper-like motifs. A cluster of basic amino acids related to the SV40 T type nuclear localization sequence is indicated by double underlining. C, helical loop analysis of two leucine-zipper-like motifs starting at Ile 271 (left) and Leu 314 (right), respectively. Amino acid residues that may be involved in the formation of a hydrophobic surface are shadowed. D, Northern blot showing MBIP mRNA in various human tissues. Multiple tissue Northern filters (CLONTECH) containing 2 μg each of poly(A)+ RNA were hybridized to the MBIP cDNA probe. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. The arrowhead indicates the position of the 1.8-kilobase (kb) MBIP mRNA.
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**MBIP Binds Specifically to MUK**—Because the clones we isolated using the yeast two-hybrid system cover only the C-terminal half of MBIP, the interaction with MUK could depend on an artificial structure created by the loss of the N-terminal half. To rule out this possibility, we tested the binding of the full MBIP and MUK. Epitope-tagged proteins were transiently overexpressed in COS-1 cells, and binding was examined by immunoprecipitation followed by Western blotting analysis. As shown in Fig. 2A, Flag-tagged MBIP co-precipitated with T7-tagged MUK when anti-Flag tag antibody was used for immunoprecipitation (lane 4, upper panel); no Flag-MBIP was detectable without T7-MUK overexpression (lane 3, upper panel). When anti-Flag antibody was used for the immunoprecipitation, T7-MUK co-precipitated with Flag-MBIP (Fig. 2B). In contrast, the MBIP binding with Cot, another JNK-activating MAPKKK, was barely detectable (Fig. 2C). These results indicate a stable and specific interaction between MBIP and MUK.

**MBIP**—A structural feature of MBIP and its deletion mutants used for the binding assay. The region coded by clone 3–3 from the yeast two-hybrid screening is also shown. B, COS-1 cells were co-transfected with an expression vector for T7-tagged MUK together with an expression vector for Flag-tagged MBIP or its deletion mutants, as indicated at the top of each panel. T7-MUK was immunoprecipitated using anti-T7 tag antibody (anti-T7, upper panel), and total cell lysate (lanes 1 and 2) and immunoprecipitated proteins (lanes 3 and 4) were analyzed by Western blotting using anti-Flag tag antibody (anti-Flag, upper panel) or anti-T7 tag antibody (anti-T7, lower panel). Note the absence of Flag-tagged MBIP in the immunoprecipitate (lane 4, upper panel). C, T7-tagged Cot was immunoprecipitated using anti-T7 tag antibody (anti-Flag), and total cell lysate (lanes 1 and 2) and immunoprecipitated proteins (lanes 3 and 4) were analyzed by Western blotting (WB) using anti-T7 tag antibody (anti-T7, upper panel) or anti-Flag tag antibody (anti-Flag, lower panel). Note the presence of T7-tagged MUK in the immunoprecipitate (lane 4, upper panel). When T7-MUK was immunoprecipitated, the C-terminal half (amino acids 1–452) (Fig. 4A) or anti-T7 tag antibody (anti-Flag, upper panel) or anti-Flag tag antibody (anti-Flag, lower panel). Note the absence of Flag-tagged MBIP in the immunoprecipitate (lane 4, upper panel).

**MBIP**—The C-terminal part of MBIP is essential for MUK binding. A, structural features of MBIP and its deletion mutants used for the binding assay. The region coded by clone 3–3 from the yeast two-hybrid screening is also shown. B, COS-1 cells were co-transfected with an expression vector for T7-tagged MUK together with an expression vector for Flag-tagged MBIP or its deletion mutants, as indicated at the top of each panel. T7-MUK was immunoprecipitated using anti-T7 tag antibody (anti-T7, upper panel) and total cell lysate (lanes 1–6) and immunoprecipitated proteins (lanes 7–12) were analyzed by Western blotting using anti-Flag tag antibody (anti-Flag, upper panel) or anti-T7 tag antibody (anti-T7, lower panel). Asterisks indicate the position of MBIP and its deletion mutants. Note the absence of MBIP/1–228 in the immunoprecipitated fraction (lane 10, upper panel). aa, amino acids; IP, immunoprecipitate; WB, Western blot.
FIG. 4. The leucine zipper-like motifs of MUK are essential for MBIP binding. A, structural features of MUK and its deletion mutants used for the binding assay. The region used as bait for the yeast two-hybrid screening is also shown. B, COS-1 cells were co-transfected with an expression vector for T7-tagged MBIP together with an expression vector for T7-tagged MUK or its deletion mutants: lanes 1 and 7, vector only; lanes 2 and 8, T7-tagged MUK; lanes 3 and 9, T7-tagged MUK-(413–496); lanes 4 and 10, T7-tagged MUK-(1–452); lanes 5 and 11, T7-tagged MUK-(1–413); lanes 6 and 12, T7-tagged MUK-(123–888). T7-tagged MUK and its deletion mutants were immunoprecipitated using anti-T7 tag antibody (anti-T7) and total cell lysate (lanes 1–6, lanes 7–12) were analyzed by Western blotting using anti-Flag tag antibody (anti-Flag, upper panel) or anti-T7 tag antibody (anti-T7, lower panel). Asterisks indicate the positions of MUK and its deletion mutants.

...results strongly suggest that MBIP interacts with MUK through the leucine-zipper-like motif.

The Interaction of MBIP and MUK in Vivo—MUK is mostly located in the cytoplasm when overexpressed in COS-1 cells (Fig. 5A), whereas much of the MBIP overexpressed in COS-1 cells is located in the nuclei forming several patch-like structures (Fig. 5F). The nuclear localization of MBIP is expected from the presence of nuclear localization sequence between the two leucine-zipper-like motifs located in the C-terminal part of MBIP. However, when MBIP was co-expressed with MUK, the nuclear localization was mostly abolished and cytoplasmic localization became apparent (Fig. 5D). Notably, some MBIP remained in the nuclei cells expressing lower amounts of MUK (arrow in Fig. 5D). Therefore, the cytoplasmic localization of MBIP depends on the amount of MUK, a dependence that may be caused by MUK covering the nuclear localization sequence or by the anchoring of MBIP in cytoplasm by MUK. In any case, this observation strongly supports the idea that the interaction between MUK and MBIP occurs in vivo.

MBIP Specifically Inhibits the Ability of MUK to Induce JNK Activation—To assess the effect of MBIP on the activity of MUK, MUK and JNK1 were transiently overexpressed in 293T cells, and the effect of MBIP co-expression on the activation of JNK1 was tested. As shown in Fig. 6A, the co-expression of MBIP inhibited the ability of MUK to induce JNK activity as estimated by Western blot analysis using an anti-active JNK antibody (lanes 3–5, uppermost panel). Importantly, the activity of Cot, another JNK-inducing MAPKKK, was not affected by MBIP (Fig. 6A, lanes 6–8), showing the specificity of MBIP action. The effect of MBIP was further confirmed using SEK1 instead of JNK1 as a reporter of MUK activity. MBIP also inhibited the ability of MUK to induce SEK1 activity as estimated by Western blot analysis using phospho-SEK1 (Thr223) antibody (Fig. 6B, lanes 3 and 4, uppermost panel). Again, no inhibitory effect of MBIP was observed with Cot (Fig. 6B, lanes 7 and 8, uppermost panel). In addition, it should be noted that the amount of overexpressed MUK as shown by the anti-T7 antibody did not change upon MBIP co-expression (Fig. 6B, lanes 3 and 4, middle panel). Instead, the mobility of MUK on SDS-polyacrylamide gel electrophoresis increased upon MBIP co-expression. This downward shift of the MUK band on SDS-polyacrylamide gel electrophoresis was more clearly observed without SEK1 overexpression (Fig. 6B, lanes 5 and 6, middle panel). Because treatment of the immunoprecipitated MUK with alkaline phosphatase also caused a similar downward shift of the MUK band on SDS-polyacrylamide gel electrophoresis, the changes in mobility are likely to depend on the phosphorylation state of MUK (data not shown). Therefore, MBIP may modify the phosphorylation state of MUK in correlation with the change in MUK activity. Taken together, these results show that MBIP is a specific negative regulator of MUK.

To test whether the MUK binding domain of MBIP is indispensable for the MUK inhibitory activity, the MBIP deletion mutants tested for MUK binding (Fig. 3) were used in experiments to assess the effect on MUK activity. As shown in Fig. 7, the overexpression of the C-terminal half of MBIP (MBIP-(172–344)), which binds to MUK, inhibits the ability of MUK to...
activate SEK1 (lanes 4, 7, and 8, uppermost panel). Notable is the complete inhibition of SEK1 phosphorylation by high dose expression of MBIP-(172–344) or the entire MBIP molecule (Fig. 7, lanes 6 and 8). On the other hand, approximately the same dose of the N-terminal part of MBIP (MBIP-(1–228)) produces no such inhibitory effect (Fig. 7, lanes 9 and 10). Therefore, both MUK binding and the inhibitory activities lie in the C-terminal half of MBIP. Despite the relatively weak binding activity of MBIP-(172–344) to MUK (Fig. 3), the inhibitory effect is comparable to that of the complete MBIP molecule (Fig. 7, lanes 4–8). This difference may be caused by the more stringent conditions of the immunoprecipitation assay used to test MUK binding (Fig. 3) compared with conditions in vivo used for the MUK inhibition assay (Fig. 7).

The Overexpression of MBIP Inhibits the JNK Activity Induced by Osmotic Stress—The specific inhibition of MUK activity by MBIP allowed us to identify the JNK-activating pathway involving MUK by testing the effect of MBIP overexpression on the activation of JNK by different kinds of extracellular stimuli. Among several JNK-activating pathways tested, the pathway induced by osmotic shock with 0.3 M sorbitol was the most sensitive to MBIP (Fig. 8). Especially, the early phase of JNK activation by sorbitol, 15 min after the addition of sorbitol, was inhibited by up to 30% from the control level, whereas the later phase was mostly unaffected (Fig. 8, A and B). Transfection of increasing amounts of MBIP expression vector inhibited osmotic shock-induced JNK activity in a concentration-dependent manner (Fig. 8B). On the other hand, no significant effect of MBIP overexpression was observed on JNK activation induced by heat shock (Fig. 8C) or TNFα (Fig. 8D) at any time. The effect of MBIP was also trivial on the activation of JNK by 10 μg/ml anisomycin (data not shown). These observations suggest the involvement of MUK in the JNK activation pathway induced by high osmolality.

Discussion

We report here the molecular cloning of MBIP that binds to and inhibits MUK/DLK/ZPK. No binding protein for MUK/DLK/ZPK has been reported other than JIP1/2, which has been proposed to act as a scaffold protein for MLK JNK/SAPK signaling (29, 30). On the other hand, relatively large numbers of proteins have been reported to bind to SH3 containing MLKs, SPRK/MLK3/PTK1, and MST/MLK2, including HPK1(22), Cdc42/Rac (21, 23, 25), KIF3A, hippocalcin, 14–3–3e (23), dynamin (31), α-tubulin, and prohibitin (32). Even if no effect on kinase activity is apparent, some of these binding proteins could be involved in the transportation of MLKs to specific compartments within cells (23, 31). JIP1/2 binds both SH3-containing and -noncontaining MLKs, and the binding activates the MLK-JNK/SAPK pathway by facilitating the interaction with downstream protein kinases, SEK2/MKK7/JNKK2/SSK4 and JNK/SAPK (29). Most of such scaffold proteins for mitogen-activated protein kinase pathways have been shown to activate kinase cascades with the exception of RIKP, which acts as an inhibitor of the Raf- mitogen-activated protein kinase/extracellular signal-regulated kinase/extracellular signal-regulated kinase signal-regulated kinase pathway by binding to and disrupting the interaction of these protein kinases (33). The possibility that MBIP also acts as a scaffold protein appears quite unlikely, because no binding of MBIP to SEK2/MKK7/JNKK2/SSK4, SEK1/MKK4/JNKK1/SSK1, or JNK1/SAPK is obvious. Therefore, MBIP may be defined as an inhibitor of MUK/DLK/ZPK.

MBIP interacts with a leucine-zipper-like motif of MUK/DLK/ZPK, and MBIP itself also contains leucine-zipper-like motifs in the C-terminal MUK binding region. Therefore, the interaction of these proteins is likely to be mediated by these structural motifs. Accordingly, Cot/Tpl-2, a JNK/SAPK-inducing MAPKKK without leucine-zipper-like motifs, does not bind to nor is it inhibited by MBIP. If a leucine-zipper-like motif is sufficient for MBIP binding, all MLK members with leucine-zipper-like motifs would be expected to bind to MBIP. However, our preliminary results show that MBIP binding to MST/MLK2, an SH3 containing MLK, is barely detectable. Furthermore, it’s ability to induce JNK activation is only moderately affected, if at all, by MBIP, whereas LZK, a non-SH3 containing MLK, is also a target of MBIP. This specificity of MBIP
action may depend on the primary structures of the leucine-zipper-like motifs of MLKs, which are significantly diverged between SH3-containing and noncontaining MLKs (18).

How does the binding of MBIP result in the inhibition of MUK/DLK/ZPK kinase activity? Even though the MBIP binding region lies outside the MUK/DLK/ZPK kinase domain, the binding of MBIP could physically block the interaction with the substrate. SEK1/MKK4/JNK1/SSK1 or SEK2/MKK7/JNK2/SSK4 with MUK/DLK/ZPK. Also MBIP binding could induce a conformational change in the MUK/DLK/ZPK protein to an inactive form. Deletion of the C-terminal half including the leucine-zipper-like motifs results in the loss of the ability of MUK/DLK/ZPK to induce JNK/SAPK activation (13). In addition, it has been suggested that dimer formation through the leucine-zipper-like motifs is essential for the activation of SPRK/MLK3/PTK1 (27). These facts indicate the importance of the leucine-zipper-like motifs in maintaining the active conformation, including the dimer of MUK/DLK/ZPK. Therefore, MBIP could inhibit MUK/DLK/ZPK activity by interfering with this function of the leucine-zipper-like motif.

MLKs, including MUK/DLK/ZPK, activate the JNK/SAPK pathway in an extracellular stimuli-independent manner when overexpressed in cultured cells (13–18). Moreover, the deletion of part of MLK other than the kinase domain never results in an increase in kinase activity (13, 19), indicating the absence of a regulatory domain that is often found in other MAPKKks, including Raf and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1 (34, 35). Because the unregulated activation of JNK/SAPK can cause apoptotic cell death in many cell lines (36–39), the amount of MLK must be tightly regulated, and the excess fraction degraded rapidly or was inactivated by other regulatory proteins that interact with MLK. MBIP appears to be such a regulatory protein for MUK/DLK/ZPK. Then the question arises as to how the activity of MBIP to suppress MUK/DLK/ZPK is regulated. One possible way is through a quantitative change in the amount of MBIP or MUK/DLK/ZPK. A decrease in the amount of MBIP or an increase in MUK/DLK/ZPK can produce free active MUK/DLK/ZPK. Another possibility is a qualitative change in MBIP or MUK/DLK/ZPK; for example, certain post-translational modifications of these molecules might disrupt the interaction. At present, we do not have data to support either of these possibilities.

Little has been reported concerning the physiological function of MLKs. MUK/DLK/ZPK mRNA is mainly detected in the brain among those human or mouse tissues that have been tested (13, 40). Therefore, MUK/DLK/ZPK may be involved in the formation of the neural network and neural cell differentiation by modulating JNK activity. On the other hand, MBIP mRNA is ubiquitously expressed in several human tissues (Fig. 1D). This discrepancy is at least partially explained by the expression pattern of LZK, another potential target of MBIP, which is found in the placenta, liver, and pancreas as well as in the brain (18). In addition, the amount of MUK/DLK/ZPK mRNA might be induced in a variety of tissues in certain physiological or pathological states including fracture repair or liver regeneration (41, 42). MBIP may serve as a pre-existing regulator for the newly synthesized MUK/DLK/ZPK.

Because the action of MBIP is quite specific to MUK/DLK/ZPK or LZK, we used this protein to identify signaling pathways involving MUK/DLK/ZPK or LZK. Among several JNK/SAPK-activating pathways tested, only that induced by osmotic shock was affected by MBIP overexpression. It should, however, be noted that the inhibition is partial and restricted to the early phase of JNK/SAPK activation (Fig. 8A). Therefore, not only MUK/DLK/ZPK but also other MAPKKks might be involved in the activation of JNK/SAPK by osmotic shock. It has been reported that osmotic shock induces the clustering of the epidermal growth factor receptor, TNF receptor, and interleukin-1 receptor in HeLa cells. The combinational activation of these receptors may cause a strong activation of JNK/SAPK (43), which may be mediated by multiple kinds of MAPKKks. One of the responses induced by osmotic shock in mammalian cell is an alteration in Golgi structure and endoplasmic reticulum-to-Golgi transport (44). Because MUK/DLK/ZPK has been reported to be localized in the Golgi apparatus (45), MUK/DLK/ZPK and MBIP could play some role in cellular response through the regulation of JNK/SAPK activation. The molecular mechanisms and physiological significance of JNK/SAPK activation by a variety of extracellular stimuli including osmotic shock are mostly unknown. MBIP may serve as a tool for use in investigating these issues.

Acknowledgment—We thank Dr. Jun Miyoshi for providing human Cot cDNA.

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J. Biol. Chem. 2000, 275:21247-21254.
doi: 10.1074/jbc.M001488200 originally published online May 8, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001488200

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