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Involvement of LOC66273 isoform 2, a novel Mth938 containing protein, in MAPK pathway

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1. ABSTRACT

Using dual-luciferase reporter assay system, our previous study showed that LI2, significantly increased AP-1 transcriptional activity. Sub-cellular localization showed that GFP-LI2 fusion protein is diffusely distributed in the cytoplasm, with some highly concentrated spots around the nucleus, suggesting that LI2 protein has a physiological role in cytoplasm. Overexpression of LI2 significantly increased AP-1 transcriptional activity. Moreover, LI2 significantly promoted transcriptional activity of Elk1 and c-jun, which might, at least partly, be associated with activated ERK1/2 and JNK/SAPK signaling pathway. These data suggest that LI2 is a novel MAPK regulating protein.

2. INTRODUCTION

In the post-genomic era, it is urgent to identify thousands of novel genes that have been deposited in the Refseq and EST databases in GenBank, most of which with unknown or poorly understood functions. Many high-throughput functional gene-screening systems based on overexpression or knockdown in transfected cell lines have been established, using plasmids, adenovirus, siRNA, microRNA and so on. Particularly, the high-throughput functional gene-screening systems based on dual-luciferase reporter genes are potent and effective tools to study gene functions in cell signal transduction (1).

Cells have developed sophisticated mechanisms to receive signals, transmit the information and orchestrate the appropriate responses, in order to be constantly aware of changes in the extracellular milieu to respond accordingly. Signal transduction mechanisms heavily rely on post-translational modifications of proteins, among which phosphorylation of protein kinases play a major role (2). In all kinds of protein kinases in eukaryotic cells, mitogen-activated protein kinases (MAPKs) seem to be involved in most signal transduction pathways (3). MAPKs are serine-threonine kinases, which mediate intracellular signaling pathways involved in a variety of cellular activities including cell proliferation, differentiation, survival, death, and transformation (4-5). The mammalian MAPK family consists of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase (also known as stress-activated protein kinase; JNK/SAPK) and p38 (6). Each MAPK signaling axis comprises at least three components: a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. MAP3Ks phosphorylate and activate MAP2Ks, which in turn phosphorylate and activate MAPKs (7). Activated MAPKs phosphorylate various substrate proteins including transcription factors such as ETS domain-containing protein 1 (Elk-1), cellular jun proto-oncogene (c-jun), cellular oncogene (c-fos), ATF2, and so on. MAPK pathways are activated either as a result of a series of binary interactions between the kinase components or through the formation of a signaling complex containing multiple kinases that is guided by a scaffold protein, such as the Ste20 subfamily (8-10).

Using dual-luciferase reporter assay system, our previous study showed that several novel genes associated with the transcription factor activator protein-1 (AP-1) pathway, including a hypothetical protein LOC66273 isoform 2 (LI2; previously identified as 1810020D17Rik, GenBank Accession No. NM_183251.3). Here we identified the mRNA and protein expression of LI2. Bioinformatics analysis indicates that LI2 is highly conserved from zebrafish to humans, but shares no obvious homology to any known genes or proteins. To our knowledge, the function of the LI2 protein has not been determined (11). Our functional investigation revealed that LI2 was involved in MAPK pathway.

3. MATERIALS AND METHODS

3.1. Reagents

Transfection Lipofectamine™ 2000, RNA extraction reagent TRIzol, ThermoSCRIPT™ RT-PCR System, and fetal bovine serum were procured from Invitrogen Life Technologies (Carlsbad, USA). PMA, rabbit antibodies against β-actin were purchased from Sigma-Aldrich (St. Louis, USA). Cell lysis buffer (10×), polyclonal antibodies against phosphorylated ERK1/2, JNK/SAPK and P38 were purchased from Cell Signaling Technology (USA). Protease inhibitor cocktail tablets were obtained from Roche Applied Science (Switzerland). IRDye™ 800-conjugated secondary antibodies against rabbit IgG were purchased from LI-COR Bioscience (NE, USA). Ionomycin was from Santa Cruz Biotechnology (USA).

3.2. cDNA cloning and vector construction

The full-length coding region of LI2 cDNA (according to GenBank Accession No. NM_183251.3) was amplified from a mouse skeletal muscle cDNA library (Clontech, USA) by PCR using the specific primers (forward 5'-GCGAACAGAAGCGCTGCATTTCAG-3'; reverse 5'-TGATGTGGAGACTGGTAAGACAG-3'). The purified PCR product was ligated into the pGEM-T Easy vector (Promega, USA). The insert was released and sub-cloned into pcDNA.3.1/myc-his (-)B (PCDB, Invitrogen, USA) and pEGFP-C3 (Clontech, USA) vectors to construct plasmids of PCDB-LI2 and pEGFP-LI2, respectively. All clones were confirmed by sequencing using an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, USA). After that, all plasmids were extracted and purified for transfection using EndoFree Plasmid Maxi Kit (Qiagen, USA).

3.3. Polyclonal anti-LI2 antibody preparation

Antibodies against LI2 were generated by immunization of rabbits with KLH-coupled LI2 peptide (SWGQMKVQGSTLTYKDCKVWPG), which were synthesized by solid phase synthesis and purified by HPLC to 90% purity (Chinese Peptide, China). Rabbit polyclonal antibody was purified using CNBr Sepharose 4B coupled with specific LI2 peptide. The antibodies were validated by ELISA, immunofluorescence and western blot analysis.

3.4. Cell line and transfection

HEK 293T cells (human embryonic kidney cell line) and 3T3-L1 fibroblasts (American Type Culture Collection, Manassas, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/L glucose supplemented with 10% calf bovine serum at 37°C in a humidified incubator with 5% CO_2. Routine cell culture procedures were strictly followed to maintain proper cell density.

Transfection of 3T3-L1 cells was performed by electroporation. Cells (2×10^6) were mixed with 10μg DNA in 350μl serum-free medium and then electroporated with a 120-V, 20-ms pulse using a BTX T820 square-wave electroporator in a 2-mm cuvette (BTX, San Diego, CA). Transfection efficiency was monitored by a pEGFP-LI2 plasmid. Cells with more than 60% transfection efficiency were used for further experiments. Transfection of 3T3-L1 cells was performed using Lipofectamine™ 2000 reagent according to the manufacturer's protocol, and the detail was shown in the following "Dual-luciferase reporter assay".

3.5. RNA Isolation and Reverse Transcription-PCR analysis

Total RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. One μg of RNA was subjected to reverse transcription with the ThermoSCRIPT™ RT-PCR System. The reactions were incubated at 42°C for 50 min. The primers used for LI2 PCR amplification were the same as the above. For GAPDH (internal control) primers, forward 5'-GTGAAGGTCGGTGTGAACGGATTT-3'; reverse 5'-

CTCCTTGGAGGCCATGTAGGCCAT-3' were used. The cycling conditions were 94°C for 5 min followed by 35 cycles (for LI2) or 25 cycles (for GAPDH): 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, then one-cycle extension at 72°C for 5 min, store at 4°C. PCR products were separated via 1.0% agarose gel electrophoresis and visualized by ethidium bromide staining.

3.6. Fluorescence microscopy

Transiently transfected 3T3-L1 cells expressing LI2-GFP were cultured on the coverslips. Cell nuclei were stained with DAPI (0.5μg/ml) for 5 min at 37°C, and observed by fluorescence confocal microscopy. Subsequently, digital images of cells were obtained using an inverted fluorescent microscope (Olympus, Japan).

3.7. Protein extraction and Immunoblot analysis

Immunoblot analysis was conducted as previously described (12) with a slight modification. Briefly, cells were washed twice with ice-cold PBS and lysed in cell lysis buffer for 30 min at 4°C. Cell lysate was clarified by centrifugation at 4°C at 16 000 g for 15 min. Protein concentration was determined using the BCA protein assay reagent (Pierce, USA). Equal amounts of protein were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membranes (Hybond™, ECL™, GE, Healthcare, USA). Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% non-fat milk for 2 hours, and incubated overnight at 4°C with the primary antibodies. After washing in TBST buffer, membranes were incubated for 1 hour at room temperature in the dark with the rabbit anti-mouse IRDye™ 800-conjugated secondary antibodies. Following another three washes with TBS-T, signals were detected using the LI-COR Infrared Imaging System (Odyssey, Lincoln, NE) and quantified with Odyssey software.

3.8. Dual-luciferase reporter assay

Dual-luciferase reporter assays were conducted as previously described (12) with a slight modification. Briefly, AP-1 luciferase activity was measured using cis-reporting system. Approximately 1.0×10^4 293T cells per well were seeded into a 96-well culture plate. After 24hours, the cells in each well were co-transfected with 50 ng of the PCDB-LI2 plasmids, 50 ng of the pAP-1-Luc plasmids containing the firefly luciferase reporter gene (PathDetect, Stratagene), and 4 ng of the pRL-TK plasmids as the internal control containing the Renilla luciferase gene (Promega, Madison, WI). The negative control was performed with PCDB plasmid. At 24 hours after transfection, the cells were stimulated with PMA (12-O-Tetradecanoylphorbol-13-acetate) (50 mM, sigma) and Ionomycin (1 mM, Calbiochem, USA) for 6 hours, and then lysed in standard lysis buffer. Using a Synergy4 Multifunction Microplate Reader (Bio-Tek Instruments, USA) the cell lysates were assayed for both firefly and renilla luciferase activities according to the manufacturer's instructions. Each independent experiment was performed three times.

Elk1, c-jun and c-fos luciferase activities were measured using trans-reporting systems. For Elk1 activity detection, approximately 1.0×10^4 293T cells/well was seeded into a 96-well culture plate. After 24 hours, the cells in each well were cotransfected with 50 ng PCDB-LI2 plasmid, 45 ng pFR-Luc plasmid, 5 ng pFA-Elk1 fusion trans-activator plasmid (PathDetect, Stratagene) and 4 ng pRL-TK plasmids as the internal control. For c-jun and c-fos activities detection, 5 ng pFA-c-jun and 5 ng pFA-c-fos were used to substitute pFA-Elk1, respectively. The negative control was performed with PCDB plasmid. At 36 hours after transfection, the cells were stimulated with PMA (50 mM) and ionomycin (1 mM) for 6 hours. The cells were collected and the cell lysates were measured as above.

4. RESULTS

4.1. Cloning and bioinformatics analysis of mouse LI2

The full-length cDNA and predicted amino acid sequences of LI2 are shown in Figure 1A. Mouse LI2 is located on chromosome 7E2, and encompasses 5 exons and 4 introns (Figure 1B). The ORF encodes 122 amino acids with a predicted molecular mass of 13.2 kDa and a pI of 7.77. Phylogenetic analysis showed that LI2 is highly conserved in human, mouse, rat, cow, chicken, and zebrafish (Figure 1C), but shares no obvious homology to any known genes or proteins. Conserved domain analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=88592) suggests that there is a predicted Mth938 (amino acid residues 5–120) domain in LI2. Mth938 is a hypothetical protein domain encoded by the Methanobacterium thermoautotrophicum (Mth) genome (13). This protein crystallizes as a dimer, although it is monomeric in solution, with one disulfide bond in each monomer. The function of the domain has not been determined (11).

4.2. Expression profiles

The mRNA level for LI2 was confirmed by RT-PCR in various mouse tissues. As shown in Figure 2A, RT-PCR analysis revealed that a ~0.5-kb transcript specific for LI2, consistent with the bioinformatics analysis (469 bp), was expressed in a variety of normal tissues, especially at a higher level in white adipose tissue and a lower level in the kidney tissue. In addition, Western blot analysis identified a band of ~13 kDa could be detected (Figure 2B), which was consistent with the calculated molecular weight of LI2 protein. The LI2 protein was expressed in various tissues, especially at a higher level in the adipose tissue and skeletal muscle. The results supported LI2 could be widely expressed, especially higher in the adipose tissue and skeletal muscle, suggesting that LI2 play an important role in the adipose tissue and skeletal muscle.

4.3. Sub-cellular localization of LI2 protein

To determine the sub-cellular localization of LI2, 3T3-L1 cells were transiently transfected with pEGFP-LI2 vector. GFP-LI2 fusion protein exhibited a diffuse cytoplasm distribution, with some highly concentrated spots around the nucleus (Figure 2C), which was consistent with the results of bioinformatics analysis (http://psort.hgc.jp/form2.html), suggesting that LI2 protein exert its physiological role in cytoplasm.

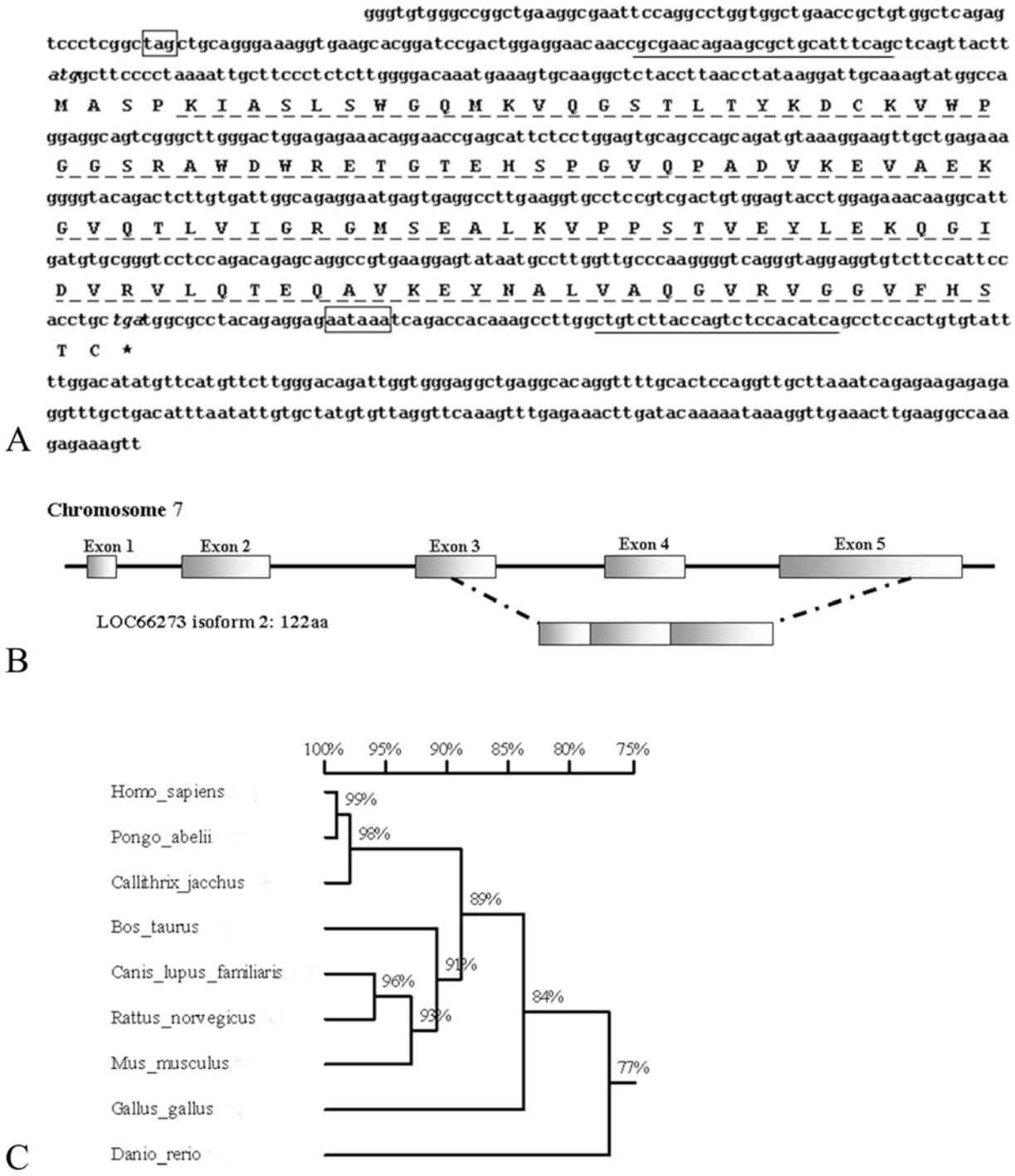

Figure 1. Identification and sequence analysis of LI2. (A) cDNA and predicted amino acid sequences of mouse LI2. Primers used to amplify the ORF are underlined. The start and stop codons are italicized. An in-frame stop codon in front of the start ATG is indicated, and bold letters in a frame at the 3' end indicate the poly (A) signal sequence. The predicted conserved domain Mth938 (amino acid residues 5–120) is indicated with a broken line. (B) The sketch map of the LI2 gene and cDNA structure. The boxes show the exons with their relative size and the positions in the LI2 gene. (C) Phylogenetic analysis of LI2.

4.4. Over-expression of LI2 promoted AP-1 activity

Using AP-1 dual-luciferase cis-reporter assay system, we found that under basal condition, over-expression of LI2 protein significantly increased AP-1 reporter gene activity compared with PCDB transfected cells (about 3-fold of that in the empty vector, $P<0.05$,

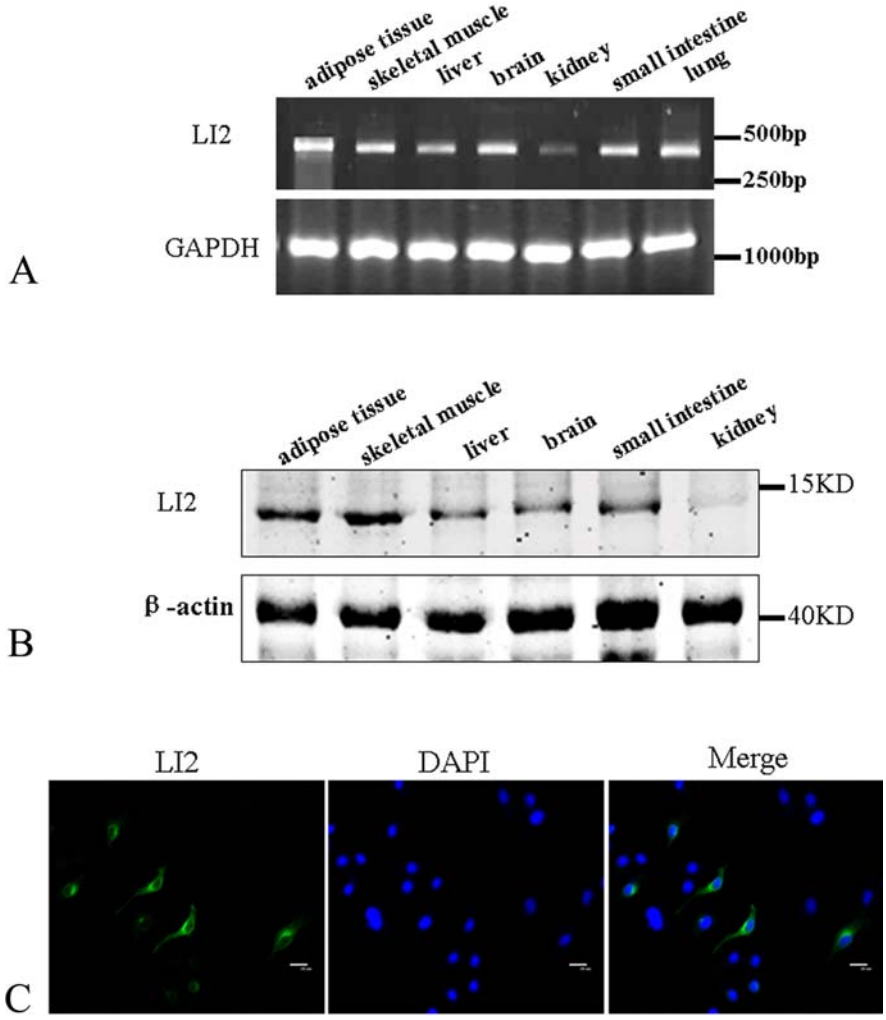

Figure 2. Expression profiles and sub-cellular localization of LI2. (A) RT-PCR analysis of LI2 mRNA expression in mouse normal tissues. GAPDH was used as an internal control. (B) LI2 endogenous protein expression was also indicated by Western blot using rabbit polyclonal anti-LI2 antibody in various mouse tissues. β-actin expression was detected as an internal control. (C) Sub-cellular localization of pEGFP-LI2. Two-color confocal microscopy analysis of pEGFP-LI2 fusion protein (green) and nuclear-specific fluorescent dye DAPI (blue) was performed after 36h transfection. Scale bars 20 mm.

Figure 3A). PMA plus ionomycin was the potent activator of AP-1 signaling (14-15). Additionally, the AP-1 activity of LI2 under stimulation was also obviously increased (approximately 2-fold, $P<0.05$, Figure 3A). Taken together, these data showed that over-expression of LI2 promoted AP-1 activity in the absence or presence of AP-1 stimulus. The AP-1 activity of LI2 was a little less than the activity of the positive control MEK1 under basal or stimulated condition.

4.5. Over-expression of LI2 increased Elk1 and c-jun transcriptional activity, but not c-fos

To identify the specific transcription factors which mediate the process LI2 promoted the AP-1 activity, dual-luciferase trans-reporter assay system were used. Under basal condition, The Elk1 and c-jun activity of LI2 were significantly increased compared with PCDB transfected cells (approximately 2.5-fold and 2-fold of that in the empty vector, respectively, $P<0.05$, Figure 3B and Figure 3C), whereas c-fos activity had hardly changed (Figure 3D). Additionally, PMA plus ionomycin treatment markedly enhanced Elk1, c-jun and c-fos luciferase activity. The Elk1 and c-jun activity of LI2 under stimulation was also significantly increased compared with the control (approximately 180% and 160%, $P<0.05$, respectively, Figure 3B and Figure 3C), while c-fos activity had nearly no difference (Figure 3D). These data suggest that LI2 might play a key role in the regulation of transcription factor Elk1 as well as c-jun, but not c-fos, under either basal or stimulated condition.

4.6. LI2 promoted phosphorylation of ERK1/2 and SAPK/JNK, but not p38

To define the mechanism by which LI2 promote Elk1 and c-jun activation, we assessed the phosphorylation status of the upstream signaling pathway of Elk-1 and c-

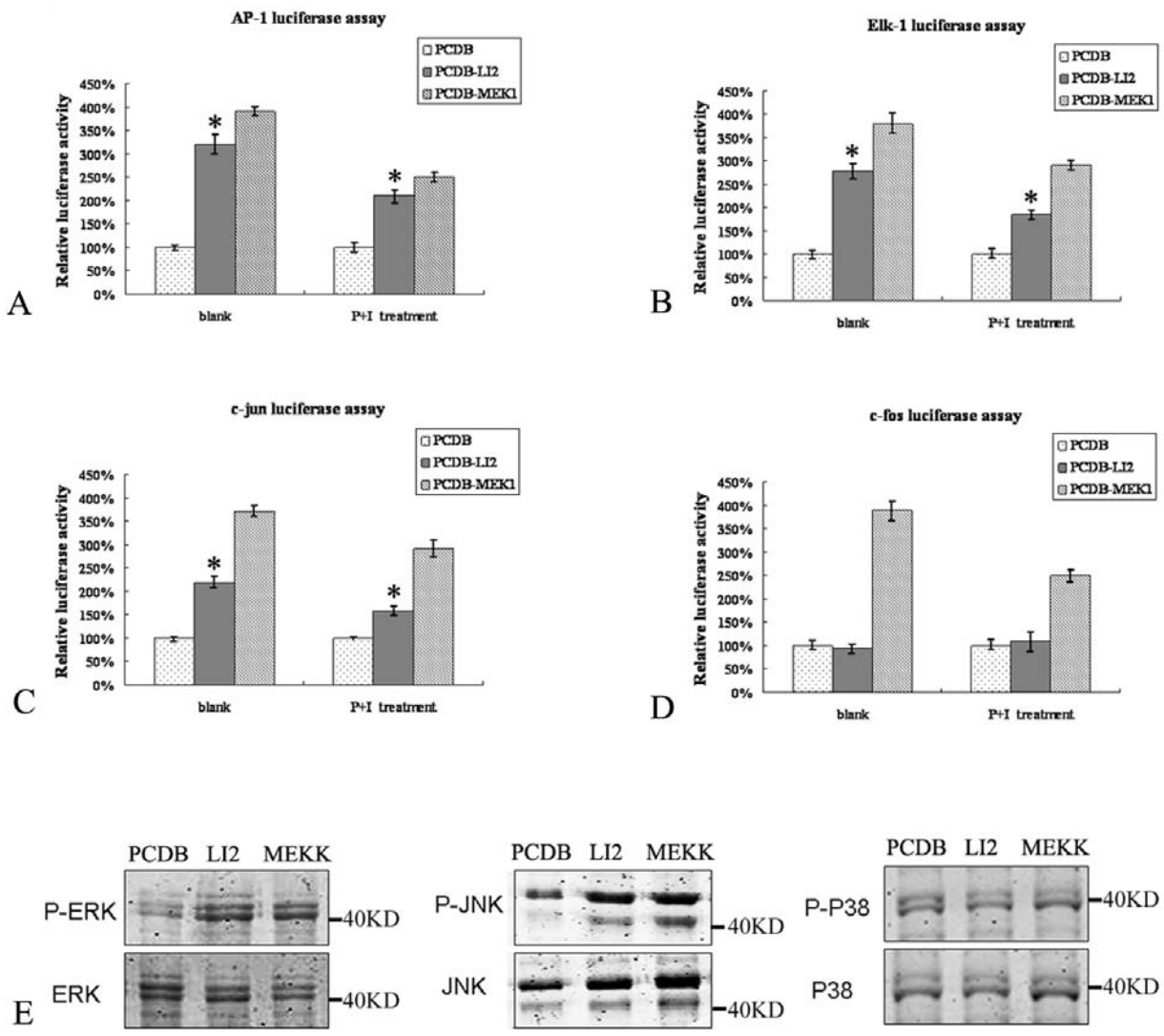

Figure 3. Overexpressed LI2 was involved in MAPK pathway. (A) Over-expression of LI2 activated AP-1 activity. AP-1 luciferase activity of LI2 overexpression was measured using cis-reporting AP-1 system. Cells were transiently cotransfected with the pAP-1-Luc plasmids and pRL-TK plasmid. Relative luciferase activity was normalized by co-transfection with pRL-TK plasmid (internal control). (B-D)Over-expression of LI2 promoted Elk1 and c-jun transcriptional activity, but not c-fos. The negative control was performed with PCDB plasmid, and PCDB-MEK1 was served as positive control. (B) Elk1 luciferase activity of LI2 overexpression was measured using trans-reporting Elk1 system. Cells were transiently cotransfected with the pFR-luc reporter plasmid, pFA-Elk1 fusion activator plasmid and pRL-TK plasmid. Relative luciferase activity was normalized by co-transfection with pRL-TK plasmid (internal control). (C) c-jun luciferase activity was measured using trans-reporting c-jun system. pFA-c-jun was used to substitute pFA-Elk1. (D) c-fos luciferase activity was measured using trans-reporting c-fos system. pFA-c-fos was used to substitute pFA-Elk1. (E) Overexpression of LI2 increased phosphorylation of ERK1/2 and JNK/SAPK, but not p38. The presence of phosphorylated proteins was monitored by Western blot using phospho-specific antibodies as indicated. Loading of equal amounts of proteins was determined by restaining of the blots with antibodies directed against Erk1/2, JNK/SAPK or p38.

jun. Because there were three main subgroups in the Mammalian MAPK family that affected Elk1 and c-jun, ERK1/2, JNK/SAPK and p38 MAPK (6), we detected the protein levels of ERK1/2, JNK/SAPK and p38 as well as their phosphorylation levels. As shown in Figure 3E, although LI2 transfected cells had hardly changed compared with the control in the protein levels of ERK1/2 and JNK/SAPK, the phosphorylation levels of ERK1/2 and JNK/SAPK of LI2-overexpressing cells are much higher than that of cells transfected with pCDB ($P<0.05$). Conversely, LI2 had no effect on both phosphorylation and protein levels of p38 MAPK ($P>0.05$). These data indicated that the activation of LI2 on Elk1 transcriptional activity was mainly mediated by ERK1/2 and JNK/SAPK MAPK, but not p38 MAPK.

5. DISCUSSION

In the present study, we confirmed that LI2 was expressed in a variety of mouse normal tissues, in both mRNA and protein levels, which was consistent with the bioinformatics prediction. Phylogenetic analysis shows that LI2 is highly conserved in human, mouse, rat, cow, chicken, and zebrafish. Conserved domain analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid =88592) suggests that there is a predicted Mth938 (amino acid residues 5–120) domain in LI2. Mth938 is a hypothetical protein domain encoded by the Methanobacterium thermoautotrophicum (Mth) genome (13). Therefore, we can suggest that LI2 play an important and fundamental role in cells. Bioinformatics analysis of sub-cellular localization suggests that LI2 protein is distributed in the cytoplasm (http://psort.hgc.jp/form2.html), and our study confirmed that GFP-LI2 fusion protein is diffusely distributed in the cytoplasm, with some highly concentrated spots around the nucleus, suggesting that LI2 protein has a physiological role in cytoplasm. LI2 shares no obvious homology to any known genes or proteins, and to our knowledge, no functional study has been performed on this gene or the Mth938 domain (11).

Using dual-luciferase reporter assay system, our previous study showed that LI2 significantly increased AP-1 transcriptional activity. Here, more functional investigations were performed to reveal the effects and the mechanism of LI2 on the AP-1 signal transduction pathways. First, we demonstrated that overexpression of LI2 promoted the transcriptional activity of AP-1 in 293T cells. Previous studies indicate that the transcription activity of AP-1 is mainly mediated by c-jun, c-fos and/or Elk-1 (16). Secondly, LI2 was identified to exert its cellular physiological role in Elk1 and c-jun signaling pathway, but not c-fos. Elk-1 is an Ets-domain transcription factor activated by MAPK that binds to the serum response element (SRE) to induce immediate early gene transcription, in response to extracellular stimuli such as serum and growth factors (17). On the other hand, c-jun encodes a protein which is highly similar to the viral protein, and which interacts directly with specific target DNA sequences to regulate gene expression. This gene is intronless and is mapped to 1p32-p31, a chromosomal region involved in both translocations and deletions in human malignancies (http://www.ncbi.nlm.nih.gov/gene/3725).

To define the mechanism by which LI2 promote Elk1 and c-jun activation, we assessed the phosphorylation status of the upstream signaling pathway of Elk-1 and c-jun, and the results illuminated that overexpression of LI2 obviously increased phosphorylation of ERK1/2 and JNK/SAPK, but not p38 in 293T cells, suggesting that the activation of LI2 on Elk1 transcriptional activity was mainly mediated by ERK1/2 and JNK/SAPK MAPK. The evolutionarily conserved MAPK family consists of serine/threonine-specific protein kinases, involved in signal transduction pathways between the membrane and the nucleus (8), which was agree with the sub-cellular location of LI2. MAPKs transduce diverse extracellular stimuli (mitogenic growth factors, environmental stresses and pro-apoptotic agents) to the nucleus via kinase cascades to regulate proliferation, DNA synthesis arrest, differentiation, and apoptosis (18-19). MAPKs become activated through phosphorylation of specific threonines and tyrosines by an upstream dual specificity kinase. Regulation of the signaling pathway occurs via the sequential phosphorylation and activation of each member of the kinase cascade (20-21).

Additionally, MAPK pathways are activated either as a result of a series of binary interactions between the kinase components or through the formation of a signaling complex containing multiple kinases that is guided by a scaffold protein (3, 7). Such scaffold proteins mediate the activation of MAPK signaling pathways consisting of specific kinase components. Among all the scaffold proteins, to our knowledge, β-Arrestin 2 acts as a scaffold protein for both the ERK1/2 and JNK/SAPK signaling pathway (22). Therefore, we can predicted that the effect of LI2 on cellular signal transduction be mediated by β-Arrestin 2. Further researches will be performed to elucidate the process.

In conclusion, we identified the expression of LI2, a novel Mth938 domain containing protein, both in mRNA and protein levels. Further functional investigations revealed that LI2 played an important role in AP-1 (including Elk1 and c-jun) transcription regulation, as well as MAPK signal transduction via the ERK1/2 and JNK/SAPK, but not p38 MAPK pathways. These present data suggest that LI2 might be a novel MAPK regulating protein, and contribute to a better understanding of the complex mechanism of LI2 as well as MAPK transcription regulation.

6. ACKNOWLEDGEMENTS

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Abbreviations: LI2: LOC66273 isoform 2, MAPKs: mitogen-activated protein kinases, ERK1/2: extracellular signal-regulated kinase 1/2, JNK/SAPK: c-Jun NH2-terminal kinase/stress-activated protein kinase, MAP3K: MAPK kinase kinase, MAP2K: MAPK kinase, Elk-1: ETS domain-containing protein 1, AP-1: activator protein-1.

Key Words: LOC66273 isoform 2; AP-1 transcriptional activity; MAPK pathway

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