Identification and characterization of a predominant isoform of human MKK3

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Abstract We have obtained a novel cDNA species of MKK3, termed MKK3b. MKK3b cDNA differs from its original form, MKK3, at the 5′-end, encoding 29 extra amino acids in the N-terminus. Analysis of MKK3 genomic DNA structure revealed that the MKK3b-unique 5′-end sequence is derived from an exon different from that of MKK3, and that they share identical sequences thereafter. This suggests that the two cDNA forms of MKK3 are either generated by differential splicing of the same gene or derived from differential promoter utilization. Northern blotting analysis showed that MKK3b mRNA is much more abundant than MKK3. Functional characterization based on the activation of p38 revealed that MKK3b is more efficient than MKK3 in mediating downstream signalling events.

Key words: p38 MAP kinase; MAP kinase kinase 3; MAP kinase kinase 3b

1. Introduction

Numerous extracellular signals are transduced through a cascade of phosphorylation, to the activation of the mitogen-activated protein kinases (MAPK). Different groups of MAP kinases are activated by distinct subsets of MAP kinase kinases. For example, Mek1/2, Sek1 (also known as MKK4 or JNKK1), and MKK3/6 are believed to directly activate Erks, SAPKs (or JNKs), and p38 (or MPK2, RK, and CSBP), respectively (reviewed in [1–10]). A cDNA encoding one of the MKKs, MKK3, has been cloned and shown to be present in all tissues and enriched in skeletal muscle [9]. MKK3 appears to specifically activate p38 MAP kinase, mediating stress-induced responses [11–13].

We initially observed that in THP-1 and HeLa cells the major protein band detected by Western blotting using anti-MKK3 antibodies raised against either a C-terminal peptide of MKK3 or recombinant MKK3 protein was approximately 47 kDa in size, indicating a discrepancy between the molecular weight deduced from the open reading frame of the identified MKK3 cDNA [9] and the apparent molecular weight of the natural MKK3 protein. This suggested that there might be more than one cDNA species for MKK3. We therefore screened a human cDNA library using the MKK3 cDNA as a probe. As a result, we obtained a novel species of MKK3 mRNA, referred to as MKK3b, which differs from the initially characterized MKK3 at the amino terminus. We have determined the multiple-exon/intron structure in the 5′-end region of the MKK3 gene, where the novel MKK3 mRNA is derived. Based on Northern blotting analysis, MKK3b is the predominant isoform in all tissues. Furthermore, we show that MKK3b is more efficient in mediating its downstream events than MKK3.

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Abbreviations: MAPK, mitogen-activated protein kinase; MKK (or Mek), MAPK kinase; ATF2, activating transcription factor-2; JNK (or SAPK), c-Jun N-terminal kinase (or stress-activated protein kinase); Erk, extracellular-regulated protein kinase; GST, glutathione *S*-transferase

2. Materials and methods

2.1. Identification of a novel form of human MKK3 cDNA

A human B lymphoma (Daudi) cDNA library in the pCDNAII vector (Invitrogen) was screened using ^{32}P-labelled MKK3 cDNA as a probe. The isolated plasmid DNA was sequenced using the dideoxynucleotide termination sequencing kit (Amersham). One of the isolated clones that appears to contain the complete coding sequence of MKK3 was subcloned into the mammalian expression vector pCDNA3. A PCR-based procedure was adopted to create a double mutant of MKK3b, MKK3b(E), by replacing the phosphorylation sites Ser^{218} and Thr^{222} with Glu as previously described [14].

2.2. Isolation of human genomic DNA of MKK3

A human placenta genomic DNA library in the λ-FixII vector (Stratagene) was screened with the MKK3 cDNA as a probe. Genomic DNA inserts were fragmented by digestion with *Eco*RI and subcloned into pBluescript for further analysis.

2.3. Northern hybridization analysis of MKK3b and MKK3

The 5′-end region that is unique to MKK3b was PCR-amplified. The MKK3b DNA fragment was ^{32}P-labelled by a random priming kit (Boehringer Mannheim), and was used to probe a multiple tissue RNA blot (Clontech). An MKK3-specific probe was prepared using two oligonucleotide primers, derived from the region unique to the original MKK3 cDNA, that partially anneal to each other to enable DNA elongation by Klenow DNA polymerase, generating a synthetic DNA probe of approximately 100 nucleotides in length with a specific activity of 2×10^9 cpm/μg.

2.4. Transient co-expression of p38 with MKK3 and MKK3b in COS-7 cells and immunokinase assays

Full-length cDNAs of flag-tagged p38, MKK3, and MKK3b were constructed into the mammalian expression vector pCDNA3 as previously described [14]. COS-7 cells were co-transfected of pCDNA3-p38 with either pCDNA3-MKK3 or MKK3b using lipofectamine according to the manufacturer's instructions. Transfection efficiency was evaluated by co-transfection with the plasmid pCMWβ-galactosidase (Clontech). After 48 h of transfection, transiently expressed p38 was immunoprecipitated by the anti-flag monoclonal antibody M2 (Kodak-IBI). Immunokinase assays were performed using the immunoprecipitates and recombinant GST-ATF2-(1–109) as a substrate as described [14].

2.5. Reporter gene expression

The GAL4-responsive reporter plasmid, pG5E1bLuc, contains five GAL4 binding sites upstream of a minimal promoter and luciferase gene. pG5E1bLuc was co-transfected into CHO cells with a construct

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expressing the GAL4 binding domain fused to ATF2-(1–505) [14,15]. Activation of an ATF-2 reporter gene by MKK3 or MKK3b was examined by co-transfection of the reporter plasmid separately with each of the expression plasmids as indicated in the text.

3. Results

3.1. Cloning of a novel MKK3 RNA species

To initially screen for a possible isoform of MKK3, its full-length cDNA fragment was ^{32}P-labelled and used to screen a human cDNA library. Sequencing analysis of cDNA inserts revealed a novel form of MKK3, which differs from the one previously characterized at the 5′-end coding sequence as well as the 5′-untranslated region (Fig. 1). The novel cDNA encodes 351 amino acids, 29 residues longer than MKK3 (Fig. 1A). The two MKK3 RNA species have identical downstream sequences. An in-frame stop codon (TAG) 36 nt upstream of the putative translation start site indicates that the cDNA is of full length (Fig. 1B). Recombinant MKK3b expressed in COS-7 cells has an apparent molecular weight close to 47 kDa, consistent with the estimated molecular weight for the natural protein detected by Western blotting (data not shown).

3.2. The gene structure of MKK3

In order to understand the origin of the novel MKK3 mRNA species, we used the cDNA fragment unique to the b-form MKK3 cDNA as a probe to screen a human placenta genomic DNA library. Seven clones were isolated and subcloned into pBluescript. The genomic DNA inserts were digested with various restriction enzymes and subjected to Southern analysis using the same DNA probe. As a result, a 5 kb *Eco*RI fragment hybridized to the b-unique probe and subsequently subcloned for sequencing analysis. As shown in Fig. 1B, the MKK3b-unique 5′-end sequence is derived from a single exon, and contains the translation initiation codon. This exon is linked to a second exon that is shared by the original MKK3 cDNA. As indicated in Fig. 1B, translation of MKK3 starts in the second exon.

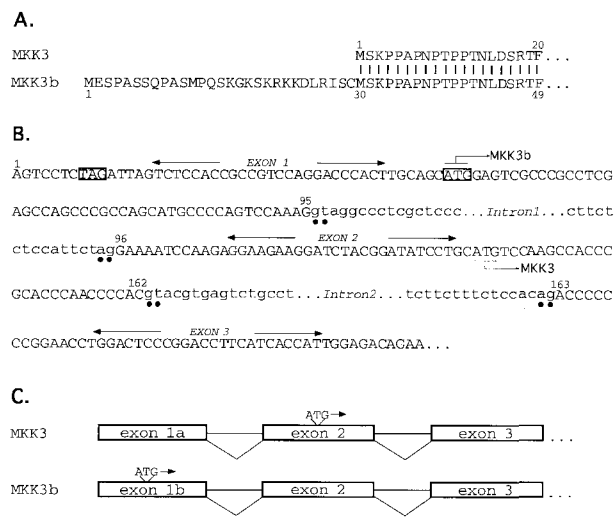

Fig. 1. The primary sequence and genomic structure of MKK3b. A: The previously identified MKK3 is compared to the novel MKK3b, showing that MKK3b is 29 amino acid residues longer than MKK3 at the N-terminus. B: The exon-intron structure of MKK3b genomic DNA. The nucleotide sequences in capital letters are exon sequences; numbers refer to the positions of the nucleotide sequence of MKK3b cDNA. The MKK3b-unique nucleotide sequence is derived from a single exon, exon 1 (1–95). Exon 1 contains the translation initiation codon (squared), as well as an in-frame stop codon (squared) upstream of the initiation codon. Partial intron sequences are shown in lower-case letters; and splicing consensus sites, GT and AG respectively for the left site and right site, are marked by black dots. The cDNA sequence of human MKK3b has been deposited in the GenBank data base with accession number U66839. C: A diagram showing MKK3 and MKK3b diverge exactly before the intron/exon junction of the putative exon 2. Exon 1a is a hypothetical denotation for the divergent 5′-end sequence of MKK3; exon 1b refers to the 5′-end exon of MKK3b. The relative locations of the translation initiation codon for each mRNA are indicated.

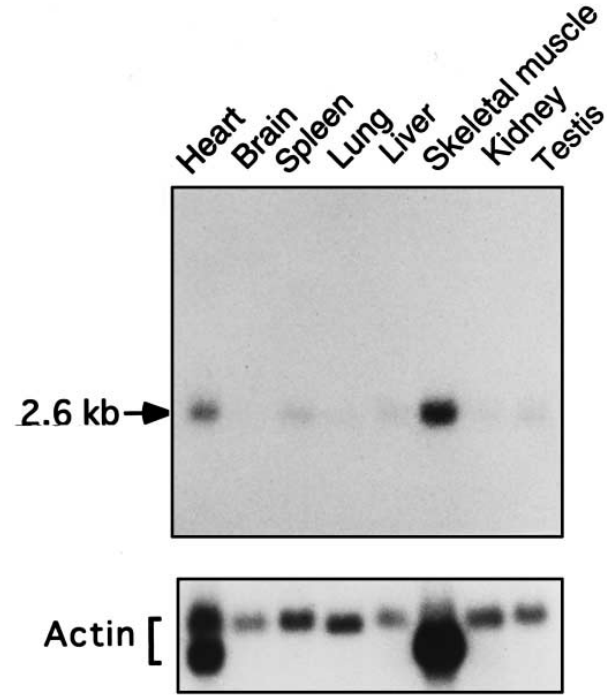

Fig. 2. Tissue distribution of MKK3b. The DNA sequence unique to MKK3b, exon 1, was radioactively labelled and utilized to probe a Northern blot containing different human tissue RNAs as indicated at the top. The arrow points to the single hybridized mRNA species of 2.6 kb in size. A β-actin probe (from Clontech) was used to probe the same blot for RNA loading (lower panel).

3.3. MKK3b is a predominant form of MKK3

We next analyzed the tissue distribution of the novel MKK3 mRNA species by hybridizing the b-unique DNA fragment as a probe to a Northern blot containing eight tissue RNA samples. Results showed that the RNA distribution is similar to that with the full-length DNA probe (Fig. 2). When we used a probe specific for the original MKK3 cDNA to hybridize the same blot, no hybridization signal was detected (data not shown). This is consistent with our observation that anti-MKK3 antibodies only reveal a 47 kDa protein in Western blotting (data not shown). Collectively, these data indicate that MKK3b is the major variant of the MKK3 gene.

3.4. MKK3b is more active in the activation of p38

In order to compare the kinase activity of the two MKK3 products, the flag-tagged p38 was co-transfected into COS-7 cells with either MKK3 or MKK3b. Cells were lysed after 48 h, and subjected to immunoprecipitation with the M-2 anti-

flag antibody. The purified p38 was assayed for its activity using ATF2 as a substrate. As shown in Fig. 3, MKK3b is capable of activating p38 kinase activity. In addition, it is evident that MKK3b is more active than MKK3. We and others have previously shown that p38 can phosphorylate the transcription factor ATF2 and increases ATF2-dependent reporter gene expression [11,14,15]. We next carried out co-transfection of the reporter gene in different combinations with p38, constitutively active mutants of MKK3 or MKK3b, MKK3(E) or MKK3b(E), respectively. Results showed that MKK3(E) alone does not activate the reporter. When co-transfected with p38, MKK3(E) activates the reporter approximately 2-fold. In contrast, MKK3b(E) exhibited much higher efficiency in activating the endogenous p38, and by itself resulted in a 7-fold increase in the reporter transcription activity. In the presence of co-transfected p38, a higher stimulation by the MKK3b(E) was observed (9-fold). These results confirm the effect of MKK3(E) on the ATF2 reporter gene expression previously reported by ourselves and others [14,15], and show that the constitutive form of the newly identified MKK3 (MKK3b(E)) is much more efficient in activating the ATF2 transcriptional activity (Fig. 4).

4. Discussion

We have obtained a novel cDNA species of the human MKK3 gene, which encodes a new MKK3 protein of 351 amino acids in length, 29 amino acid residues longer than the MKK3 previously identified [9]. The nature of the origin of the two MKK3 mRNA species is unclear. They may either be a product of differential splicing or are derived from differential promoter utilization. We favor the second possibility because they diverge completely in their 5′-ends, and because we failed, by 5′-end extension experiments, to obtain any further upstream sequences that may be identical to both the mRNAs. Therefore, the transcription initiation site may be different between the two transcripts.

Northern blotting analysis revealed that the distribution pattern of the novel mRNA species almost entirely mirrors that using the full-length MKK3 DNA as a probe, being most abundant in skeletal muscle and nearly undetectable in brain. The new probe hybridized to a mRNA of the same molecular weight, approximately 2.6 kb in length. Furthermore, the probe unique to the previously identified MKK3 failed to detect any mRNA on the same Northern blot. Based on the analysis of the MKK3 genomic DNA, it was found that the 5′-untranslated sequence of the previously identified MKK3 is

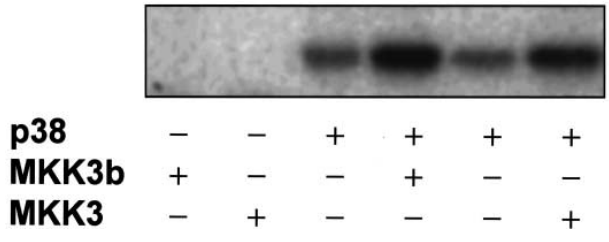

Fig. 3. Activation of p38 by MKK3 and MKK3b. COS-7 cells were co-transfected with the flag-tagged p38 together with pCDNA3 constructs expressing MKK3, MKK3b, or the empty vector as a control. The kinase activity of p38 isolated by immunoprecipitation was measured with GST-ATF2(1-109) as substrate. Similar results were obtained in three experiments.

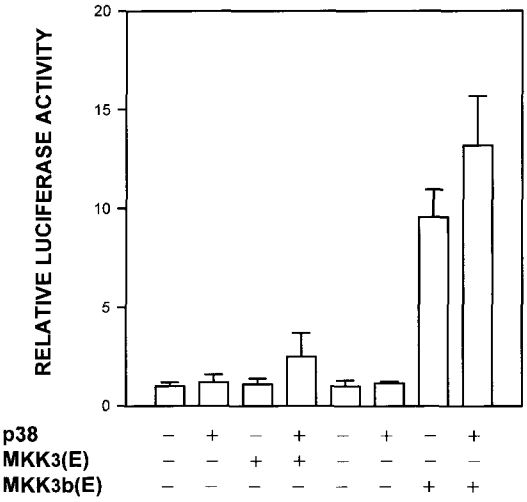

Fig. 4. Stimulation of ATF2-dependent gene expression by MKK3 and MKK3b. An ATF2-dependent reporter gene was co-transfected into CHO cells with β-galactosidase expression vector pCMV-β-gal and a different combination with p38, the constitutive form of MKK3 (MKK3(E)) or MKK3b (MKK3b(E)). Empty vector pCDNA3 was used to normalize the total DNA to 1 μg per transfection. The ratio of luciferase activity to β-galactosidase activity is presented as the mean ± S.D. ($n = 3$).

linked, but not contiguous, to the new MKK3b sequence. Together with the fact that the two cDNA species diverge exactly at the intron-exon junction, it is unlikely that the original sequence is a cloning artefact. It is of interest to extend the analysis of the MKK3 tissue distribution, as it is possible that MKK3 may be present in as yet unidentified cell types.

Although MKK3b has a much stronger effect on p38 activation than MKK3, it is possible that MKK3 may display a stronger kinase activity in the phosphorylation of other substrates. The N-terminal sequence difference of MKK3 and MKK3b may allow differential subcellular localization of the proteins. Indeed, there is a cluster of positively charged amino acid residues (Arg/Lys residues) in the unique N-terminus of MKK3b, which is identical to a putative nuclear localization site [16]. Whether this sequence has any role in the physiological function of MKK3b still awaits further investigation. Understanding the functional difference between the two forms of MKK3 would provide more insight into the mechanism by which the p38 MAP kinase cascade operates.

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