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Identification and characterization of functional domains in a mixed lineage kinase LZK

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Abstract The mixed lineage kinase (MLK) family is a recently described protein kinase family. The MLKs contain a kinase domain followed by a dual leucine zipper-like motif. We previously reported the molecular cloning of LZK (leucine zipper-bearing kinase), a novel MLK, and that LZK activated the c-Jun NH_2 terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway through MKK7 in cells. Here, we reveal that LZK forms dimers/oligomers through its dual leucine zipper-like motif, and that this is necessary for activation of the JNK/SAPK pathway. We also identify the C-terminal functional region of LZK, which is indispensable for the activation of SEK1, but not that of MKK7.

Key words: Signal transduction; Mixed lineage kinase; c-Jun NH_2 terminal kinase; Stress-activated protein kinase; Mitogen-activated protein kinase; Leucine zipper

1. Introduction

Mitogen-activated protein kinase (MAPK) pathways are one type of the most important intracellular signal transduction pathways transmitting extracellular stimuli to the nuclei of cells [1–5]. Each MAPK pathway consists of at least three protein kinase components, MAP kinase, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). Upon activation, MAPKKK phosphorylates and activates MAPKK, and then MAPKK phosphorylates MAPK, followed by nuclear translocation of the activated MAPK. This leads to alteration of the expression patterns of specific subsets of genes, making cells appropriately respond to extracellular stimuli. The c-Jun NH_2 terminal kinase (JNK) pathway is one of the MAPK pathways, and is activated when cells are exposed to proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1, and extracellular stresses such as UV irradiation and osmotic shock [6–10]. As with other MAPK pathways, this pathway consists of MAPK (JNK), MAPKKs (MKK7 and SEK1/MKK4), and a variety of MAPKKKs including MEKK1 (mitogen-activated protein or extracellular signal-regulated protein kinase kinase kinase 1) [11], transforming growth factor-β-activated kinase 1 [12], ASK1 (apoptosis signal-regulated kinase 1) [13], and mixed lineage kinase family protein kinases (MLKs) [14–18]. The MLKs comprise a recently described protein kinase family consisting of MLK1, 2 and 3 [19–21], MAPK-upstream kinase (MUK)/dual leucine zipper-bearing kinase (DLK) [14,22], and leucine zipper and sterile α motif kinase [23]. Kinases in this family share structural features such as a kinase catalytic domain, which contains the characteristic amino acid sequences of both serine/threonine kinase and tyrosine kinase, followed by a dual leucine zipper-like motif.

We previously reported the molecular cloning of a novel MLK, LZK (leucine zipper-bearing kinase), from the human brain [24]. LZK shows serine/threonine kinase activity and activates the JNK pathway when overexpressed in cells, but the molecular mechanism underlying MAPKK activation by LZK remains unclear. As dimerization/oligomerization is believed to be necessary for activation of the downstream MAPK pathway by many MAPKKKs including MLKs [25–27], we here examined the significance of dimer/oligomer formation by LZK as to its signal transduction.

In this study, we first reveal that LZK forms dimers/oligomers through its dual leucine zipper-like motif. Next, we demonstrate the functional significance of the dimerization/oligomerization. Furthermore, we identify the unique and essential region for the downstream MAPKK activation by LZK.

2. Materials and methods

2.1. Plasmids, antibodies and reagents

To construct a mammalian expression vector for hemagglutinin (HA)-tagged LZK, a fragment encoding the His-tag of pcDNA His-LZK was replaced by a fragment encoding a HA epitope tag. To construct an expression vector for a mutant LZK which lacks a dual leucine zipper-like motif, pcDNA His-LZK ΔZip, the cDNA fragment of LZK encoding amino acids 1–431, was amplified by polymerase chain reaction and then inserted into the *Nhe*I–*Bam*HI fragment of pcDNA His-LZK. The oligonucleotides used were; 5′-TAATACGACTCACTATAGGG-3′ and 5′-GATTGGATCCCTTCTCTCCATTCAGCCTGAGACT-3′, as sense and antisense primers, respectively. The expression vector for kinase-negative LZK was constructed by site-directed mutagenesis, AAG being converted into GCG, which resulted in substitution of lysine 195 with alanine. As lysine 195 is most likely an ATP binding residue, LZK K195A was expected to be a kinase-negative mutant [28]. The sequence of the oligonucleotide used was 5′-GAGGTGGCCATCGCGAAAGTGAGA-3′. The mismatched nucleotides for mutagenesis are underlined.

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Abbreviations: MLK, mixed lineage kinase; LZK, leucine zipper-bearing kinase; JNK, c-Jun NH_2 terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEKK1, mitogen-activated protein or extracellular signal-regulated protein kinase kinase kinase 1; MUK, MAPK-upstream kinase; DLK, dual leucine zipper-bearing kinase; ASK1, apoptosis signal-regulated kinase 1; HA, hemagglutinin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis

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The expression construct pcDNA His-LZK, and pSRα HA-JNK, pSRα Myc-MKK7, pSRα Myc-SEK1 and pEF MEKK1ΔN were described previously [12,24,29,30]. The *Escherichia coli* expression construct for glutathione *S*-transferase (GST)-c-Jun (1–79) was kindly provided by Dr. Masahiko Hibi (Osaka University, Japan). The expression constructs for His-tagged serial deletion mutants of LZK and GST kinase-negative JNK will be described elsewhere (Ikeda et al., submitted for publication).

Anti-His antibodies were purchased from Qiagen. Anti-Myc, anti-HA and anti-MEKK1 were from Santa Cruz, and anti-Flag was from Sigma. Anti-phosphorylated JNK was from Promega.

2.2. Cell culture and transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and kanamycin. For transfection, cells were subcultured and grown overnight, and then transiently transfected with various expression constructs using LipfectAMINE (Gibco BRL) according to the manufacturer's protocol. After 24 h, the cells were lysed and then the lysates were subjected to immunoprecipitation or directly to Western blotting as described in the figure legends.

2.3. Immunoprecipitation and Western blotting

Cells transiently transfected with various plasmids were lysed in cell

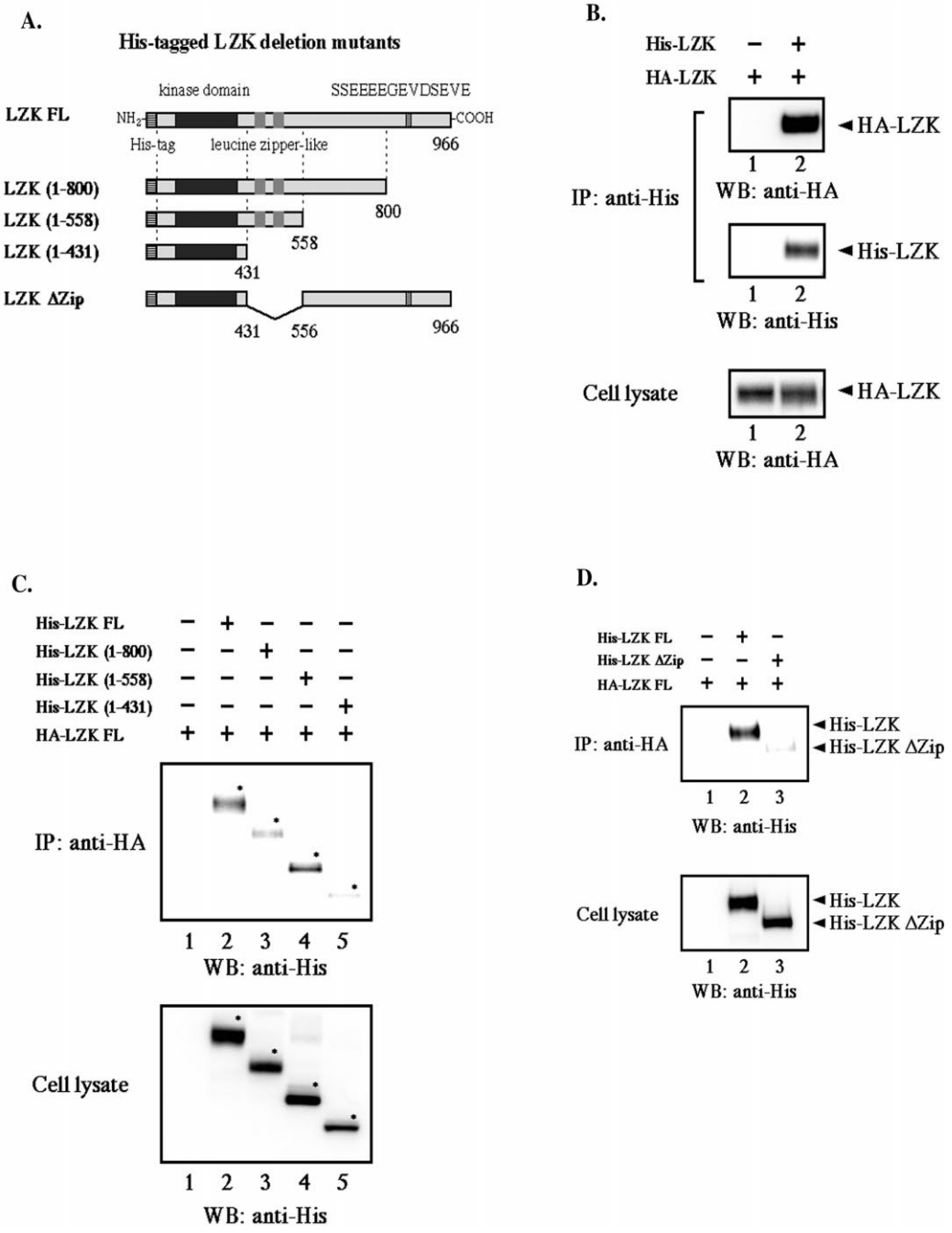

Fig. 1. LZK forms dimers/oligomers in cells. A: His-tagged LZK and its deletion mutants used in this study are schematically represented. B: HA-LZK was expressed with or without His-LZK in COS7 cells, and His-LZK was immunoprecipitated from each cell lysate. The presence of HA-LZK and His-LZK in the immunoprecipitate was examined by Western blotting with anti-HA or anti-His antibodies, respectively (top and middle). The expression of HA-LZK in cells was examined by Western blotting (bottom). C: Serial C-terminal deletion mutants of LZK were co-expressed with HA-LZK, followed by immunoprecipitation and Western blotting as in B. The representative results of three independent experiments are shown. The specific bands corresponding to LZKs are indicated by asterisks. D: His-LZK FL or His-LZK ΔZip was co-expressed with HA-LZK, followed by immunoprecipitation and Western blotting as in B.

lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 25 mM β-glycerophosphate, 50 mM sodium fluoride, 10% glycerol, 1% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). After centrifugation, the clarified lysates were subjected to immunoprecipitation for 2 h at 4°C. The beads were washed three times with lysis buffer, and twice with wash buffer (Tris-buffered saline, pH 7.5, 0.05% Tween 20, and 1 mM dithiothreitol), and then the absorbed proteins were solubilized with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. Western blotting was carried out as described previously [24].

2.4. In vitro kinase kinase assay

Cells were transfected with expression plasmids, as indicated in the figure legends. After 24 h, cell lysates were prepared and JNK, MKK7 and SEK1 activity was measured by means of in vitro kinase assay as described elsewhere (Ikeda et al., submitted for publication). Briefly, each kinase was immunoprecipitated from cell lysate prepared as above, and kinase reactions were carried out by incubating immunoprecipitates with kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP and either 3 μg of GST-c-Jun (1–79) (JNK assay) or 5 μg of GST-KN-JNK (MKK7 or SEK1 assay) for 20 min at 30°C. Reactions were terminated by the addition of SDS–PAGE sample buffer. Proteins were resolved by SDS–PAGE and then the phosphorylated substrate was visualized using a phosphorimage analyzer Fuji BAS2000.

3. Results and discussion

To determine whether or not LZK forms dimers or oligomers in cells, we performed a co-immunoprecipitation assay. HA-tagged LZK was co-expressed with His-tagged LZK in COS7 cells, followed by immunoprecipitation with anti-HA antibodies, and the co-immunoprecipitated His-tagged LZK was detected by Western blotting with anti-His antibodies as the primary antibodies. As shown in Fig. 1B, HA-LZK was co-immunoprecipitated with His-LZK, indicating that LZK forms homo-dimers or oligomers in cells. Similar results were observed when anti-HA was used for immunoprecipitation and anti-His for Western blotting.

Next, we examined the functional region in the LZK molecule which is responsible for the dimerization/oligomerization, by means of a similar assay using a series of His-tagged

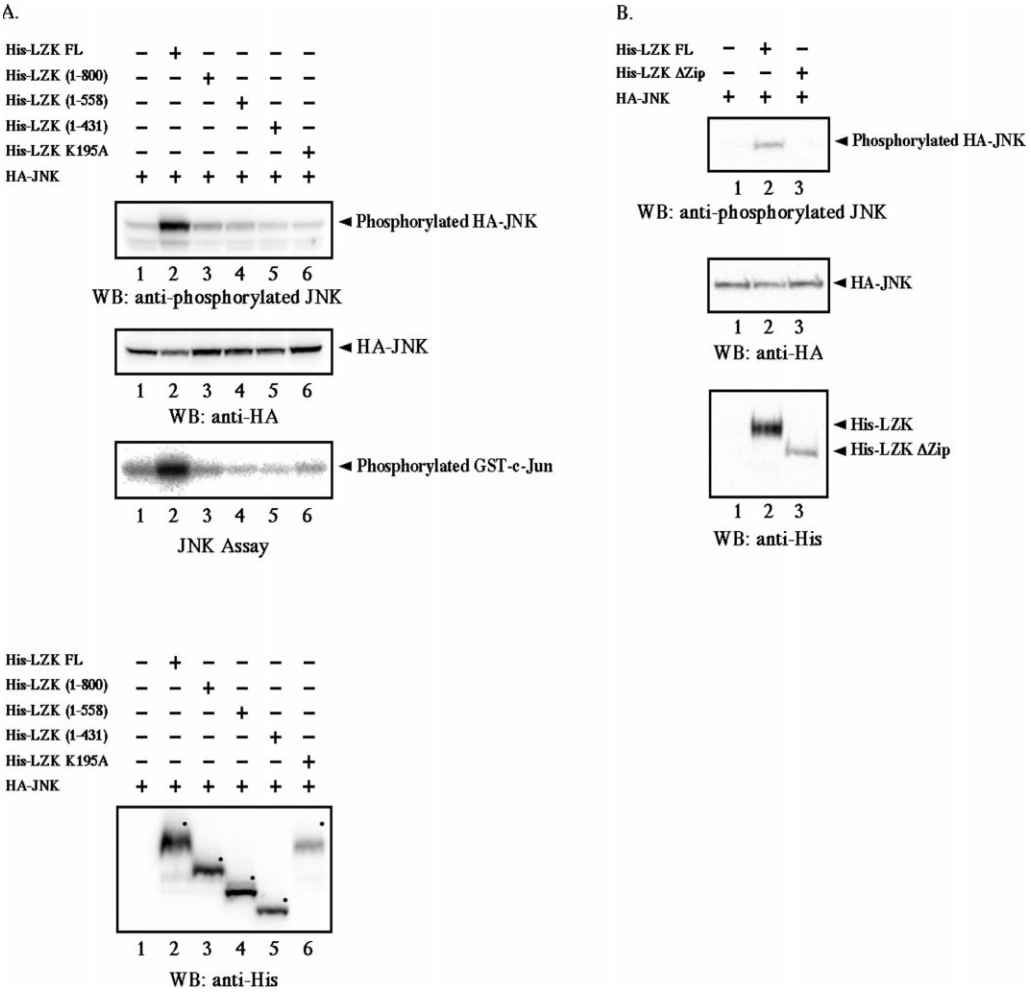

Fig. 2. C-terminal deletion mutant LZKs are incapable of activation of JNK. A: Serial deletion mutants of LZK were co-expressed with HA-JNK, and at 24 h post-transfection, the cells were lysed by the addition of SDS–PAGE sample buffer, and then the amount of dually phosphorylated JNK was examined by Western blotting with anti-phosphorylated JNK antibodies (top). After the antibodies have been stripped off the membrane, the same membrane was reprobed with anti-HA antibodies to examine the total amount of overexpressed JNK in each transfection (second from the top). The JNK activity was also measured by in vitro kinase assay with GST-c-Jun (1–79) as a substrate (third from the top). In a parallel experiment, the amount of LZK in each lysate was determined by Western blotting with anti-His antibodies (bottom). The representative results of three independent experiments are shown. B: His-LZK FL or His-LZK ΔZip was co-expressed with HA-JNK in cells, followed by Western blotting as in A.

LZK deletion mutants, as shown in Fig. 1A. All the mutants containing a dual leucine zipper-like motif were co-immunoprecipitated with full length HA-tagged LZK (Fig. 1C, lanes 2–4). On the other hand, LZK (1–431), a mutant that lacks the dual leucine zipper-like motif, failed to be co-immunoprecipitated efficiently with LZK FL (lane 5), although a faint but reproducible signal was observed. Densitometric analysis of the data revealed that the amount of co-immunoprecipitated LZK (1–431) was about 13% that of LZK (1–558), when normalized to the amounts of HA-LZK in the anti-HA immunoprecipitates, which were almost identical among the experiments (data not shown). This suggests that the dual leucine zipper-like motif was necessary for LZK to form dimers/oligomers effectively. To confirm this, we repeated this assay using a deletion mutant, His-LZK ΔZip, which lacks the amino acids 432–555 corresponding to the dual leucine zipper-like of the LZK molecule. His-LZK ΔZip also failed to be co-immunoprecipitated effectively with HA-LZK (Fig. 1D), suggesting again strongly that LZK forms homo-dimers/oligomers via the dual leucine zipper-like motif. As LZK binds to a scaffold protein, JIP-1, via its N-terminal region containing the kinase catalytic domain (A. Ikeda et al., submitted for publication), the region necessary for dimerization/oligomerization is distinct from the JIP-1 binding region.

Knowing that LZK forms dimers or oligomers, we next investigated the functional significance of the dimer/oligomer formation. First we examined whether or not each of these LZK deletion mutants activates JNK. Full length or a mutant LZK was co-expressed with HA-JNK in COS7 cells, and then the cells were lysed at 24 h post-transfection. The amount of activated JNK in each cell lysate was determined by Western blotting with anti-phosphorylated JNK as the primary antibodies. The JNK activity was also measured by in vitro kinase assay with GST-c-Jun (1–79) as a substrate. The in vitro kinase assay gave essentially the same results as those of the Western blot with anti-phosphorylated JNK antibodies (see Fig. 2A). As expected, full length LZK remarkably activated JNK [24] (Fig. 2A, lane 2). However, to our surprise, all the C-terminal deletion mutants failed to activate JNK like the kinase-negative mutant LZK (LZK K195A), even though they were able to form dimers/oligomers when co-expressed with full length LZK (Fig. 2A, lanes 3–6, see also Fig. 1C). Mutant LZKs were expressed in the transfected cells properly (Fig. 2A, bottom panel). Among these deletion mutants, the one that contained the largest C-terminal portion lacked only 166 amino acid residues from the C-terminal end (amino acids 801–966). This region contains the 14 amino acid stretch of unknown function, which is conserved in LZK and MUK/DLK [24].

LZK ΔZip, which contains the C-terminal end region but fails to form dimers/oligomers, was also completely unable to activate JNK (Fig. 2B), indicating that dimerization/oligomerization is indispensable for LZK to activate the JNK pathway.

Therefore, dimer/oligomer formation is the primary requirement for activation of the JNK pathway by LZK and the C-terminal end region (amino acids 800–966) of LZK also participates in the activation of the JNK pathway. These results led us to examine why these C-terminal deletion mutant LZKs failed to activate JNK. We next investigated whether or not LZK (1–800) activates two distinct MAPKKs of the JNK pathway, MKK7 and SEK1. His-LZK FL or His-LZK (1–800) was co-expressed with either Myc-tagged MKK7 or SEK1, followed by in vitro MAP kinase kinase assay using GST kinase-negative JNK (GST-KN-JNK) as a substrate. In both experiments, MEKK1ΔN (a constitutive active form of MEKK1) was used as a positive control for MAPKKK. As shown in Fig. 3A, both LZK FL and LZK (1–800) activated MKK7 efficiently. However, LZK (1–800) failed to activate SEK1 even though LZK FL considerably activated it (Fig.

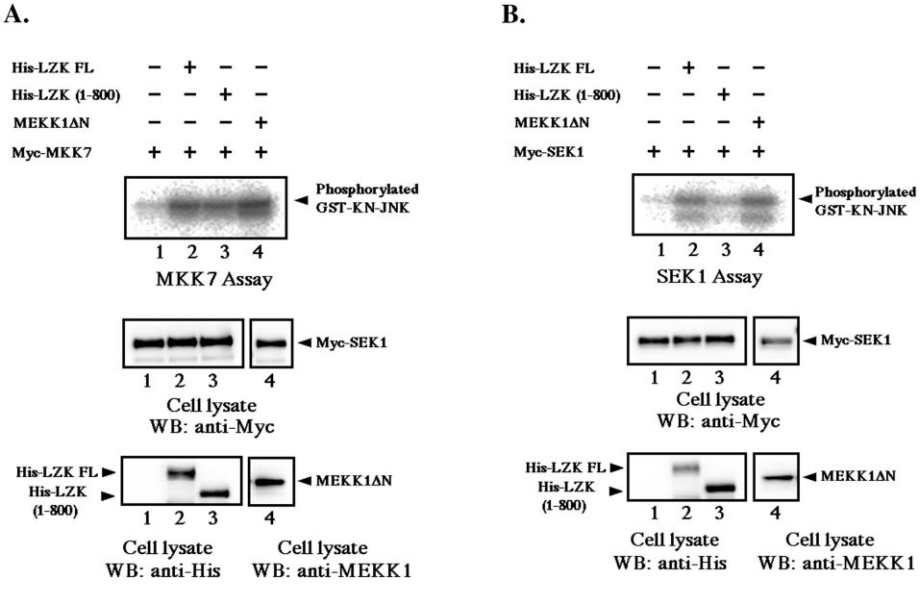

Fig. 3. The C-terminal region of LZK is indispensable for the activation of SEK1, but not that of MKK7. A: His-tagged LZK FL, LZK (1–800), or MEKK1ΔN was co-expressed with Myc-MKK7. 24 h after transfection, the cells were lysed and Myc-MKK7 was immunoprecipitated from the cell lysate, and then the kinase activity of MKK7 was determined by in vitro MAP kinase kinase assay using GST-KN-JNK as the substrate (top). The amounts of overexpressed Myc-MKK7, His-LZKs, and MEKK1ΔN were determined by Western blotting (middle and bottom). B: His-tagged LZK FL, LZK (1–800), or MEKK1ΔN was co-expressed with Myc-SEK1 in cells. SEK1 activity, and the amounts of Myc-SEK1, His-LZKs and MEKK1ΔN were determined as in A.

3B, upper panel; compare lanes 2 and 3). The amounts of expressed MKK7 or SEK1 were almost identical in the lysate (middle panels). These results indicate that the C-terminal end of LZK is essential for the activation of SEK1 MAPKK, but not for that of MKK7 MAPKK. As shown in Fig. 2A, lane 3, LZK (1–800) failed to activate JNK, but it efficiently activated MKK7 (Fig. 3A), which is indicative of an activation of the JNK pathway. The reason for this apparent discrepancy is currently not clear. However, it should be noted that the assay conditions used were different between these two experiments: MKK7 was overexpressed in the Fig. 3A experiment, but only endogenous MKK7 was present in the Fig. 2 experiment.

For MUK/DLK, the MLK that exhibits the highest sequence similarity with LZK, the dual leucine zipper-like motif itself is enough for the formation of homo-dimers/oligomers [26]. This suggests that the corresponding region of LZK is similarly necessary for the formation of homo-dimers/oligomers. MUK/DLK forms hetero-dimers/oligomers with LZK less efficiently than homo-dimers/oligomers, suggesting the presence of an interaction between these two MLKs in vivo [26]. However, it is likely that the N-terminal region rather than the dual leucine zipper-like motif mediates the interaction between LZK and MUK/DLK, as the leucine zipper region of MUK/DLK was not co-immunoprecipitated with LZK [26]. The facts that LZK is expressed in most of human organs, with some variation in the amount [24], and on the other hand MUK/DLK is a brain-specific protein [14,22], suggest that LZK forms homo-dimers or oligomers in some tissues.

For some MAPKKKs such as ASK1 [31] and MLK3 [25], dimerization is believed to be essential for efficient signal transduction to the downstream MAPKKs. Our results strongly suggest that dimerization or oligomerization is also indispensable for LZK to activate the JNK pathway. MLK3 and MUK/DLK are believed to form disulfide-linked homo-dimers/oligomers [25,26,32], but we could not clarify in this study whether or not LZK forms disulfide bridged dimers/oligomers.

In addition to the dual leucine zipper-like motif as the dimerization/oligomerization domain, the C-terminal end region of LZK has been identified as an essential region for the activation of SEK1/MKK4 but not that of MKK7. As mentioned above, this region contains the 14 amino acid sequence completely conserved in LZK and MUK/DLK. To our knowledge, this is the first report suggesting the possible role of this sequence.

Therefore, LZK includes distinct functional regions such as the JIP-1 binding region within or before the N-terminal region of its kinase catalytic domain, a dual leucine zipper-like motif as the dimerization/oligomerization domain, and a SEK1-activating region at its C-terminal end (see Fig. 4). These results provide important clues as to how the kinase activity of LZK or other MLKs is regulated in vivo.

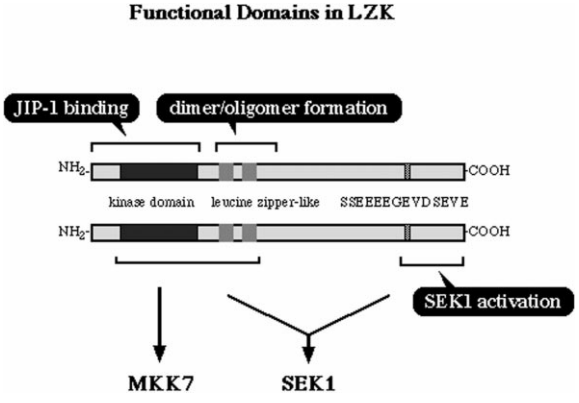

Fig. 4. Schematic representation of the functional domains in LZK. The three functional domains in LZK are shown. LZK contains a JIP-1 binding region at its N-terminus, a region for dimer/oligomer formation in the middle, and a region essential for SEK1 activation at its C-terminus.

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