Genomics Insights

ORIGINAL RESEARCH

Cloning and Initial Functional Characterization of Mlk4α and Mlk4β

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Abstract: We have cloned a novel human mixed-lineage kinase gene, *MLK4*. Two alternatively spliced forms, *MLK4α* (580 aa) and *MLK4β* (1036 aa), have been identified and mapped to chromosomal band 1q42. *MLK4* shows high amino acid homology to the kinase catalytic domain of *MLK3* (72%), *MLK1* (71%) and *MLK2* (69%). Strong expression of *MLK4* was detected in the human pancreas and kidneys. pCMV-MLK4β c-myc-tagged protein (human) was expressed in the cytoplasm and nucleus of transiently transfected COS-1 cells, while pCMV-MLK4α c-myc-tagged protein (human) was expressed in cytoplasm only. Both *MLK4* isoforms reduced the colony formation ability of MCF7 cells by 85%–95% and almost totally suppressed cell proliferation in the CyQUANT cell proliferation assay. Human pCMV-MLK4β transgenic mice expressed the *MLK4β* in all tissues examined but no phenotypic abnormalities were observed. Thus, in this work, we present the cloning and sequencing of *MLK4α* and *MLK4β* for the first time; the data obtained suggest that *MLK4* may function as a MAP kinase.

Keywords: MLK, protein kinase, gene cloning, tumor suppressor gene

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Introduction

Protein kinases play an important role in many signaling pathways, and therefore have the potential to contribute to various diseases ranging from cancer and inflammation to diabetes and cardiovascular disorders.

Mixed-lineage kinases (MLKs) are serine/threonine protein kinases that regulate signalling by the c-Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways. MAPK cascades exist in all eukaryotes and orchestrate diverse cellular activities, including mitosis, programmed cell death, motility and metabolism. Substrates for MAPKs include transcription factors, phospholipases, other protein kinases, cytoskeleton-associated proteins and membrane receptors.[1]

MAPK cascades typically consist of a MAPK, a MAPKK (MAP kinase kinase) and a MAPKKK (MAP kinase kinase kinase).[2] Transmission of the signals is achieved by sequential phosphorylation and activation of the cascade components.[3] At least three independent MAPK pathways have been identified in mammalian cells. They include the extracellular signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK)/stress activated protein kinases (SAPK) pathway and the p38 pathway. The ERK pathway regulates cell proliferation, cell differentiation and developmental processes.[4] The JNK/SAPK and p38 pathways are involved in cellular stress responses and apoptosis.[5]

The MLKs function as MAPKKKs. The MLKs are characterized by the unique catalytic domain that is a hybrid between those found in serine/threonine and tyrosine kinases.[6] The MLKs can be divided into three subfamilies.[1,7]

The first subgroup, which includes *MLK1–MLK4*, is characterized by an amino-terminal SRC-homology (SH3) domain, followed sequentially by a kinase domain, a leucine-zipper region and a Cdc42/Rac-interactive binding (CRIB) motif that interacts with the RHO-family GTPase Rac and Cdc42. *MLK1–MLK4* share 69%–72% sequence identity within their catalytic domains and approximately 64%–92% sequence identity with their other domains and motifs. The carboxyl termini of these proteins are proline-rich but have different sequences, indicating that these regions might serve different regulatory functions.

The second subfamily includes two genes, dual leucine zipper-bearing kinase (*DLK*) and leucine zipper bearing kinase (*LZK*).[8,9]

The third subgroup of *MLK*s consists of zipper sterile-α-motif kinases (*ZAK*s), *ZAKα* and *ZAKβ*.[10,11]

Some *MLK*s are restricted in their cell and tissue expression (like *MLK1* and *MLK2*), whereas several others are widely produced (*MLK*3, *ZAK*α). The diverse regulatory domains that are present in the MLKs indicate that these proteins are probably regulated differently by different upstream stimuli and participate in selective interactions with other proteins that target MLKs to different subcellular localizations.[1,12]

The physiological roles of *MLK*s have not been defined and it seems that the initial picture of *MLK*s as comparatively selective regulators of the *JNK* group of *MAPK*s is no longer valid; in fact, *MLK*s may have a more general upstream role than was initially postulated.[1,12]

It was shown that *MLK*s might contribute to neurogenerative diseases, and they have been suggested as attractive therapeutic targets for the treatment of diseases such as Parkinson's and Huntington's.[1,13,14] Still, *MLK2*, *MLK3*, *DLK* and *LZK* are the only *MLK*s that have been studied in detail.[6,9,15] In this work, we present the cloning of *MLK4α* and *MLK4β* for the first time, and an analysis of their genomic structure, chromosomal localization, expression patterns and initial functional characterization.

Results

Cloning and nucleotide sequence analysis of *MLK4α* and *MLK4β*

The *Not*I linking clone NR5-DM9 (partial sequence 480 bp, GenBank accession No. AJ311799) showed 87% identity over 63 nucleotides with human *MLK3* cDNA (GenBank accession No. NM_002419) and 83% identity over 87 nucleotides with human *MLK1* cDNA (GenBank accession No. AF251442). BLASTN analysis revealed that this *Not*I linking clone was identical to part of the PAC clone RP5-862P8 (GenBank accession No. AL133380). Based on the similarity between the PAC clone RP5-862P8 and the human *MLK3* gene, we designed the primers KINB and KINC and amplified a part of the human *MLK4* gene (630–1734 bp) from Heart Marathon-Ready™ cDNA using polymerase chain reaction (PCR) (Fig. 1).

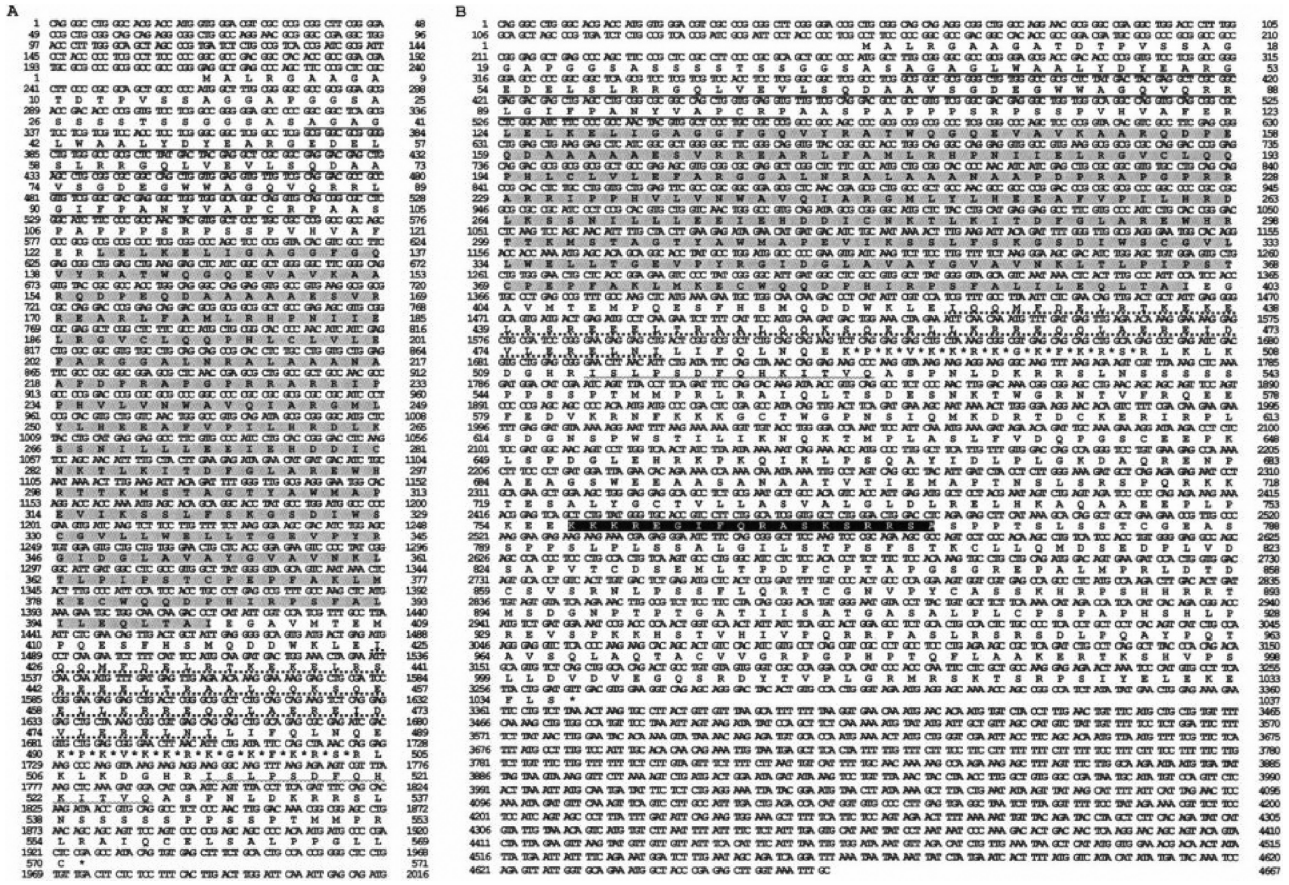

Figure 1. Nucleotide and amino acid sequences of *MLK4α* (**A**) and *MLK4β* (**B**). The following features are indicated: kinase catalytic domain (shaded), the SH3 domain (underlined), the leucine zipper domain (···), the basic domain (*), the CRIB motif (≈) and the bipartite nuclear localization signal (black box). *MLK4α* and *MLK4β* have an identical N-terminal region (1–558 aa) and share all domains, except the bipartite nuclear localization signal.

The 3′-RACE product of human *MLK4α* gene was obtained as described in the Materials and Methods section. It contained a poly(A) tail of 30 adenine nucleotides and a typical polyadenylation site (AATAAA) 18 nucleotides upstream from the poly(A) tail. The 5′-end of the *MLK4α* gene was cloned from the Heart Marathon-Ready cDNA. The cloned sequence revealed that the first ATG (Met) codon is situated at 262 nucleotides and this codon is situated within the sequence CCCATGG, which is consistent with the Kozak model.[16]

A BLASTN search of the EMBL and EST databases for *MLK4α* nucleotide sequences revealed a human EST (GenBank accession No. AW408639) representing a different spliced form of *MLK4* at the 3′-end. Using the PAC clone RP5-862P8, the human *MLK3* and sequences of several ESTs (GenBank accession Nos. AL135711 and BE867187) that are situated downstream from the 3′UTR of *MLK4α* cDNA on the PAC clone, we designed the primers KIND (derived from the common part of *MLK4α* and AW408639) and KINF (derived from the AL135711 and BE867187 sequences). With these primers, a 3′ segment of human *MLK4β* was cloned from the Heart Marathon-Ready cDNA. *MLK4β* cDNA has four additional exons at the 3′-end and an open reading frame that is 466 aa longer than *MLK4α*.

The primers KIN5/KIN3a and KIN5/KIN3b were used to amplify complete coding sequences of spliced variants of *MLK4α* (84–2171 bp, 570 amino acids) and *MLK4β* (84–3476 bp, 1036 amino acids).

Sequence analysis revealed that *MLK4α* and *MLK4β* genes consist of 6 and 10 exons and span more than 46.3 kb and 56.2 kb of genomic DNA, respectively. The complete sequence of the inserts in the plasmids containing *MLK4α* (3910 bp; GenBank accession No. AJ311797) and *MLK4β* (4667 bp; GenBank accession No. AJ311798) is shown in Figure 1(A, B) and its different predicted domains are shown in Figure 2.

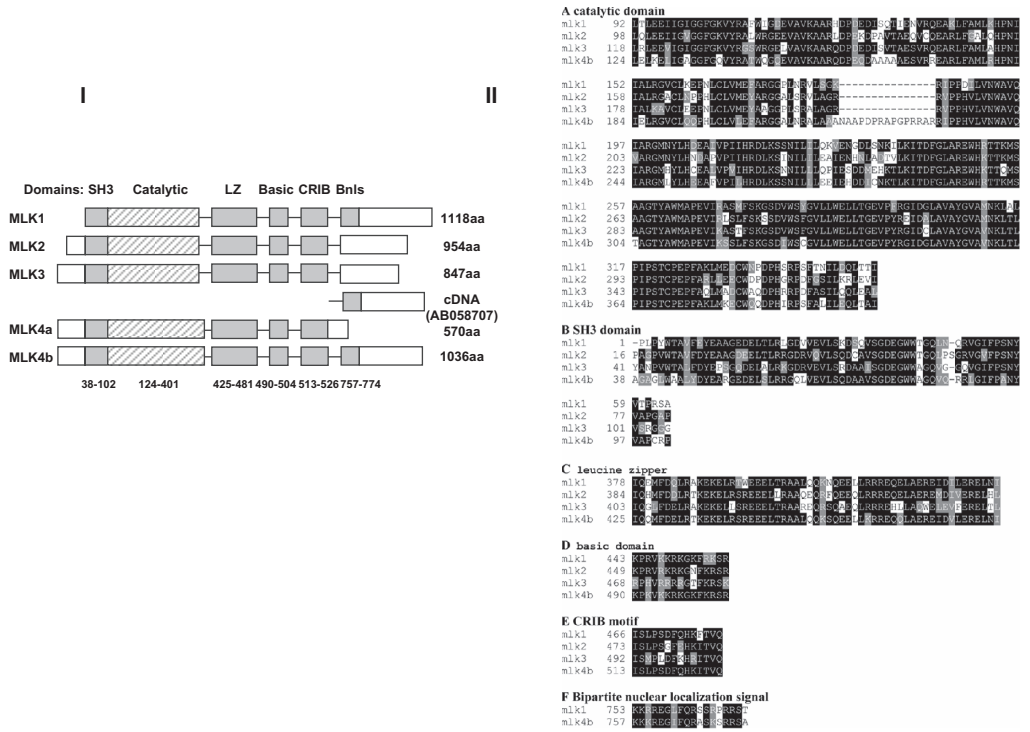

Figure 2. Functional domains of MLK4. **I**) Composite structure of human MLKs, showing the relative positions of the Src-homology-3 (SH3), catalytic, leucine-zipper (LZ), basic, and Cdc42/Rac-interactive binding (CRIB) domains, and the Bipartite nuclear localization signal (Bnls). cDNA (AB058707) was cloned by Nagase et al.[21] See text for discussion. **II**) Alignment of the deduced MLK4β sequences domains with those of MLK1, MLK2 and MLK3. **A**) Alignment of the kinase catalytic domains; **B**) Alignment of the SH3 domains; **C**) Alignment of the double leucine zipper domains; **D**) Alignment of the basic domains; **E**) Alignment of the CRIB motifs; **F**) Alignment of the bipartite nuclear localization signals. Black and gray boxes indicate identical and similar amino acid residues, respectively.

Expression Patterns of *MLK4*

To determine the distribution of *MLK4α* and *MLK4β* mRNA within the tissues, a multiple human tissue Northern blot (Clontech, Palo Alto, CA, USA) was hybridized to the non-coding 3′-end of specific DNA fragments of *MLK4α* (1970–3910 bp) and *MLK4β* (2087–4667 bp). As shown in Figure 3A, one transcript of about 7 kb was observed for the *MLK4β* gene. This transcript was detected in the pancreas, kidney, skeletal muscle, liver and heart. Northern blot hybridization for *MLK4α* did not result in any specific signal (data not shown), indicating a low abundance of *MLK4α* mRNA variant.

To investigate the possibility that *MLK4α* still was expressed at low levels, we checked its expression by reverse transcription PCR (RT-PCR) using multiple tissue cDNA panels. Figure 3B shows that both *MLK4α* and *MLK4β* mRNAs were expressed in the pancreas, kidney, liver, lung and brain. Expression in the heart and placenta was detected at a lower level. Only *MLK4β* was expressed in skeletal muscle.

Chromosomal Localization of *MLKα* and *MLK4β*

Using fluorescent in situ hybridization (FISH) we assigned the *Not*I linking clone NR5-DM9 (insert size 5 kb) to chromosomal band 1q42 (Fig. 4). A number of diseases have been localized to this region, including a predisposition to prostate cancer[17] and papillary renal cell carcinoma.[18]

Subcellular Localization of *MLKα* and *MLK4β*

According to the sequence data, a bipartite nuclear localization signal is present only in the longer *MLK4β*. To study the localization of both *MLK4* variants in the cell, *MLK4α* and *MLK4β* were cloned into a pCMV-Tag3 vector (pCMV-MLK4α and pCMV-MLK4β) for the c-myc-tagged expression of both proteins. COS-1 cells were transfected with either pCMV-MLK4α or pCMV-MLK4β and immunostained with α-myc antibody 9E10 (Fig. 5A, B). The data thus obtained showed that

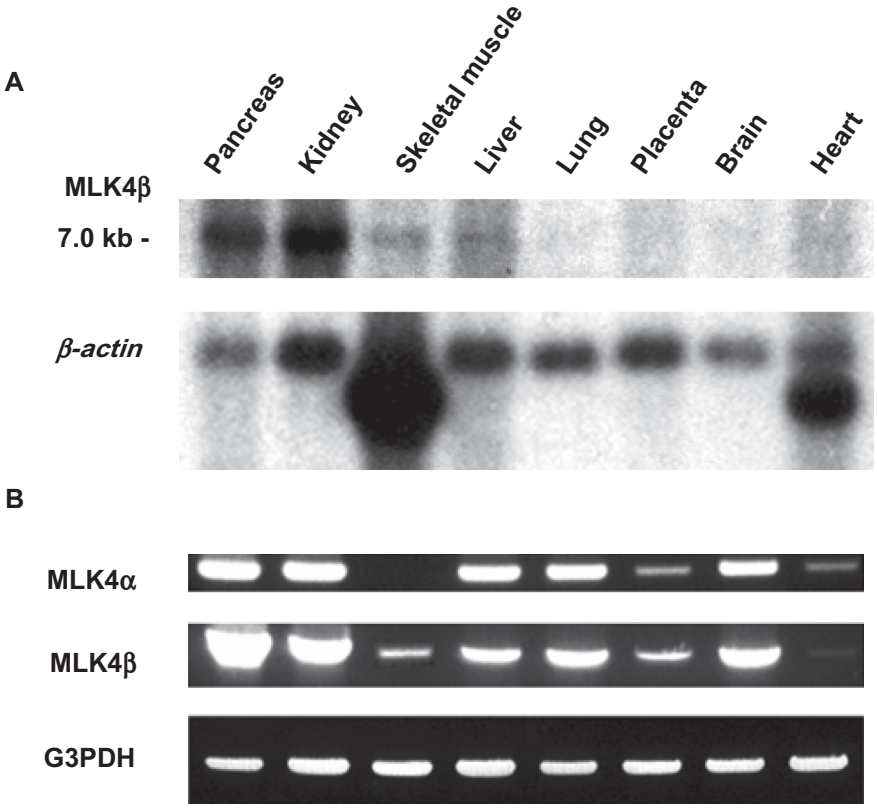

Figure 3. Expression analysis of *MLK4α* and *MLK4β*: **A**) Northern blot hybridization for *MLK4β*, **B**) PCR with multiple tissue cDNA panel for *MLK4α* and *MLK4β*.

pCMV-MLK4β fusion protein was localised in the cytoplasm and nucleus of transiently transfected COS-1 cells, while pCMV-MLK4α was present in the cytoplasm only.

Functional Analyses of *MLKα* and *MLK4β* in Vitro

To study the possible effect of *MLK4* expression on a cell's viability and proliferation, colony formation tests and cell proliferation rate analyses were performed for *MLK4*-transfected cells. Human breast cancer cells (MCF7) were transfected with either pCMV-MLK4α or pCMV-MLK4β constructs and used for the experiments. To evaluate the viability of the transfected cells, they were seeded on 10-cm cell culture dishes at the same cell density; the number of colonies growing on each plate was counted after two weeks in the presence of G418 (Fig. 6A). Both *MLK4* isoforms reduced colony formation ability by 85% for *MLK4*α and 95% for *MLK4*β compared to cells transfected with the empty vector.

To study the influence of the *MLK4* gene on MCF7 cell proliferation, we measured the DNA accumulation rate in cell clones stably transfected with *MLK4α*- and *MLK4β* (see Methods) using the CyQUANT NF Cell Proliferation Assay (Fig. 6B). The CyQUANT NF Cell Proliferation Assay is based on measuring cellular DNA content via fluorescent dye binding (see Methods). The assay is designed to produce an analytical linear response from at least 100–20,000 cells per well in most cell lines. Almost no cell proliferation was shown both for *MLK4α*- and *MLK4β*-transfected cells compared to the control MCF7 cells transfected with the vector only. The strong inhibition of the viability and cell proliferation by *MLK4* suggests the possible involvement of *MLK4* in programmed cell death/apoptosis, as was shown for *MLK1*, *MLK2* and *DLK*.[1,19]

Transgenic *MLK4* Mice

To study the effect of *MLK*4 expression at the organism level, *MLK4β*-transgenic mice were produced

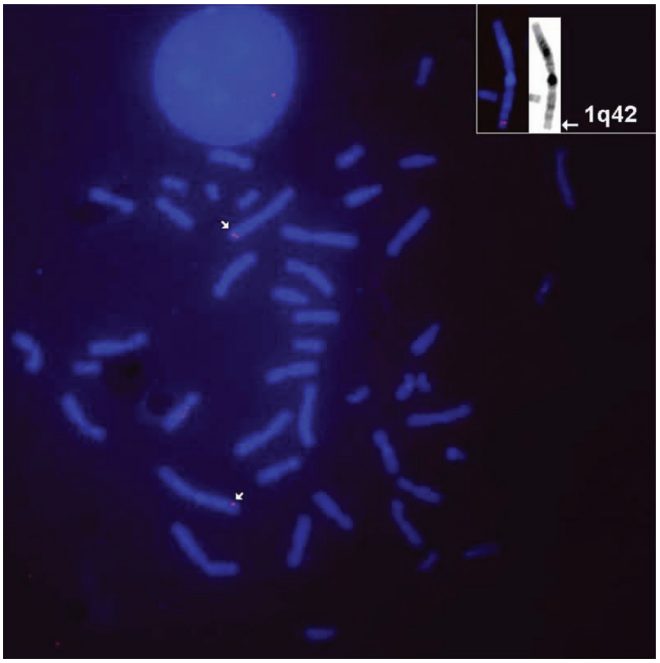

Figure 4. FISH-based assignment of *MLK4* to human chromosome band 1q42.

(see Methods). We chose *MLK4β* because almost all coding information in *MLK4α* (except for 12 amino acids in the C-terminal end) is present in *MLK4β*. The aim of this experiment was to see rapid, strong changes in transgenic mice. Fifty-six offspring were genotyped by PCR using human *MLK4* primers and seven *MLK4*-positive mice were found. A different human *MLK4* expression level was shown for the different mouse tissues, ranging from the highest expression being found in the thymus to weaker expression in the heart, kidney and liver (Fig. 7). Surprisingly, no phenotypical abnormalities were observed for the human *MLK4*-transgenic mice compared to the non-transgenic mice after 12 months' observation.

Discussion

In this work, we present cloning and sequencing of *MLK4α* (3910 bp, GenBank accession No. AJ311797) and *MLK4β* (4667 bp, GenBank accession No. AJ311798) for the first time.[20] Using FISH, we assigned the *MLK*4 to chromosomal band 1q42. Two *MLK4* isoforms have different 3′-sequences and an identical 5′-region (1–1936 bp). The 5′UTR of 261 bp contains an in-frame stop codon at position 118. The open reading frames of the *MLK4α* and *MLK4β* genes consist of 1710 and 3108 bp, respectively. They code for 570 aa and 1036 aa proteins. The 3′UTR length is 1938 bp for *MLK4α* cDNA and 1297 bp for *MLK4β* cDNA. A Poly(A) tail was identified for the *MLK4α* gene only. Independently, a cDNA clone (KIAA1804) containing 476 aa from the 3′-end of the *MLK4β* was cloned by Nagase et al.[21] (GenBank accession No. AB 058707). This clone missed 560 aa of MLK4β from the 5′-end and thus lost all functional domains except bipartite nuclear localization signal (Fig. 2 and see below). We ran a comparison between the RefSeq entry NM_034535 and *MLK4β,* and found nine differences. These differences are likely to be polymorphisms because they are present both in *MLK4α* and *–4β*.

MLK4α and *MLK4β* cDNAs encode amino acid polypeptides with a calculated molecular mass of 62.9 kDa and 113.8 kDa, respectively. Both of them have an identical N-terminal region (1–558 aa), which contains the SRC homology (SH3) domain (38–102 aa), the kinase catalytic domain (124–401 aa), the leucine zipper domain (425–481 aa), the basic domain (490–504 aa) and the Cdc42/Rac interactive binding (CRIB) motif (513–526 aa) (Fig. 2). A search for significant matches against the PROSITE library of protein profiles was done via the ISREC server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html).

The *MLK4* catalytic domain is a hybrid between those in the serine/threonine and tyrosine protein kinases with 69%–72% homology to *MLK3* (GenBank accession No. NP_002410), *MLK1* (GenBank accession No. AAG44591) and *MLK2* (GenBank accession No. Q02779); see Figure 2.

The SRC homology 3 (SH3) domain of *MLK4* is similar to that of *MLK3* (68% identity), *MLK1* (66% identity) and *MLK2* (64% identity) (Fig. 2). It is generally assumed that the SH3 domain is involved in specific protein–protein interactions and recognizes proline-rich sequences containing the core PxxP.[22]

The leucine zipper domain of *MLK4* (425–481 aa) displays 84% identity to *MLK1*, 73% identity to *MLK2* and 66% identity to *MLK3* (Fig. 2). The domain is able to promote homodimerization and binding to other proteins with similar domains.

The basic domain of *MLK4* (490–504 aa) is located C-terminally to the leucine zipper domain, in contrast to location of basic domain in the transcription factors.[23] The basic domain of *MLK4* is similar to that

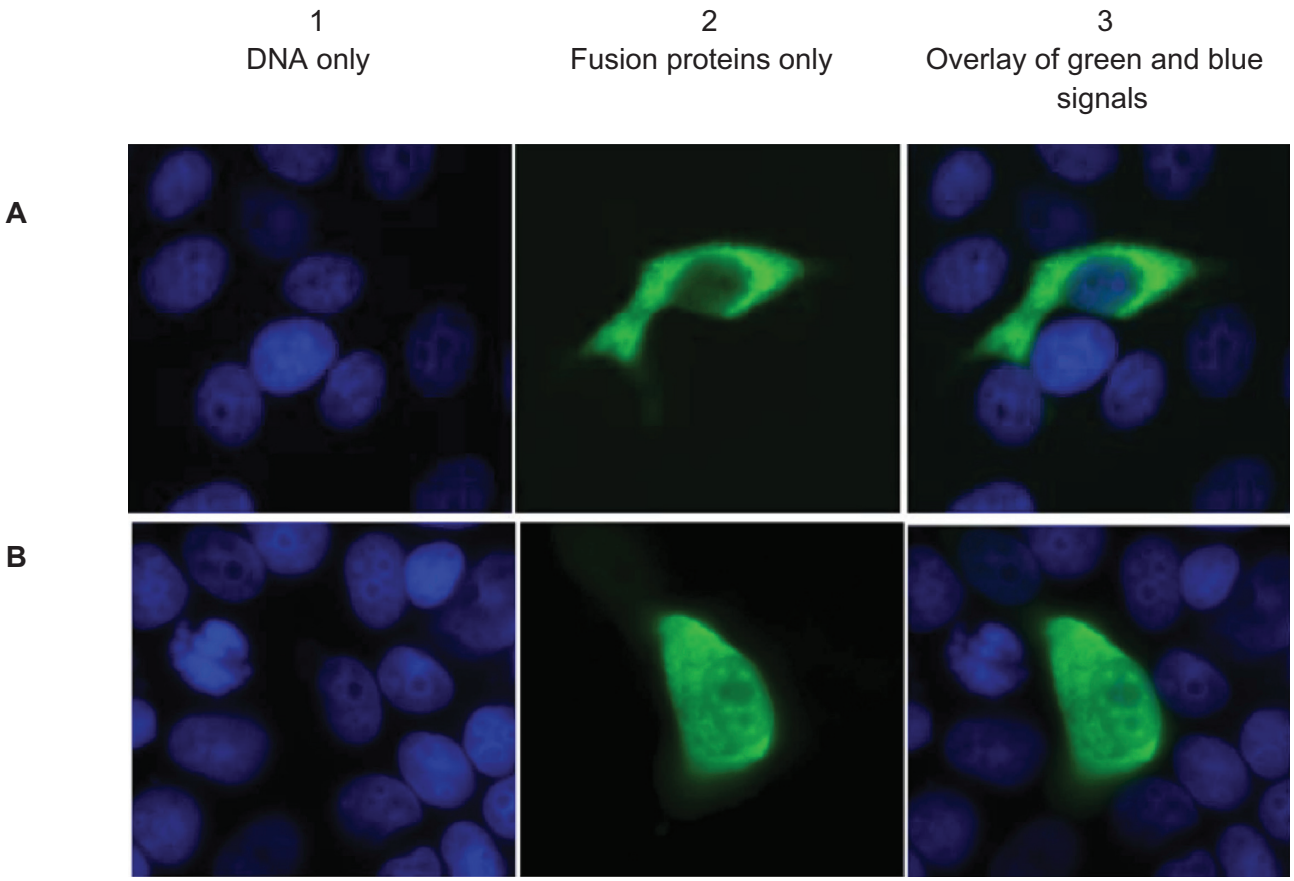

Figure 5. Cellular localization of **A**) MLK4α and **B**) MLK4β c-myc fusion proteins. COS-1 cells were transfected with pCMV-MLK4α and pCMV-MLK4β constructs, and the c-myc-tag was stained with mouse α-c-myc monoclonal antibody (green). DNA is stained blue. (A1 and B1) DNA only; (A2 and B2) fusion proteins only; (A3 and B3) overlay of green and blue signals.

of *MLK1* and *MLK2* with 80% identity but has only 53% identity to *MLK3* (Fig. 2).

The CRIB motif is highly similar to that of *MLK1* (92% identity), *MLK2* (85% identity) and *MLK3* (71% identity) (Fig. 2). It was shown that *MLK2* and *MLK3* interact with Rac and Cdc42 in a GTP-dependent manner.[24] The CRIB motif of *MLK3* is required for its activation.[25]

In addition, *MLK4β* has a bipartite nuclear localization signal (757–774 aa). The nuclear localization signal of the *MLK4*α protein, however, was not found (Fig. 2). Within the *MLK1*, *MLK2* and *MLK3* genes, the bipartite nuclear localization signal was found in *MLK1* only.

The presence of this bipartite nuclear localization signal is the most likely explanation for the difference in the subcellular localization of the two *MLK4* proteins: pCMV-MLK4β fusion protein was expressed in the cytoplasm and nucleus of transiently transfected COS-1 cells, while pCMV-MLK4α was expressed in cytoplasm only. As it was mentioned in the Introduction, different localization of the other *MLK* family members was reported: a cytoskeletal localization for *MLK2*[24] and nuclear localization for ZAKα/MLTKα.[26] It is likely that the two *MLK4* variants, which have different localizations, could have different functions.

Despite a high level of domain homology at the N-terminal region, the C-terminal regions of the MLKs are divergent.[11] The C-terminal region of *MLK4*β is rich in proline, which is similar to *MLK1*, *MLK2* and *MLK3*. This proline-rich region contains several general consensus motifs (PxxP) for SH3 binding sequences[22] and may be involved in the protein–protein interaction with the SH3[27] and WW[28] domains of other proteins (WW domains bind proline-rich proteins containing the PPXY or PPLP core motifs), or with its own SH3 domain.

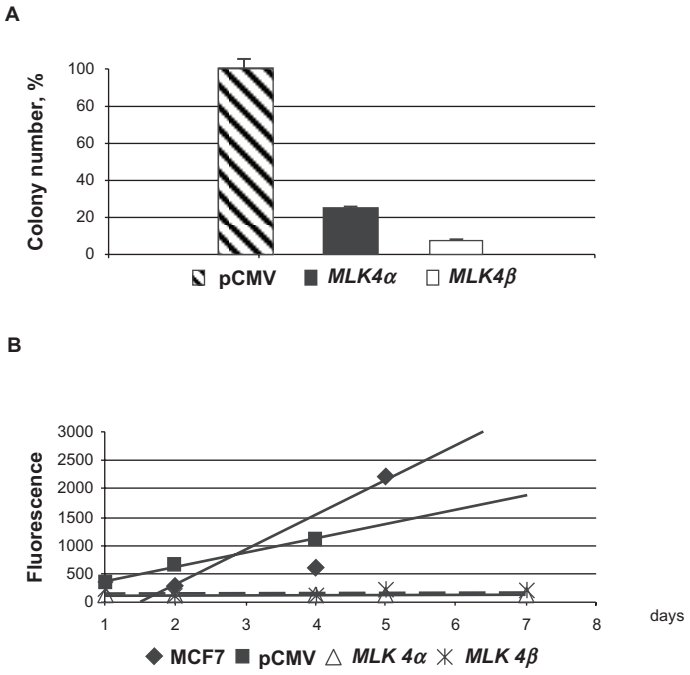

Figure 6. Colony formation and cell proliferation assays with pCMV-MLK4α and pCMV-MLK4β. **A**) Colony formation. The number of G418-resistant colonies in MCF7 cells transfected with empty pCMV-Tag3 vector was set at 100%. **B**) The CyQUANT NF Cell Proliferation assay was used to measure the cell proliferation rate. Two stably transfected clones for each construct (pCMV-MLK4α and pCMV-MLK4β; see Methods) for were used in the experiment, which was done in triplicate. Original MCF7 cells and MCF7 cells stably transfected with pCMV-Tag3A plasmid were used as controls.

MLK4 displays a high level of amino acid identity and a very similar domain structure to *MLK1*, *MLK2* and *MLK3*. It has been shown that all tested MLKs (*MLK2, MLK3, DLK* and *LZK*) function as MAPKKKs.[1] The data therefore suggest that *MLK4* may also function as a MAPKKK.

Our experiments clearly indicate that both forms of *MLK4* inhibit viability and cell growth in vitro (colony formation experiments and CyQUANT NF proliferation assay).

On the other hand, it was shown that wild-type *MLK3* overproduction induces transformation of NIH3T3 fibroblasts, whereas catalytically inactive *MLK3* does not.[29] *ZAKα/MLTKα* was also reported to function as an oncogene-inducing neoplastic cell transformation.[26] Another report indicated that *MLK3* inhibits Rac-mediated cellular transformation.[30]

The controversial results of the possible involvement of *MLK4* and other MLKs in cell proliferation suggest that a more detailed functional study of the MLK family will be necessary.

Mutational analysis of the *MLK4* kinase domain in 147 colorectal cancers revealed a rather high frequency (6.8%) of somatic mutations.[31] In this study, our *MLK4* sequence was used (GenBank database, accession No. AJ311798). However, Shao *et al.* 2007 did not find any mutations in the MLK4 kinase domain in 46 colorectal samples isolated from Japanese patients and only two of 24 cell lines in the study harbored the mutation R470C in the *MLK4* gene.[32] No mutations in *MLK4* were found in gastric and hepatocellular carcinomas from Korean patients either, suggesting that the *MLK4* kinase domain mutations are rare and may not contribute to the development of these carcinomas.[33]

The relevance of these observations is unclear and currently, no evidence suggests that any *MLK* family member functions as an oncogene in humans. *MLK4β*-transgenic mice were produced but, surprisingly, no obvious phenotypical abnormalities were observed compared to the non-transgenic mice after 12 months' observation. This result is in disagreement with the strong effect of *MLK4* in vitro. It is interesting that *MLK3*–/– knockout mice were also viable and healthy.[34] These results indicate that we are in the initial stages of understanding the physiological role of *MLK*4 and other *MLK*s, and further work is necessary to clarify the functional significance of MLK4α and MLK4β proteins.

Conclusions

In summary, in this work, we present the cloning and sequencing of *MLK4α* and *MLK4β* for the first time. Using FISH, we assigned the *MLK*4 gene to chromosomal band 1q42. A number of diseases have been localized to this region, see for example.[35,36] pCMV-MLK4β fusion protein was localized in the cytoplasm and nucleus of transiently transfected COS-1 cells, while pCMV-MLK4α was present in cytoplasm only. Both forms of *MLK4* strongly inhibited viability and MCF7 cell proliferation in vitro. These data showed that *MLK4* genes could be important not only for basic science but could also be promising avenues of cancer diagnostics and therapeutics.

Materials and Methods

*Not*I linking library construction

The construction and sequencing of *Not*I linking libraries has been described previously.[37–40] In brief,

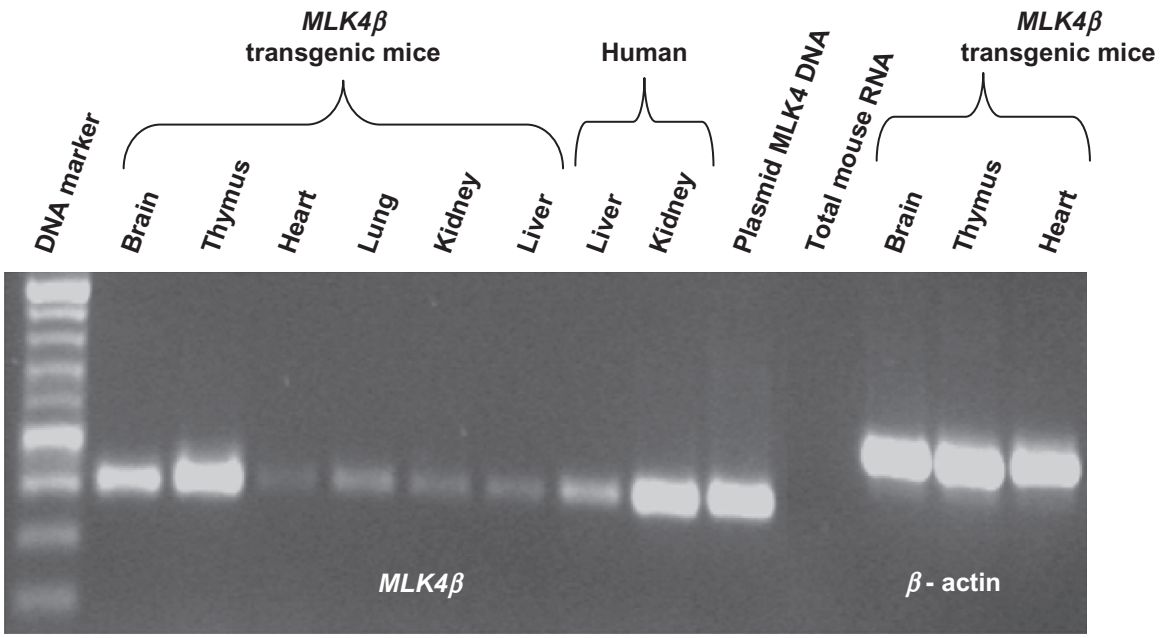

Figure 7. RT-PCR analysis of *MLK4β* expression in the different transgenic mouse tissues. M—DNA marker; 1–6—transgenic mouse tissues (1 = brain, 2 = thymus, 3 = heart, 4 = lung, 5 = kidney, 6 = liver); 7—human liver; 8—human kidney; 9—plasmid *MLK4* DNA (positive control), 10—total mouse RNA (negative control), 11–13—actin expression control for the transgenic mouse tissues (11 = brain, 12 = thymus, 13 = heart).

genomic DNA of the CBMI-Ral-Sto cell line was completely digested with *Bam*HI and self-ligated with T4 ligase at a low concentration. To eliminate any remaining linear molecules, the sticky ends were partially filled-in with Klenow enzyme in the presence of dATP and dGTP.[41]. Subsequently, DNA was digested with *Not*I and ligated to λSK17 and λSK22. To convert the library into plasmid form, 2 µg of λDNA was digested with *Sal*I, self-ligated with T4 ligase and transformed into E. coli XL1-Blue MRF cells (Stratagene, La Jolla, CA, USA). The restriction enzymes, Klenow enzyme and T4 ligase used in this work have been manufactured by Roche, (Mannheim, Germany). All molecular biology and microbiology procedures were performed according to standard methods.

Molecular Cloning of Human *MLK4*

MLK4 gene fragment (630–1734 bp) from Heart Marathon-Ready cDNA (Clontech, Palo Alto, CA, USA) has been obtained by PCR, using the primers KINB and KINC (for primer sequences, see Table 1) and according to the manufacturer's instructions.

PCR product was cut and extracted from agarose gel using the JETquick gel extraction kit (Genomed GmbH, Bad Oeynhausen, Germany) and cloned using the TOPO TA cloning kit for sequencing (Invitrogen BV, Groningen, Netherlands). Plasmid DNA was isolated with the GFX™ micro plasmid prep kit (AmershamPharmaciaBiotech, Uppsala, Sweden). Sequencing was done using an ABI 310 Sequencer with the ABI Prism® BigDye™ terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA) as previously described.[40]

3′-RACE was performed with Heart Marathon-Ready cDNA using two gene-specific primers combined with two adaptor primers, AP1 and AP2. PCRs were done according to the manufacturer's protocol with the Advantage® 2 PCR enzyme system (Clontech). For 3′-RACE of *MLK4α*, the KIND and KINE primers were used.

To amplify the 3′-end of *MLK4β* the KIND and KINF primers were applied.

To obtain sequences from the 5′-end of *MLK4*, including the first ATG (Met), we used the KINA and KING primers.

To obtain gene fragments of *MLK4α* cDNA (84–2171 bp, as in AJ311797) and *MLK4β* cDNA (84–3476 bp, as in AJ311798), which contain a complete open reading frame, the following primers were used: KIN5 and KIN3a for *MLK4α*, and KIN5 and KIN3b for *MLK4β*.

Table 1. Primer sequences.

Primers	Sequences
KINB	5′GCTGGAGCTGAAGGAG CTCATCG3′
KINC	5′GGGCTTCTCCTGGTTTA GCTGGAA3′
KIND	5′CAAGCTCATGAAAGAATGCTG GCAACAAG3′
KINE	5′CAGTTGACTGCTATTGAGGG GGCAGTGAT3′
KINF	5′GCAAATTTACCAAGCTCTCGGGTAG CCATT3′
KINA	5′CAGGGCCTGGGCACGACCATG3′
KING	5′CGAACTCCAGCACCAGG CAGAGGT3′
KIN5	5′GGCCGAGGCTGGACCCTTT3′
KIN3a	5′GGCCAGGTCGCATCACCAA3′
KIN3b	5′CACAGCTTTTGAAAACACAGCAGC ATGAAA3′
KINI	5′GGATAAGACCTCTCTCCGATGGCA ACAGTC3′
G3PDHF	5′GGGCGCCTGGTCACAA3′
G3PDHR	5′AACATGGGGGCATCAGCAGA3′
KINRTF	5′CACAGTCTTTCGACAAGA AGAATTTG3′
KINRTR	5′AGACTATTCGTAGGAGCCA TCTCAATG3′
ActinHS-Fov	5′GAGCAAGAGAGGCATCCTCACC3′
ActinHS-Rev	5′GGAAGGAAGGCTGGAAGAGTGC3′

Expression Patterns and Chromosomal Localization of *MLK4* Gene

Northern blot hybridization with human multiple tissue #7760-1 Northern blot (Clontech) and PCR with human multiple tissue cDNA panels (Clontech) were used to determine the expression patterns of *MLK4α* and *MLK4β*. Human multiple tissue Northern blot was consecutively hybridized at 42 °C for 16 hours with three [^{32}P] labeled probes: i) the 3′-end of *MLK4α* cDNA (1970–3910 bp), ii) the 3′-end of *MLK4β* cDNA (2087–4667 bp) and iii) β-actin cDNA as previously described.[42]

PCR with multiple tissue cDNA panels (Clontech) was done with primers KIND and KIN3a for *MLK4α*, KINF and KINI for *MLK4β*, and G3PDHF and G3PDHR for *G3PDH*. To amplify the gene fragments of *MLK4α*, *MLK4β* and *G3PDH*, 40, 30 and 20 cycles of amplification were performed, respectively. PCR was carried out with 30 sec denaturation at 95 °C, 20 sec annealing at 60 °C and 1 min extension at 72 °C. Initial denaturation was done for 4 min at 95 °C.

The FISH of the *Not*I linking clone NR5-DM9 (insert size 5 kb) (GenBank accession No. AJ324357) with normal metaphase chromosomes was done as described previously.[43] Sixty metaphase spreads with specific signals have been analyzed.

Subcellular Localization of the *MLK4* Gene by Immunostaining

For cellular localization of the corresponding proteins, we cloned *MLK4* gene into the pCMV-Tag3 vector (GenBank accession No. AF072997) to express c-myc-MLK4 fusion proteins. The full-length *MLK4* coding sequences from pCR4-TOPO clones (for *MLK4α*, the plasmid was digested by NcoI and EcoRI, and for *MLK4β*, by NcoI and SpeI) were blunted by Klenow enzyme (Invitrogen BV, Groningen, Netherlands) and reinserted into a pCMV-Tag3A digested by EcoRV.

COS-1 cells (http://phage.atcc.orghttp://phage.atcc.org) were cultured in IMDM with 10% fetal bovine serum (Gibco, Paisley, UK) and transfected with either pCMV-MLK4α or pCMV-MLK4β constructs using Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's protocol. The immunostaining was done as described.[44] We used mouse monoclonal antibody 9E10 (Abcam, Cambridge, UK) against c-myc-tag, fused with *MLK4α* or *MLK4β* and the FITC-conjugated swine anti-mouse secondary antibody F0205 (Dako, Glostrup, Denmark). Hoechst 33258 (Sigma-Aldrich, St. Louis, USA) was used for DNA counterstaining at a concentration of 0.4 μg/mL.

Colony Formation Assay

MCF7 breast cancer cells were also cultured in IMDM. Transient transfection with either pCMV-MLK4α, pCMV-MLK4β or original pCMV-Tag3A vector was done using Lipofectamine Plus Reagent. Transfected cells were stripped 24–48 h after transfection with trypsin/EDTA (Sigma) and plated on 100 mm cell culture dishes at 500–1000 cells per plate. After selection with 500 μg/mL G418 (Cayla, Toulouse, France) for two weeks, Giemsa-stained colonies were photographed and counted.

Cell Proliferation Assay

To obtain MCF7 cells stably transfected with pCMV-MLK4α or pCMV-MLK4β (transfection

was performed as described above), selection with 500 µg/mL G418 was done for 10 weeks due to very slow cell growth. Two PCR positive clones for each construct were used to measure the cell proliferation rate using the CyQUANT NF Cell Proliferation Assay (Invitrogen) according to the manufacturer's protocol. Briefly, cells were plated at a density of 100–500 cells per well in a 96-well plate (8–12 identical wells in total). Parental MCF7 cells stably transfected with the pCMV-Tag3A vector were used as a control. The number of cells per well was counted every 24 hours: growth medium was removed, 80 µL of green-fluorescent CyQUANT GR dye (which exhibits strong fluorescence enhancement when bound to cellular nucleic acid) was added to the well and incubated for 30 min at 37 °C. The fluorescence intensity of each sample was measured using a fluorescence microplate reader with excitation at 485 nm and emission detection at 530 nm (SpectraMax Gemini, Molecular Devices, Sunnyvale, USA). Plotted data points represent averages of triplicate samples, and the plotted line is a linear regression fit of all data points. The assay is designed to produce a linear analytical response from at least 100–20,000 cells per well in most cell lines.

Transgenic Mice

The pCMV-MLK4β construct(4 ng/µL) was linearized with *Bst*BI and microinjected into the male pronucleus of eggs from B6CBA F1 crosses of common house mice (*Mus musculus* L.). The eggs were transferred into the pseudopregnant D1 recipient strain. The injection buffer contained 10 mM Tris-HCl, pH 7.5 and 0.1 mM EDTA. A total of 136 eggs were transferred, 56 offspring were genotyped by PCR and seven of them were found to be positive. The transgenic founders were crossed with CD1 mice and F1 progeny were used for the analysis of *MLK4β* expression in different mouse tissues. Both PCR and RT-PCR were carried out with KINRTF and KINRTR primers for 25 cycles with 25 sec denaturation at 95 °C, 20 sec annealing at 60 °C and 1 min extension at 72 °C; β-actin was used as a housekeeping control (primers ActinHS-Fov and ActinHS_Rev). Animal experiments were approved by North Stockholm Ethical Committee (decision 150/08) on 29 May of 2008.

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Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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