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Mixed Lineage Kinase LZK Forms a Functional Signaling Complex with JIP-1, a Scaffold Protein of the c-Jun NH_2-Terminal Kinase Pathway[1]

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Leucine zipper–bearing kinase (LZK) is a novel member of the mixed lineage kinase (MLK) protein family, the cDNA of which was first cloned from a human brain cDNA library [Sakuma, H., Ikeda, A., Oka, S., Kozutsumi, Y., Zanetta, J.-P., and Kawasaki, T. (1997) *J. Biol. Chem.* 272, 28622–28629]. Several MLK family proteins have been proposed to function as MAP kinase kinase kinases in the c-Jun NH_2 terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway. In the present study, we demonstrated that, like other MLKs, LZK activated the JNK/SAPK pathway but not the ERK pathway. LZK directly phosphorylated and activated MKK7, one of the two MAPKKs in the JNK/SAPK pathway, to a comparable extent to a constitutive active form of MEKK1 (MEKK1ΔN), suggesting a biological role of LZK as a MAPKKK in the JNK/SAPK pathway. Recent studies have revealed the essential roles of scaffold proteins in intracellular signaling pathways including MAP kinase pathways. JIP-1, one of the scaffold proteins, has been shown to be associated with MLKs, MKK7, and JNK [Whitmarsh, A.J., Cavanagh, J., Tournier, C., Yasuda, J., and Davis, R.J. (1998) *Science* 281, 1671–1674], suggesting the presence of a selective signaling pathway including LZK, MKK7, and JNK. Consistent with this hypothesis, we provided evidence that LZK is associated with the C-terminal region of JIP-1 through its kinase catalytic domain. In addition, LZK-induced JNK activation was markedly enhanced when LZK and JNK were co-expressed with JIP-1. These results constituted important clues for understanding the molecular mechanisms regulating the signaling specificities of various JNK activators under different cellular conditions.

Key words: JNK/SAPK pathway, MLK family, scaffold protein, signal transduction.

Cells respond to various extracellular signals that can change their fate to growth, differentiation, apoptosis, and so on. To transduce extracellular signals to their nuclei precisely, cells have developed various elaborate intracellular signaling systems, among which are the mitogen-activated protein kinase (MAPK) pathways (*1–4*). MAPK pathways consist of three classes of protein kinases, MAPK, MAPK kinase (MAPKK), and MAPKK kinase (MAPKKK). When cells receive extracellular signals, sequential activation of MAPKKK, MAPKK, and MAPK occurs, followed by nuclear translocation of the activated MAPK. The translocated MAPK regulates the expression of specific subsets of genes through phosphorylation of target molecules such as transcription factors. The c-Jun NH_2 terminal kinase (JNK, also known as SAPK) is one of the four MAP kinases that have been identified in mammalian cells (*5–9*). JNK is activated when cells are exposed to proinflammatory cytokines, growth factors, and environmental stresses such as tumor necrosis factor (TNF)-α, transforming growth factor (TGF), epidermal growth factor (EGF), osmotic shock, and UV irradiation. Like other MAPK pathways, the JNK pathway consists of three classes of protein kinases. Upon activation, JNK is dually phosphorylated at Thr and Tyr residues within the kinase catalytic domain by a MAPKK, MKK7, or SEK1/MKK4. Each MAPKK is phosphorylated and activated by a MAPKKK such as MEKK1 (*10*), TGF-β acti-

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Abbreviations: LZK, leucine zipper-bearing kinase; MLK, mixed lineage kinase; JNK, c-Jun NH_2 terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MEKK, mitogen-activated protein or extracellular signal–regulated protein kinase kinase kinase; MUK, MAPK-upstream kinase; DLK, dual leucine zipper–bearing kinase; PCR, polymerase chain reaction; RT, reverse transcription; HA, hemagglutinin; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; SAPK, stress-activated protein kinase; TBS, Tris-buffered saline; ERK, extracellular signal–regulated kinase; PTB domain, phosphotyrosine-binding domain.

vated kinase1 (TAK1) (*11*), Tpl2/Cot (*12*), apoptosis signal-regulated kinase1 (ASK1) (*13*), and mixed lineage kinases (MLKs) (*14–19*). Among these three classes of protein kinases, the greatest variety is observed in MAPKKK rather than MAPKK or MAPK. However, little is known about the physiological significance of this variation and the mechanisms maintaining accurate signal transduction to various MAPKKKs from upstream molecules.

Recently, non-kinase type components of these pathways have been cloned and characterized. These components physically associate with multiple kinases involved in a specific MAPK pathway, and serve as a scaffold or anchoring proteins, which facilitate accurate and efficient signal transduction (*20–24*). JNK Interacting Proteins (JIPs) were shown to associate directly with a specific set of kinases involved in the JNK pathway and to enhance the signal transduction efficiency of the pathway, which leads to JNK activation. Thus, JIPs, consisting of JIP-1, 2, and 3, are thought to serve as scaffolding proteins in the JNK pathway (*25, 26*). Each JIP scaffold protein is believed to be associated selectively with JNK, MKK7, and MLK family kinases, and to enhance MLK-induced JNK activation. It has been reported that the JIP group of scaffolding proteins forms homo- or hetero-oligomeric complexes (*25*), which accumulate in the peripheral cytoplasmic projections or growth cones of developing neurites (*25, 27*). JIP-1, the first identified member of the JIP protein group, forms a multi-molecule scaffolding complex, and allows accurate and efficient signal transduction from MLK3 to MKK7 and JNK. Thus, JIP-1 seems to play important roles in the effective utilization of kinase components in the JNK pathway.

We previously reported the molecular cloning of LZK from a human brain cDNA library (*28*). The structural properties of LZK, such as its hybrid-type kinase domain and dual leucine zipper-like motif, strongly suggest that it belongs to the MLK family. Since LZK exhibits remarkably high sequence similarity in this region with MUK/DLK, we concluded that LZK and MUK/DLK form a subfamily of the MLK family. In this report, we demonstrate that LZK selectively activates JNK through phosphorylation and activation of MKK7, suggesting the existence of a specific signaling pathway from LZK to JNK *via* MKK7. We also provide evidence that LZK associates with JIP-1 and activates JNK with higher efficiency, suggesting that signal transduction efficiency of LZK and MUK/DLK subfamily kinases, as well as that of MLK3, is upregulated through interaction with the JIP-1 scaffold protein.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—To construct a mammalian expression plasmid for His-tagged LZK, a cDNA fragment encoding His-tagged full-length LZK was cloned into the pcDNA 3.1 vector (Invitrogen). Expression plasmids for deletion mutants of LZK were constructed with the pcDNA 3.1 vector and cDNAs encoding different parts of LZK as below. To construct expression plasmids for His-LZK (1–800) and LZK (1–558), the *Nhe*I–Aor51HI or *Nhe*I–*Bam*HI digested fragment of pcDNA His-full-length LZK was inserted into the pcDNA 3.1 vector. To construct pcDNA His-LZK (1–431), a PCR fragment encoding this part of the LZK molecule was digested with *Nhe*I and *Bam*HI, and then subcloned into the pcDNA 3.1 vector. The sequences of the primers used were: 5′-TAATACGACTCACTATAGGG-3′ (sense primer) and 5′-GATTGGATCCTATTCTCTCCATTCAGCCTGAGACT-3′ (antisense primer). To construct an expression plasmid for a mutant LZK that lacks the dual leucine zipper like-motif, pcDNA His-LZK ΔZip, the cDNA fragment of LZK encoding amino acids 1 to 431 was amplified by PCR and then inserted into the *Nhe*I–*Bam*HI fragment of pcDNA His-LZK. The oligonucleotides used as sense and antisense primers, respectively, were: 5′-TAATACGACTCACTATAGGG-3′ and 5′-GATTGGATCCCTTCTCTCCATTCAGCCTGAGACT-3′. To construct an expression vector for a mutant LZK that lacks both the kinase domain and dual leucine zipper-like motif, His-LZK ΔKD Zip, *Nhe*I–*Bgl*II fragment of pcDNA His-full-length LZK was inserted into the *Nhe*I–*Bam*HI digested pcDNA His-full-length LZK. EF1α promoter-driven mammalian high expression plasmid pEF His was a kind gift from Dr. Shigeo Ohno (Yokohama City University). A pEF-based mammalian expression vector for His-LZK was constructed by inserting a cDNA fragment encoding His-tagged LZK into the pEF vector. The expression plasmid for kinase negative mutant LZK, pEF His-LZK ΔKD, was constructed by removing the *Hap*II–*Ehe*I restriction fragment corresponding to amino acids 189 to 321. This region includes the invariant lysine residue (Lys195) located within the kinase domain, which is supposed to be essential for kinase catalytic activity (*29*). To construct a mammalian expression plasmid for JIP-1, a cDNA fragment encoding full-length JIP-1b was amplified by the RT-PCR technique using P8 mouse brain oligo-dT primed cDNA as a template and then cloned into the pcDNA 3.1 vector. The synthetic oligonucleotides used were: 5′-GGTAGATCTAGGCCCCCAGACCCTGCAGAACCCACCTCCA-3′ (sense primer) and 5′-TTCTCTAGATTACTACTCCAAGTAGATATCTTCTGTAGG-3′ (antisense primer). To construct a Flag-tagged version of the JIP-1 expression plasmid, the cDNA fragment encoding JIP-1 was first subcloned into the pEF His vector, and then the fragment encoding the His-tag was replaced with that encoding the Flag-epitope tag. To construct an expression plasmid for JIP-1 (284–707), JIP-1 (465–707), JIP-1 (582–707), and JIP-1 (465–581), a PCR fragment encoding these regions of JIP-1b was ligated with the *Xba*I–*Not*I digested pEF Flag vector. The synthetic oligonucleotides used as sense primers were: 5′-GGTTCTAGAAGGCCCCCAGACCCTGCAGAACCCACCTCCA-3′ for JIP-1 (284–707), 5′-CCGCTCTAGATCCTCCAGTGCTGAGTCCTTT-3′ for JIP-1 (465–707) and JIP-1 (465–581), and 5′-ACATCTAGACTCTGTGCTGCTATGCAAAAGAT-3′ for JIP-1 (582–707). As an antisense primer, the same oligonucleotide was used as for the cloning of full-length JIP-1b, except that the oligonucleotide 5′-AATGCGGCCGCCTAGACATCATTGCCCTTGTGATAA-3′ was used for the construction of the expression plasmid for JIP-1 (465–581). The expression plasmid for JIP-1 (1–277), *Eco*RI–*Bgl*II digested fragment of pEF Flag-JIP-1, was inserted into the *Eco*RI–*Bam*HI digested pEF Flag vector. Mammalian expression constructs pSRα HA-JNK, pSRα HA-ERK, pSRα Myc-MKK7, and pEF MEKK1ΔN were described previously (*11, 30, 31*). Anti-His antibodies were purchased from Qiagen. Anti-Myc, anti-HA, and anti-MEKK1 were from Santa Cruz Biotechnologies, and anti-Flag was from Eastman Kodak. Anti-phosphorylated JNK was from Promega. Myelin basic protein (MBP) was from Upstate

Biotechnologies, and GST-c-Jun (1–79) was from Santa Cruz Biotechnologies.

Preparation of GST-Fused JNK from Escherichia coli—A kinase-negative form of GST-fused JNK (GST-KN-JNK) and GST-fused wild type MKK7 (GST-MKK7) were produced in *E. coli* using the pGEX expression system (Amersham Pharmacia). The GST-KN-JNK protein was affinity-purified on glutathione-Sepharose beads, and eluted with a buffer containing 10 mM reduced glutathione and 50 mM Tris/HCl, pH 8.0. Then the eluted protein was dialyzed against a buffer comprising 20 mM Tris/HCl, pH 7.5, 1 mM EGTA, and 1 mM dithiothreitol, and stored at –80°C until use. GST-MKK7 protein was affinity-purified and dialyzed as above, and stored at –20°C until use.

Cell Culture and Transfection—COS7 cells were cultured in DMEM supplemented with 10% fetal calf serum and kanamycin. For transfection, cells were subcultured and grown overnight, then transiently transfected with various expression constructs using LipfectAMINE (Gibco BRL) according to the manufacturer's protocol. For the experiments in Figs. 1 and 2, cells were lysed at 24 h post-transfection and then subjected to the *in vitro* kinase assay (see below). For the experiments in Figs. 3, 4, and 5, cells were lysed at 24 to 48 h post-transfection, and lysates were subjected to immunoprecipitation and Western blotting. For the experiment in Fig. 6, cells were lysed in Laemmli's SDS-PAGE sample buffer and the lysate was directly subjected to SDS-PAGE, followed by Western blotting.

In Vitro Kinase Assay—Cells were transfected with expression plasmids as indicated in the figure legends. After 24 h, cell lysates were prepared as described previously in lysis buffer A comprising 20 mM Hepes, pH 7.5, 25 mM β-glycerophosphate, 150 mM NaCl, 10% glycerol, 0.5% Triron X-100, 1.5 mM $MgCl_2$, 2 mM EGTA, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium vanadate, and 20 μg/ml aprotinin. After centrifugation, cleared lysates were subjected to immunoprecipitation. The beads were washed three times with wash buffer (TBS containing 0.05% Tween 20 and 1 mM dithiothreitol) and once with kinase reaction buffer (20 mM Tris/HCl, pH 7.5, 25 mM β-glycerophosphate, and 2 mM EGTA). Kinase reactions were carried out by incubating immunoprecipitates with a kinase reaction buffer containing 1 μCi of [γ-^{32}P]ATP and 3 μg of either GST-c-Jun (JNK assays) or myelin basic protein (ERK assays) for 20 min at 30°C. Reactions were terminated by addition of Laemmli's SDS-PAGE sample buffer. Then the proteins were resolved by SDS-PAGE, and phosphorylated substrates were visualized with a Fuji BAS2000 image analyzer. To measure the MKK7 phosphorylating activity of LZK, LZK was immunoprecipitated as above, and the kinase reaction was carried out with 5 μCi of [γ-^{32}P]ATP and 1.2 μg of GST-MKK7, followed by SDS-PAGE and Fuji BAS2000 detection.

Immunoprecipitation and Western Blotting—Cells transfected with various plasmids were lysed in lysis buffer B (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. The beads were washed three times with lysis buffer B, then the absorbed proteins were solubilized with 30 μl of Laemmli's SDS-PAGE sample buffer. Western blotting was carried out as described previously (*28*).

RESULTS

LZK Activates JNK but Not ERK—To determine whether LZK activates ERK or JNK, HA-epitope tagged ERK or JNK was co-expressed with either His-tagged wild-type LZK or kinase-negative mutant LZK (LZK ΔKD). At 24 h post-transfection, cells were lysed, and each MAPK was immunoprecipitated and subjected to the *in vitro* kinase assay. In cells transiently transfected with wild-type LZK, JNK activity was increased (Fig. 1A). However, LZK ΔKD, a kinase-negative mutant form of LZK with in-frame deletion of amino acids 189 to 321, a region including the invariant Lys residue in the kinase domain (*29*), failed to activate JNK. On the other hand, LZK overexpression had no effect on ERK activity (Fig. 1B). In both experiments, the amounts of immunoprecipitated JNK and ERK were almost identical (data not shown). Western blot analysis confirmed that the amounts of wild-type LZK and LZK ΔKD expressed in COS7 cells were essentially the same. Therefore, we concluded that LZK selectively activated the JNK pathway, but not the ERK pathway, and that the

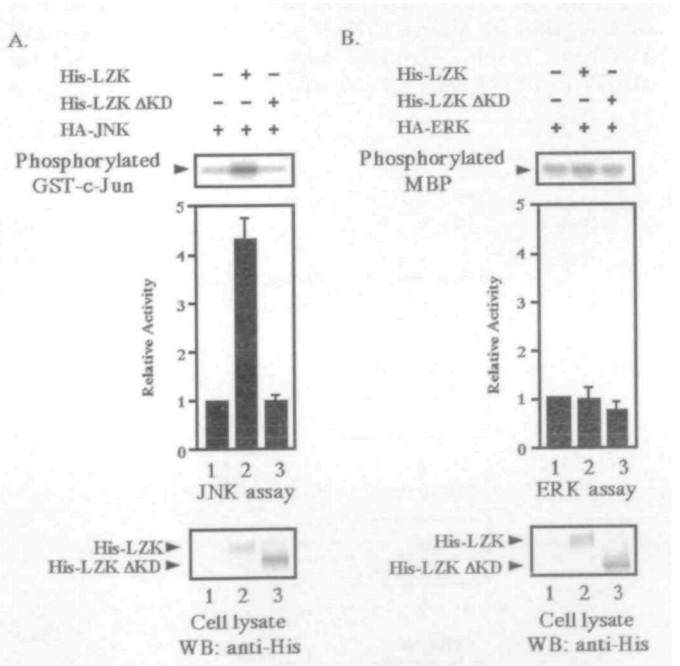

Fig. 1. **LZK activates JNK but not ERK.** A: His-tagged LZK or its kinase-negative mutant, LZK ΔKD, was co-transfected with HA-tagged JNK in COS7 cells. After 24 h, HA-JNK was immunoprecipitated from cell lysates, and kinase activity was measured by the *in vitro* kinase assay using GST-c-Jun as a substrate. B: COS7 cells were co-transfected with LZK or LZK ΔKD and HA-tagged ERK. After 24 h, HA-ERK was immunoprecipitated and kinase activity was measured by the *in vitro* kinase assay using myelin basic protein (MBP) as a substrate. Representative autoradiograms obtained in the *in vitro* kinase assay are shown (upper panels). The amount of His-LZK or His-LZK ΔKD in each lysate was determined by immunoblotting with anti-His antibodies (lower panels). Bar graphs show the mean increase in substrate phosphorylation relative to the empty vector control. The mean values for three independent experiments are shown with standard deviations.

kinase catalytic activity of LZK was required for JNK activation by LZK.

LZK Phosphorylates and Activates MKK7—Analysis of the primary structures of MLK family kinases revealed that LZK forms a subfamily with MUK/DLK, and that MLK1, 2, and 3 form another (*28*). MUK/DLK, as well as MLK2, have been shown to preferentially activate MKK7 rather than SEK1/MKK4 (*32, 33*). This prompted us to examine whether LZK also activates MKK7. To test this, we first examined whether LZK phosphorylates recombinant MKK7 *in vitro*. LZK or LZK ΔKD was expressed in COS7 cells and immunoprecipitated, and then kinase activity was measured using GST-MKK7 as a substrate. Figure 2A shows that wild-type LZK, but not LZK ΔKD, phosphorylates GST-MKK7. This result correlates well with the fact that wild-type LZK, but not LZK ΔKD, activates JNK in COS7 cells (see Fig. 1A). To confirm that LZK enhanced the activity of MKK7 within cells, we next carried out *in vitro* assay similar to the assay described in Fig. 1. Myc-tagged MKK7 was co-expressed with either His-tagged LZK or MEKK1ΔN, a constitutive active form of MEKK1 (*11*), as a positive control, and MKK7 was immunoprecipitated from each cell lysate. MKK7 kinase activity was measured by the *in vitro* kinase assay with GST-kinase–negative JNK as a substrate. MKK7 activity was stimulated about 6 to 8-fold on co-expression of LZK compared to the vector control (Fig. 2B). The MKK7 activation caused by LZK was comparable to that by MEKK1ΔN (Fig. 2B, compare lanes 2 and 3). These results strongly suggest that LZK activates MKK7 in COS7 cells to a reasonable extent, serving as a MAPKKK in the JNK/SAPK pathway.

LZK Is Associated with JIP-1 in COS7 Cells—Recently, several scaffold proteins which interact with multiple components of a particular signaling pathway and enhance the signal transduction of the pathway have been cloned and intensively characterized (*20–27*). The JIP-1 protein has been shown to associate selectively with JNK, MKK7, MUK/DLK, and MLK3, and to serve as a scaffold protein in the JNK/SAPK pathway (*21*). JIP-1 is thought to control accurate and rapid signal transduction leading to JNK activation. As mentioned above, LZK exhibits high sequence similarity to MUK/DLK in its kinase domain and dual leucine zipper-like motif, implying that LZK selectively activates JNK through interaction with the JIP-1 protein. To investigate this possibility, we performed a co-immunoprecipitation experiment. His-tagged LZK was co-expressed with Flag-tagged JIP-1 in COS7 cells, and then the JIP-1 protein was immunoprecipitated with anti-Flag antibodies. As shown in Fig. 2B, the amounts of His-LZK and Flag-JIP-1 were almost identical. The presence of His-LZK in the immunoprecipitates was examined by Western blotting with anti-His antibodies as the primary antibodies. As shown in Fig. 3A, His-tagged LZK was co-immunoprecipitated with Flag-JIP-1, suggesting that LZK was specifically associated with the JIP-1 protein in COS7 cells.

Since it became clear that LZK was associated with JIP-1, we next examined the JIP-1 binding region in the LZK molecule by using various deletion mutants of LZK. Figure 4A shows the schematic structures of LZK and its deletion mutants used in this experiment. As previously reported,

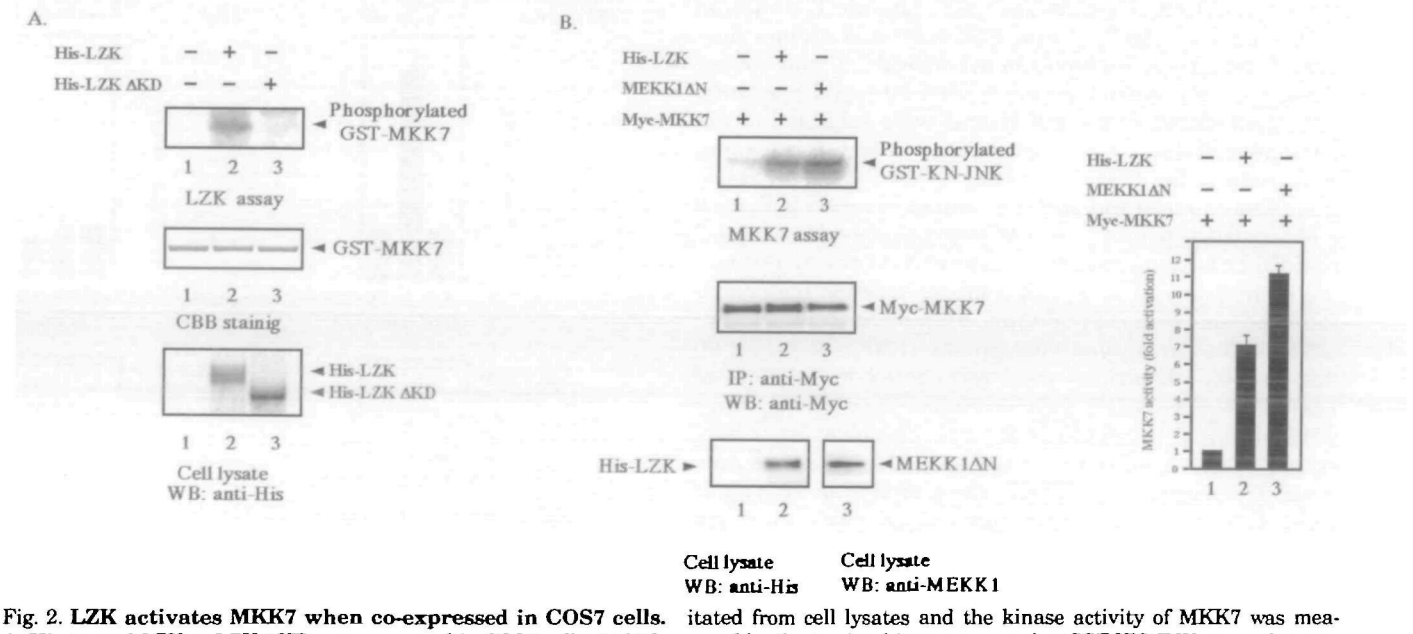

Fig. 2. **LZK activates MKK7 when co-expressed in COS7 cells.** A: His-tagged LZK or LZK ΔKD was expressed in COS7 cells. At 24 h post-transfection, LZK or LZK ΔKD was immunoprecipitated from each cell lysate and kinase activity of LZK was measured by the *in vitro* kinase assay using GST-MKK7 as a substrate. A representative autoradiogram is shown (top). The amounts of GST-MKK7 included in each reaction mixture as a substrate were essentially identical (middle). The expression of LZK or LZK ΔKD was determined by Western blotting with anti-His antibodies (bottom). B: His-tagged LZK or MEKK1ΔN was co-expressed with Myc-tagged MKK7 in COS7 cells. At 24 h post-transfection, Myc-MKK7 was immunoprecipitated from cell lysates and the kinase activity of MKK7 was measured by the *in vitro* kinase assay using GST-KN-JNK as a substrate. A representative autoradiogram of three independent experiments is shown (top). The amount of immunoprecipitated Myc-tagged MKK7 was determined by immunoblot analysis with anti-Myc antibodies (middle), and the amounts of His-LZK and MEKK1ΔN expressed in COS7 cells were determined by immunoblot analysis with anti-His or anti-MEKK1 antibodies, respectively (bottom). Representative results of three independent experiments are shown. The amount of phosphorylated GST-KN-JNK was measured by Fuji BAS2000 and shown as mean ± SE from three independent experiments (graph).

LZK has several distinct structural features, such as a kinase catalytic domain, a dual leucine zipper-like motif, and a C-terminal acidic 14 amino acid sequence of unknown function (SSEEEEGEVDSEVE) that is completely conserved in the LZK and MUK/DLK subfamily of the MLK family. Three deletion mutants LZK (1–800), LZK (1–558), and LZK (1–431), lack this 14 amino acid sequence. LZK (1–431) also lacks the dual leucine zipper-like motif, through which LZK was shown to form dimer/oligomers (*34*). A similar leucine zipper–like motif is believed to be essential for the dimerization of other MLKs such as MLK3 and DLK (*35, 36*). LZK ΔZip also lacks the dual leucine zipper–like motif, but contains most of the C-terminal half of the LZK molecule. LZK ΔKD Zip lacks the region containing the kinase catalytic domain and dual leucine zipper–like motif. To investigate the JIP-1–binding region in the LZK molecule, we carried out a co-immunoprecipitation assay, as in Fig. 3. The amount of Flag-JIP-1 was almost identical in each sample (data not shown). Four of these deletion mutants, LZK (1–800), LZK (1–558), LZK (1–431), and LZK ΔZip as well as full-length LZK were shown to be associated with JIP-1, but LZK ΔKD Zip failed to associate with JIP-1. These results indicate that the kinase catalytic domain of LZK is essential for the interaction of LZK with JIP-1, but the dual leucine zipper–like motif is not. During the preparation of this manuscript, Nihalani *et al.* reported that the N-terminus of DLK is essential for its interaction with JIP-1 (*36*). These results taken together indicate that the conserved JIP-1 binding motif resides within the N-terminus of MLKs.

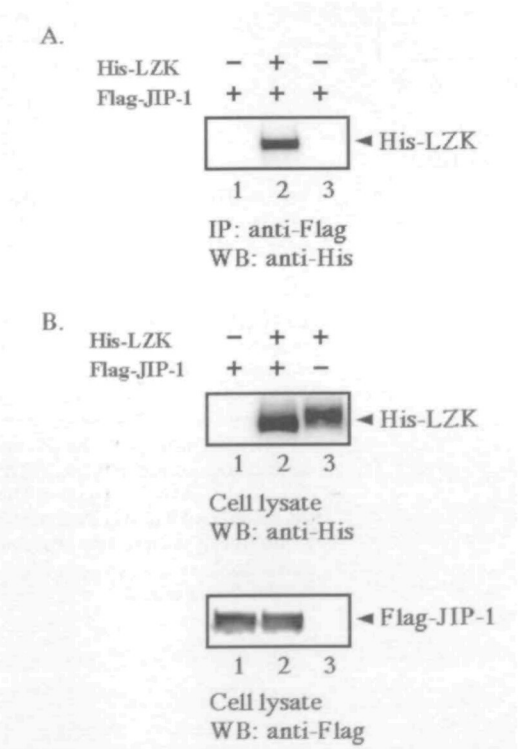

Fig. 3. **LZK associates with JIP-1 when co-expressed in COS7 cells.** A: COS7 cells were co-transfected with His-tagged LZK and/or Flag-tagged JIP-1. At 24–48 h post-transfection, the cells were lysed in lysis buffer. The JIP-1 protein was immunoprecipitated from cell lysates using anti-Flag antibodies and protein G-Sepharose beads. The presence of His-LZK in the JIP-1 immunoprecipitates was examined by immunoblot analysis with anti-His antibodies. B: The presence of His-LZK and/or Flag-JIP-1 in cell lysates was examined by immunoblotting with anti-His or anti-Flag antibodies, respectively.

We next examined the LZK-binding region in the JIP-1 molecule by using deletion mutants of JIP-1. Figure 5A shows schematic structures of JIP-1 and its deletion mutants used in this experiment. The amount of LZK in each sample was almost identical (data not shown). As shown in Fig. 5B, JIP-1 (465–707) co-immunoprecipitated with LZK as well as full-length JIP-1 or JIP-1 (284–707), suggesting that LZK binds to the C-terminus of JIP-1. JIP-1 (582–707) co-immunoprecipitated with LZK, but JIP-1 (465–581) did not, indicating that the LZK-binding region of JIP-1 is within amino acids 582 to 707, which corresponds to the PTB domain of the JIP-1 molecule. These results correlate well with a previous report by Whitmarsh *et al.*, which showed that JIP-1 binds to MLK3 or DLK at its C-terminus (*21*). Although we observed the co-immunoprecipitation of LZK and MKK7, as described above, the finding that JIP-1 (465–707), which bound to LZK, failed to co-immunoprecipitate with MKK7 suggested that LZK binds directly to JIP-1 rather than through MKK7 (data not shown).

JIP-1 Enhances LZK-Induced JNK Activation—In the next experiment, we examined whether JIP-1 serves as an enhancer of LZK-induced activation of the JNK/SAPK pathway. It was previously reported that JIP-1 enhances MLK3-induced JNK activation (*21*), but it remains unclear whether this effect is restricted to the MLK1, 2, 3 subfamily, or the JNK-activating potency of the LZK, MUK/DLK subfamily kinases is also augmented. We examined whether LZK-induced JNK activation is enhanced by JIP-1 by determining the amount of activated JNK by Western blot analysis using anti-phosphorylated JNK antibodies. COS7 cells were co-transfected with HA-JNK and either His-LZK or a control vector with or without His-JIP-1. After 22 h, culture media were replaced with serum-deprived media for 2 h to reduce the background JNK phosphorylation. Then the cells were lysed in Laemmli buffer, followed by SDS-PAGE and Western blot analysis with anti-phosphorylated JNK antibodies as the primary antibodies. In a parallel experiment, the total amount of JNK in each sample was determined using anti-HA antibodies.

In the presence of LZK, phosphorylation of JNK was induced even in the absence of JIP-1 (Fig. 6A, lane 3). However, co-expression of the JIP-1 protein markedly enhanced LZK-induced JNK phosphorylation (Fig. 6A, compare lanes 3 and 4). The results of the densitometric measurement of the immunoreactivity to anti-phosphorylated JNK are shown in Fig. 6C. Data represent the mean ± SE of three independent experiments. Under the conditions used, no apparent JNK phosphorylation signal was observed without LZK, regardless of JIP-1 expression, suggesting that the JNK phosphorylation observed was solely due to LZK co-expression. Western blot analysis with anti-HA confirmed that the amounts of overexpressed JNK in each sample were almost identical (Fig. 6B). We also confirmed that the amounts of His-LZK and His-JIP-1 were almost identical in each sample (data not shown). These results taken together indicate that JIP-1 enhanced LZK-induced JNK activation *in vivo* through physical association with LZK, MKK7, and JNK.

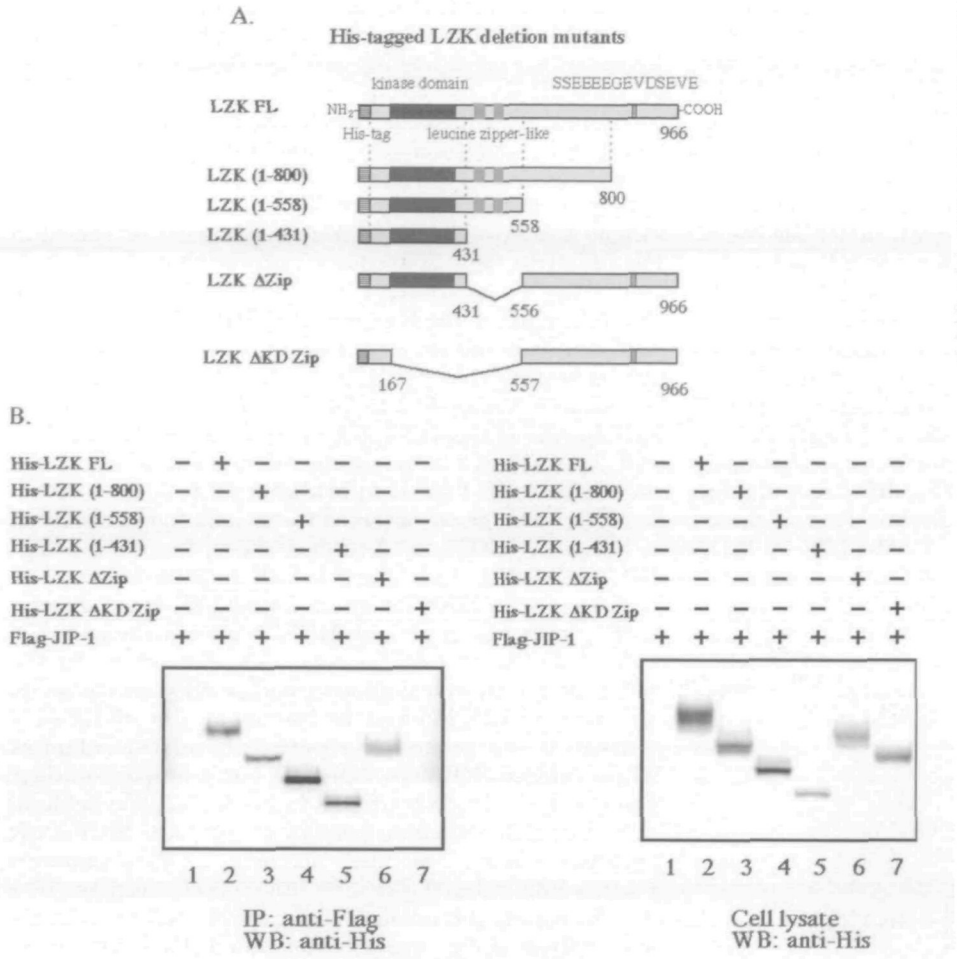

Fig. 4. **LZK associates with JIP-1 *via* its kinase catalytic domain.** A: His-tagged LZK and its deletion mutants used in this experiment are schematically presented. B: Each deletion mutant was co-expressed with Flag-tagged JIP-1 in COS7 cells, and JIP-1 was immunoprecipitated from each cell lysate. The presence of LZK in JIP-1 immunoprecipitates was examined by immunoblotting with anti-His antibodies (left). In a parallel experiment, a whole-cell lysate of each transfectant was subjected to SDS-PAGE, and LZK was immunodetected with anti-His antibodies (right).

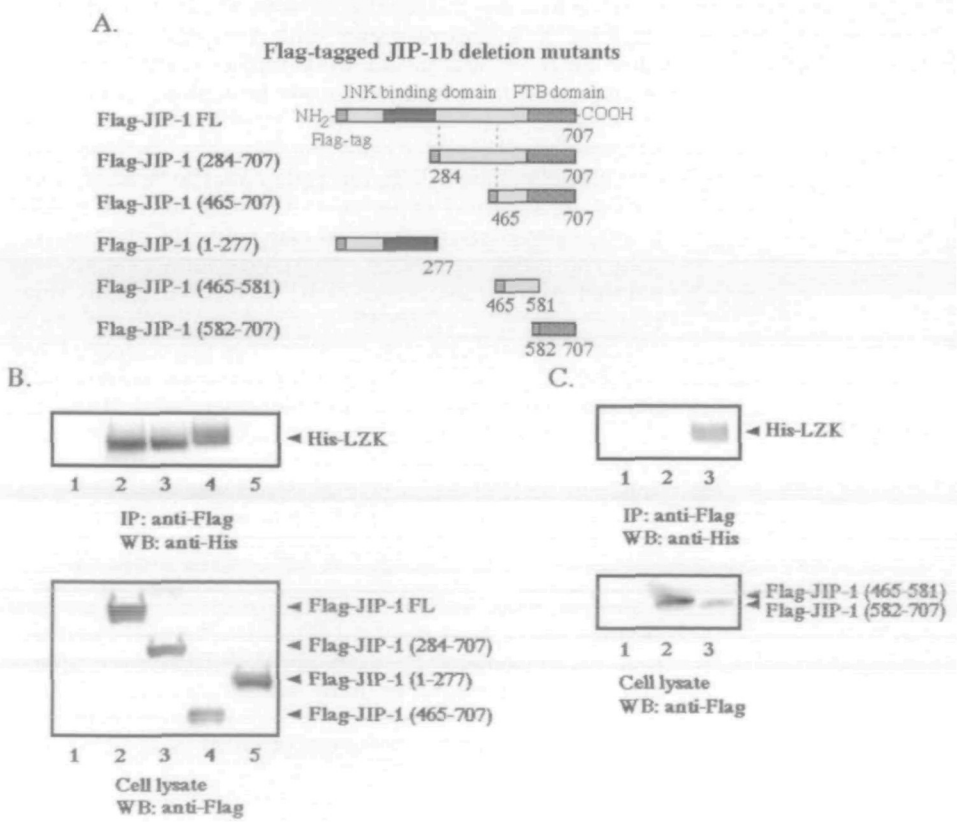

Fig. 5. **JIP-1 associates with LZK *via* its C-terminal region.** A: Flag-tagged JIP-1b and its deletion mutants used in this experiment are schematically presented. B and C: His-tagged full-length LZK was co-expressed with Flag-tagged deletion mutants of JIP-1 in COS7 cells, and JIP-1 was immunoprecipitated from each cell lysate. The presence of LZK in JIP-1 immunoprecipitates was examined by immunoblotting with anti-His antibodies (upper panel). The presence of His-LZK and Flag-JIP-1 mutants in cell lysates was examined by immunoblotting with anti-Flag antibodies (lower panel).

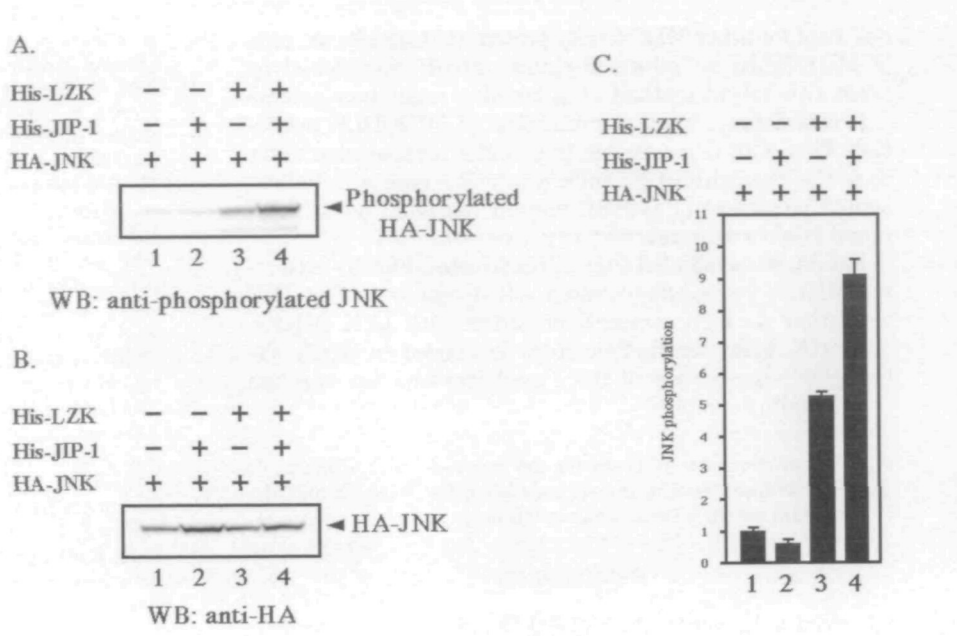

Fig. 6. **JIP-1 enhances LZK-induced JNK activation.** A: COS7 cells were co-transfected with HA-JNK and His-tagged LZK and/or JIP-1. After 24 h, the cells were lysed by the addition of SDS-PAGE sample buffer, and the amount of dually phosphorylated JNK was determined by immunoblot with anti-phosphorylated JNK antibodies. B: The same lysates as used in A were subjected to SDS-PAGE, then immunoblotted with anti-HA antibodies to determine the total amount of overexpressed JNK in each transfection. C: The extent of JNK activation was represented as a graph. To determine the extent of JNK activation under each set of conditions, the immunoreactivity to anti-phosphorylated JNK antibodies in Western blots was measured densitometrically when equal amounts of anti-HA immunoreactivity were loaded. Data represent the mean ± SE of three independent experiments, expressed in arbitrary units relative to the mean of negative control (JNK without LZK and JIP-1, lane 1) as one unit. Co-expression of JIP-1 significantly enhances LZK-induced JNK activation under the experimental conditions used.

DISCUSSION

In this report, we described that LZK is a MAP kinase kinase kinase which selectively activates the JNK/SAPK pathway by phosphorylation and activation of MKK7, and that scaffold protein JIP-1 enhances LZK-induced JNK activation through association with LZK, MKK7, and JNK. These results are well consistent with previous reports that MUK/DLK, MLK2, and MLK3 activate the JNK/SAPK pathway but not the ERK pathway, indicating that MLK family proteins are associated with the JNK pathway rather than the ERK pathway (*14–19*). MUK/DLK was reported to activate MKK7 more efficiently than SEK1/MKK4 (*32*). Since LZK exhibits high sequence similarity in its kinase domain and dual leucine zipper-like motif with MUK/DLK, it is possible that LZK has a preference for MKK7 rather than SEK1/MKK4, although we have not yet defined the substrate specificity of LZK. We also observed that LZK activated p38 MAP kinase in COS7 cells when expressed without JIP-1 (Ikeda *et al.*, unpublished observation), suggesting that LZK is able to activate SEK1/MKK4 and/or MKK3, MKK6 under certain conditions. Since JIP-1 selectively associates with LZK, MKK7, and JNK, it is possible that JIP-1 plays the role of a "signaling selector," which restricts the substrates of LZK to MKK7 and thus restricts the function of LZK or other MLKs to the activation of the JNK/SAPK pathway.

The tissue distributions of mRNA expression of JIP-1 and its splice variant IB1 (Islet Brain 1) are similar to that of LZK (*37*). We detected transcripts of both JIP-1 and LZK in rat pancreas on RT-PCR. In addition, we observed that the LZK protein is localized in Langerhans islets of the pancreas (Ikeda *et al.*, manuscript in preparation). Since the expression of IB-1 is restricted to β cells of Langerhans islets in the pancreas (*38*), and the gene encoding IB-1 has been reported to be a candidate gene for type 2 diabetes (*39*), the interaction of these molecules in the β cells may be physiologically significant. On the contrary, JIP-1 homologue JIP-2 was expressed in a brain-specific manner (*25*). MUK/DLK is also a brain-specific protein, so it is possible that JIP-1 utilizes LZK as a MAPKKK while JIP-2 utilizes MUK/DLK under physiological conditions.

Our results presented in this report clearly demonstrated that the kinase catalytic domain of LZK associates with the C-terminal region of JIP-1 protein, which contains a PTB domain. The PTB domain of JIP-1 is reported to be associated with p190 rhoGEF, a guanine nucleotide exchange factor for a small GTP-binding protein Rho (*27*). The binding site on p190 rhoGEF for the JIP-1 PTB domain was shown to be a modified consensus site in which a phenylalanine residue substitutes for a phospho-tyrosine residue in a conservative consensus site. However, the kinase domain of LZK contains neither a conservative nor a modified consensus sequence. Thus, it is possible that a novel recognition mechanism that does not involve a PTB-domain and a phospho-tyrosine- or phenylalanine-containing consensus site, regulates the association of JIP-1 and LZK.

In the ERK pathway, Raf Kinase Inhibitor Protein (RKIP) has been reported to bind to Raf-1 (MAPKKK), MEK (MAPKK), and ERK (MAPK) (*40*). This interaction seems analogous to that of JIP-1, but RKIP has been shown to inhibit Raf-induced activation of MEK and ERK. It is therefore important to understand the biological significance of the different modes of regulation by these scaffold/anchoring proteins.

As shown in Figs. 1 and 2, LZK expressed in COS7 cells was able to activate JNK and phosphorylate MKK7 without any additional stimulation, indicating that LZK is already activated at least partially, even if not fully. This observation raised an important question about the regulatory mechanisms of the activation of LZK, to which we currently do not have any clear solutions. In this regard, it should be noted that the similar phenomena were also

observed for other MLK family proteins (*14–19*). In the case of MUK/DLK, an inhibitor protein MBIP was cloned by yeast two-hybrid method as a possible regulatory protein (*41*) associated with the initial step of MUK/DLK activation. Therefore it is possible that under certain conditions, as in the case with COS7 cells, where this type of inhibitors should be absent or present only at marginal levels, LZK might behave as a constitutive active form.

Finally, we concluded that LZK activates JNK by activating MKK7, and scaffold protein JIP-1 enhances this JNK activation through complex formation with LZK, MKK7, and JNK. However, further study is needed to clarify the biological significance of the signal transduction through this signaling complex.

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