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(71) Demandeur/Applicant:
CEPHALON, INC., US

(72) Inventeurs/Inventors:
ANGELES, THELMA S., US;
DURKIN, JOHN T., US;
HOLSKIN, BEVERLY P., US;
MEYER, SHERYL L., US;
SPAIS, CHRYSANTHE M., US

(74) Agent: SMART & BIGGAR

(54) Titre : NOUVEAU POLYPEPTIDE DE KINASE 7 DE LIGNEE MELANGEE (MLK7), POLYNUCLEOTIDES LES
CODANT, ET LEURS METHODES D'UTILISATION

(54) Title: NOVEL MIXED LINEAGE KINASE (7) (MLK7) POLYPEPTIDE, POLYNUCLEOTIDES ENCODING THE SAME,
AND METHODS OF USE THEREOF

(57) **Abrégé/Abstract:**

The present invention provides isolated mixed lineage kinase (7) (MLK) polynucleotides, expression vectors, host cells, isolated polypeptides, methods of producing polypeptides, isolated antibodies, compositions having the foregoing, methods for identifying a compound that binds a polypeptide or polynucleotide, and to methods for identifying a compound that modulates the activity of a polypeptide.

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(71) Applicant (*for all designated States except US*): **CEPHALON, INC.** [US/US]; 145 Brandywine Park, West Chester, PA 19380 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **ANGELES, Thelma, S.** [US/US]; 1509 Grovenor Court, West Chester, PA 19380 (US). **DURKIN, John, T.** [US/US]; 47 Wellington Road, Ardmore, PA 19003 (US). **HOLSKIN, Beverly, P.** [US/US]; Apt. 31 D, West Tower, Philadelphia, PA 19106 (US). **MEYER, Sheryl, L.** [US/US]; 274 Level Road, Collegeville, PA 19426 (US). **SPAIS, Chrysanthé, M.** [US/US]; 6 West Corwen Terrace, West Chester, PA 19380 (US).

(74) Agents: **MILLER, Suzanne, E.** et al.; Woodcock Washburn LLP, One Liberty Place - 46th Floor, Philadelphia, PA 19103 (US).

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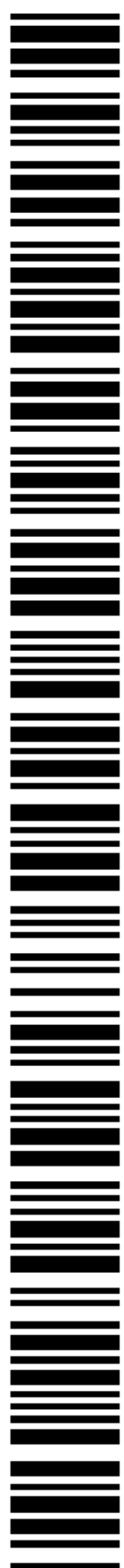
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(54) Title: NOVEL MIXED LINEAGE KINASE (7) (MLK7) POLYPEPTIDE, POLYNUCLEOTIDES ENCODING THE SAME, AND METHODS OF USE THEREOF

(57) Abstract: The present invention provides isolated mixed lineage kinase (7) (MLK) polynucleotides, expression vectors, host cells, isolated polypeptides, methods of producing polypeptides, isolated antibodies, compositions having the foregoing, methods for identifying a compound that binds a polypeptide or polynucleotide, and to methods for identifying a compound that modulates the activity of a polypeptide.



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**NOVEL MIXED LINEAGE KINASE 7 (MLK7) POLYPEPTIDE,
POLYNUCLEOTIDES ENCODING THE SAME, AND METHODS
OF USE THEREOF**

5 CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims priority to U.S. provisional application Serial No. 60/293,381 filed May 24, 2001, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

10 The present invention is directed, in part, to a novel mixed lineage kinase 7 (MLK7) polypeptide, polynucleotides encoding the same, methods for identifying compounds that bind to or modulate the activity of the polypeptide, and methods for assaying the enzymatic activity of MLK7.

15 BACKGROUND OF THE INVENTION

 The MLK family comprises a group of enzymes in which the protein sequence of the kinase domains of the family members show homology with both tyrosine and serine/threonine protein kinases. In particular, this region contains many amino acids highly conserved among tyrosine kinases, but the homology to serine/threonine protein
20 kinases in the catalytic domain, subdomains VIb and VIII, is consistent with the known serine/threonine kinase activity of MLK3 (Gallo *et al.*, *J. Biol. Chem.*, **1994**, 269, 15092-15100). In addition, all family members contain a leucine zipper, which is an α -helical structure characterized by a periodic repeat of leucine or isoleucine every seventh amino acid for a stretch of at least 22 amino acids to form a parallel coiled-coil structure

(Landschultz *et al.*, *Science*, **1988**, 240, 1759-1764). These kinases comprise a portion of very complex kinase cascades such as, for example, the stress-signaling cascade, which involves modulation of, *inter alia*, c-Jun N-terminal kinase (JNK), which in turn modulates, *inter alia*, transcription factors including c-Jun, ATF2 and ELK1. JNK is
5 described in U.S. Patents 5,534,426, 5,593,884, 5,605,808, and in international publication WO 95/03324.

The MLK family consists of 6 reported family members: 1) mixed lineage kinase 1 (MLK1); 2) mixed lineage kinase 2 (MLK2); 3) mixed lineage kinase 3 (MLK3); 4) dual leucine zipper bearing kinase (DLK); 5) leucine zipper bearing kinase (LZK), and 6)
10 MLK-like mitogen activated protein triple kinase/mixed lineage kinase 6 (MLTK/MLK6). A partial sequence has been reported for MLK1 (Dorow *et al.*, *Eur. J. Biochem.*, **1993**, 213, 701-710). Mixed lineage kinases 1, 2 and 3 have, in addition to the features described above, a src-homology-3 (SH3) domain N-terminal to the kinase domain (Dorow *et al.*, *J. Protein Chem.*, **1994**, 13, 458-460; Dorow *et al.*, *Eur. J. Biochem.*, **1995**,
15 234, 492-500; and Ing *et al.*, *Oncogene*, **1994**, 9, 1745-1750). Their leucine zipper region is just C-terminal to the kinase domain and is comprised of 2 repeats of 4 alpha helical regions separated by a small spacer region, termed a dual leucine zipper. Deletion analysis suggests a functional role of this domain in homodimerization (Leung *et al.*, *J. Biol. Chem.*, **1998**, 273, 32408-32415). This domain is followed by a small region comprised
20 of a preponderance of basic amino acids similar to nuclear localization signals from several proteins (Dorow *et al.*, *Eur. J. Biochem.*, **1995**, 234, 492-500). Immediately adjacent to that is a Cdc42/Rac interactive binding (CRIB) domain (Burbelo *et al.*, *J. Biol. Chem.*, **1995**, 270, 29071-29074). The C-terminus has generally a low level of complexity, where 3 amino acids (proline, serine and glycine) comprise up to 41%
25 (MLK3) of the primary amino acid sequence. MLK2 is also known as MST (Kato *et al.*, *Oncogene*, **1995**, 10, 1447-1451). MLK3 is also known as scr-homology 3 (SH3) domain-containing proline-rich kinase (SPRK) (Gallo *et al.*, *J. Biol. Chem.*, **1994**, 269, 15092-15100) and protein tyrosine kinase 1 (PTK1) (Ezoe *et al.*, *Oncogene*, **1994**, 9, 935-938).

DLK and LZK share the same general features, but do not have the SH3 and
30 CRIB domains and their spacer region in the dual leucine zipper is about 18 amino acids longer than in MLKs 1-3 (Holtzman *et al.*, *J. Biol. Chem.*, **1994**, 269, 30808-30817; Sakuma *et al.*, *J. Biol. Chem.*, **1997**, 272, 28622-28629). DLK is also known as leucine-

zipper protein kinase (ZPK) (Reddy *et al.*, *Biochem. Biophys. Res. Commun.*, **1994**, 202, 613-620) and MAPK-upstream kinase (MUK) (Hirai *et al.*, *Oncogene*, **1996**, 12, 641-650).

MLTK/MLK6 is even more divergent. They share a kinase domain and putative
5 leucine zipper region, but like the DLK/LZK group, there is no SH3 binding domain or CRIB domain, nor is there a basic amino acid region. Rather, the two cDNA sequences diverge after the putative leucine zipper region, resulting in two different isoforms, alpha and beta. Furthermore, unlike the other family members containing the "dual" 4 + 4 repeats, the putative leucine zipper region contains six contiguous alpha-helical repeats.
10 In addition, unlike other family members, the kinase domain is essentially at the N-terminus (Gotoh *et al.*, *J. Biol. Chem.*, **2001**, 276, 4276-4286). MLTK α /MLK6 α is also known as leucine-zipper sterile alpha motif kinase (ZAK) (Liu *et al.*, *Biochem. Biophys. Res. Commun.*, **2000**, 274, 811-816).

Members of the MLK family are also described in, for example, U.S. Patent
15 5,676,945; U.S. Patent 5,554,523; international publication WO 93/15201; Canadian Patent 2,148,898; Diener *et al.*, *Proc. Natl. Acad. Sci. USA*, **1997**, 94, 9687-9692; DeAizpurua *et al.*, *J. Biol. Chem.*, **1997**, 272, 16364-16373; Tung *et al.*, *Oncogene*, **1997**, 14, 653-659; Sells *et al.*, *Trends in Cell Biol.*, **1997**, 7, 161-167; Mata *et al.*, *J. Biol. Chem.*, **1996**, 271, 16888-16896; Hirai *et al.*, *J. Biol. Chem.*, **1997**, 272, 15167-15173;
20 Fan *et al.*, *J. Biol. Chem.*, **1996**, 271, 24788-24793; Blouin *et al.*, *DNA and Cell Biol.*, **1996**, 15, 631-642; Pombo *et al.*, *Nature*, **1995**, 377, 750-754; Kiefer *et al.*, *EMBO J.*, **1996**, 15, 7013-7025; Hu *et al.*, *Genes & Dev.*, **1996**, 10, 2251-2264; and Su *et al.*, *EMBO J.*, **1997**, 16, 1279-1290. Portions of the kinase domain for human kinase homologs have also been reported in U.S. Patent 5,817,479. In addition, nucleotide sequences for a mixed
25 lineage kinase mRNA have been reported in the Genbank database (*see*, Accession Numbers AF238255 and AF251441).

Due to the inadequacies of screening compounds that modulate members of the stress signaling cascade and promote either cell death or cell survival, there continues to be a need for new, selective methods of screening compounds. In addition, there
30 continues to be a need for screening assays for therapeutics which may be useful in treating inflammation and neurodegenerative disorders. The present invention is directed to these, as well as other, important ends. In particular, the present invention describes an

additional member of the MLK family, a seventh family member, termed herein as MLK7, identified from human genomic sequences that encode a kinase domain 73-76% homologous to the MLK 1-3 subfamily. MLK7 contains an SH3 domain N-terminal to the kinase domain. MLK7 also contains a dual leucine zipper C-terminal to the kinase domain, followed by a small region comprised of a preponderance of basic amino acids similar to nuclear localization signals from several proteins. This was used to clone a cDNA that encodes a functional kinase protein. The novel polypeptide of the present invention can be used, for example, to phosphorylate MAP kinase kinase 4 (MKK4) and MKK7, and play a role in JNK stress-activated kinase pathway activation. Thus, the polypeptide of the present invention can be used, for example, to identify inhibitors of the same and such inhibitors can be used to promote neuronal cell survival. Compounds that decrease activity of the MLK polypeptides are useful for, for example, promoting cell survival, treating neurodegenerative disorders, and treating inflammation.

15 SUMMARY OF THE INVENTION

The present invention provides a novel MLK7 polypeptide and polynucleotides encoding the same.

In particular, the present invention is directed to isolated polynucleotides comprising a nucleotide sequence set forth in SEQ ID NO:1, polynucleotides homologous to SEQ ID NO:1, polynucleotides that encode a polypeptide comprising SEQ ID NO:2, and polynucleotides that encode a polypeptide comprising an amino acid sequence homologous to SEQ ID NO:2.

The present invention is also directed to polynucleotides comprising a nucleotide sequence complementary to at least a portion of SEQ ID NO:1.

25 The present invention is also directed to expression vectors comprising the polynucleotides of the invention and host cells comprising the same.

The present invention is also directed to isolated polypeptides encoded by the polynucleotides of the invention and to methods of producing polypeptides comprising SEQ ID NO:2 and polypeptides homologous thereto, by introducing a recombinant expression vector comprising a polynucleotide of the invention into a compatible host cell, growing the host cell under conditions for expression of the polypeptide, and recovering the polypeptide.

The present invention is also directed to isolated antibodies that bind to an epitope on a polypeptide of the invention.

The present invention is also directed to compositions comprising a polynucleotide, expression vector, polypeptide, or antibody and a carrier or diluent, and to
5 kits comprising a polynucleotide, expression vector, polypeptide, or antibody.

The present invention is also directed to methods for identifying a compound that binds a polypeptide of the invention by contacting the polypeptide with the compound and determining whether the compound binds to the polypeptide.

The present invention is also directed to methods for identifying a compound that
10 binds a polynucleotide of the invention by contacting the polynucleotide with the compound and determining whether the compound binds the polynucleotide.

The present invention is also directed to methods for identifying a compound that modulates the activity of a polypeptide of the invention by contacting the polypeptide with the compound and determining whether the polypeptide activity has been modulated.

15 The present invention is also directed to compounds identified by the identification methods of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a representative kinase subdomain alignment and functional
20 domains for MLK1 and MLK3 from the world wide web of the internet at sdsc.edu/Kinases/pkr/pk_catalytic/pk_hanks_seq_align_long.html#OPK-XI. Other proteins aligned by DNASTar program using Clustal method with PAM250 residue weight table. Amino acids in bold are highly conserved amino acids in protein tyrosine kinases; amino acids in bold italics are
25 conserved in protein serine-threonine kinases; amino acids in bold italics underlined are highly conserved in both (Hanks *et al.*, *Meth. in Enzymol.*, **1991**, 200, 38-62). Conserved amino acids for tyrosine kinases altered in these proteins are shown in bold above the sequence (capital letters for conserved, small letters for preferred).

Figure 2 depicts a representative phylogenetic tree of the kinase domains in the
30 human mixed lineage kinase family. This analysis was performed using the MegAlign feature of the DNASTar program (Clustal method with PAM250 residue weight table). The proteins are, from top to bottom, MLK3, MLK7, MLK1, MLK2, DLK, LZK and

MLTK/MLK6. This alignment organizes the seven proteins into three subfamilies: the MLK1-3 subfamily (MLKs 1, 2, 3 and 7), the DLK subfamily (DLK, LZK) and the MLTK/MLK6 subfamily.

Figure 3 shows a representative domain structure of MLK7 polypeptide and the predicted molecular weight (MW) of the polypeptide.

Figure 4 shows the sequence of the putative leucine zipper region that follows the kinase domain. The leucine/isoleucine residues spaced every seventh amino acid (one alpha-helical turn) are shown in bold and are underlined. The seventh amino acid preceding and following this region begin and end the shown sequence to demonstrate that this pattern does not continue.

Figure 5 shows that overexpression of MLK7 increases JNK activity in CHO cell lysates, and that six-hour treatment with CEP11004 partially inhibits JNK activity driven by MLK7.

Figure 6 illustrates the phosphorylation of myelin basic protein by baculoviral GST-MLK7_{KD} (open circles) and GST-MLK7_{KD/LZ} (filled-in circles) using a radioactive multiscreen trichloroacetic acid precipitation assay. The entire data set containing the positive enzyme control GST-MLK3_{KD} (filled-in diamonds) is shown in the inset.

Figure 7 shows the phosphorylation of (A) GST-MKK4(K113A) and (B) GST-MKK7(K149A) by baculoviral GST-MLK7_{KD} (open circles) and GST-MLK7_{KD/LZ} (filled-in circles) using an ELISA-based assay with time-resolved fluorescence readout. GST-MLK3_{KD} (filled-in diamonds) was used as the positive enzyme control.

Figure 8 demonstrates the *in vitro* activation of the JNK pathway by baculoviral GST-MLK7_{KD} and GST-MLK7_{KD/LZ}. Inactivated forms of MKK4 and MKK7 were separately phosphorylated by baculoviral GST-MLK7_{KD}. Subsequently, these two MKK proteins were activated and proceeded to catalyze the phosphorylation of GST-JNK1 β 1(K55A) as probed by a phospho-specific JNK antibody. Similar results were observed for GST-MLK7_{KD/LZ} and GST-MLK3_{KD} (enzyme control), albeit to different efficiencies. Inset shows the entire data set, including controls.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention provides purified and isolated polynucleotides (e.g., DNA sequences and RNA transcripts, chimeric RNA/DNA molecules, both sense and

complementary antisense strands, both single- and double-stranded, including splice variants thereof) encoding a human MLK polypeptide referred to herein as “MLK7.” DNA polynucleotides of the invention include genomic DNA, cDNA, and DNA that has been chemically synthesized.

5 As used herein, “activity” means a variety of measurable indicia suggesting or revealing binding, either direct or indirect; affecting a response, such as having a measurable affect in response to some exposure or stimulus, including, for example, the affinity of a compound for directly binding a polypeptide or polynucleotide of the invention, or, for example, measurement of amounts of upstream or downstream proteins
10 (e.g. JNK) or activity thereof, or other similar functions after a stimulus or event.

As used herein, “antibody” means complete, intact antibodies, and Fab, Fab’, F(ab)₂, and other fragments thereof. Complete, intact antibodies include monoclonal antibodies, chimeric antibodies, and humanized antibodies.

As used herein, “binding” means the physical or chemical interaction between
15 two polypeptides or polynucleotides or compounds or associated proteins or compounds or combinations thereof. Binding includes, but is not limited to, ionic, non-ionic, Hydrogen bonds, Van der Waals, hydrophobic interactions, etc. Binding can be either direct or indirect through or due to the effects of another protein or compound.

As used herein, “compound” means any chemical or molecule including, but not
20 limited to, small molecules, peptides, proteins or polypeptides, sugars, nucleotides, or nucleic acids, and the like. The compounds can be natural or synthetic.

As used herein, “contacting” means bringing together, either directly or indirectly, a compound into physical proximity to a polypeptide or polynucleotide of the invention. The polypeptide or polynucleotide can be in any number of buffers, salts,
25 solutions, etc. Contacting includes, but is not limited to, placing the compound into a beaker, microtiter plate, cell culture flask, or a microarray, or the like, which contains the MLK7 polypeptide or fragment thereof, polynucleotide encoding the same, or antibody binding to the same.

As used herein, “homologous nucleotide sequence,” or “homologous amino acid
30 sequence,” or variations thereof, means sequences characterized by a homology, at the nucleotide level or amino acid level, of at least a specified percentage. Homologous nucleotide sequences include nucleotide sequences encoding for a protein of a species

other than humans, including, but not limited to, mammals. Homologous nucleotide sequences also include, but are not limited to, naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. A homologous nucleotide sequence is not, however, identical to the nucleotide sequences encoding other known
5 MLK polypeptides. Homologous amino acid sequences include those amino acid sequences that encode conservative amino acid substitutions, as well as polypeptides having kinase activity. A homologous amino acid sequence is not, however, identical to the amino acid sequences encoding other known MLK polypeptides. Percent homology can be determined by, for example, the Gap program (Wisconsin Sequence Analysis
10 Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using default settings, which uses the algorithm of Smith and Waterman (*Adv. Appl. Math.*, **1981**, 2, 482-489, which is incorporated herein by reference in its entirety).

As used herein, “modulates” or “modifies” means an increase or decrease in the
15 amount, quality, or effect of a particular activity or polypeptide.

As used herein, “treating” means to having a therapeutic effect and at least partially alleviating or abrogating an abnormal condition in an organism.

As used herein, “therapeutic effect” means inhibition or activation of factors causing or contributing to an abnormal condition. A therapeutic effect alleviates, at least
20 to some extent, one or more of the symptoms of the abnormal condition. In regard to treatment of abnormal conditions, a therapeutic effect can refer to one or more of the following: i) an increase in the proliferation, growth, and/or differentiation of cells; ii) inhibition (i.e., slowing or stopping) of cell death; iii) inhibition of degeneration; iv) relieving to some extent one or more of the symptoms associated with the abnormal
25 condition; and v) enhancing the function of the affected population of cells.

As used herein, “abnormal condition” means a function in cells or tissues of an organism that deviates from their normal functions in that organism. An abnormal condition can be any condition that is not desired by an individual. An abnormal condition can relate to cell proliferation, cell differentiation, cell signaling, or cell
30 survival. Abnormal differentiation conditions include, but are not limited to, neurodegenerative or inflammatory disorders. Abnormal cell survival conditions can also

relate to conditions in which programmed cell death (apoptosis) pathways are activated or abrogated.

As used herein, "administering" means a method of incorporating a compound into a cell or tissue of an organism. For cells within an organism, administration techniques include, but are not limited to, oral, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly, intraventricularly, and transdermally, as well as by inhalation or suppository. For cells outside an organism, administration techniques include, but are not limited to, cell microinjection, transformation, and carrier techniques.

As used herein, "organism" means a mammal such as a mouse, rat, rabbit, guinea pig or goat, more preferably a primate such as a monkey or ape, and most preferably a human.

As used herein, "stringent hybridization conditions" or "stringent conditions" means conditions under which a probe, primer, or oligonucleotide will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. Target sequences are generally present in excess. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30C for short probes, primers or oligonucleotides (e.g. 10 to 50 nucleotides) and at least about 60C for longer probes, primers or oligonucleotides. Stringent conditions can also be achieved with the addition of destabilizing agents, such as formamide.

The polynucleotides of the invention can include genomic DNA which comprises the protein coding region for a polypeptide of the invention and is also intended to include allelic variants or splice variants thereof. Splice variants of the invention are encoded by the same original genomic DNA sequences but arise from distinct mRNA transcripts. Allelic variants are modified forms of a wild-type gene sequence, the modification resulting from recombination during chromosomal segregation or exposure to conditions which give rise to genetic mutation. Allelic variants, like wild-

type genes, are naturally occurring sequences, as opposed to non-naturally occurring variants which arise from *in vitro* manipulation.

In the present invention, a polynucleotide encoding MLK7 polypeptide is set forth in SEQ ID NO:1. A preferred polypeptide of the invention comprises a double stranded molecule. Also preferred are other polynucleotides encoding particular MLK7 polypeptides of the invention which differ in sequence from the particular polynucleotide set forth in SEQ ID NO:1 by virtue of the well-known degeneracy of the universal nuclear genetic code.

The invention is further directed to additional species homologs, preferably mammalian, of the human MLK7 polynucleotides. Species homologs, sometimes referred to as “orthologs,” in general, share at least 35%, at least 40%, at least 45%, at least 50%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% homology with human polynucleotides of the invention. Generally, percent sequence “homology” with respect to polynucleotides of the invention can be calculated as the percentage of nucleotide bases in the candidate sequence that are identical to nucleotides in the MLK7 sequence set forth in a particular polynucleotide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity.

Examples of related polynucleotides include human and non-human genomic sequences, including allelic variants, as well as polynucleotides encoding polypeptides homologous to MLK7 polypeptides and structurally related polypeptides sharing one or more biological, immunological, and/or physical properties of MLK7 polypeptides. Non-human species genes encoding proteins homologous to MLK7 polypeptides can also be identified by Southern and/or PCR analysis and are useful in animal models for MLK polypeptide disorders. Polynucleotides of the invention are also useful in hybridization assays to detect the capacity of cells to express MLK7 polypeptides. Polynucleotides of the invention can also provide a basis for diagnostic methods useful for identifying a genetic alteration(s) in an MLK7 locus that underlies a disease state or states, such information being useful both for diagnosis and for selection of therapeutic strategies.

The disclosure herein of a full-length polynucleotide encoding a MLK7 polypeptide makes readily available to the worker of ordinary skill in the art every possible fragment of the full length polynucleotide. MLK7 polynucleotides, therefore,

encompass fragments comprising at least 14, and preferably at least 16, 18, 20, 25, 50, 75, 100, 150, 200, 250, 400, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, or 3000 consecutive nucleotides of a polynucleotide encoding MLK7. Preferably, fragment polynucleotides of the invention comprise sequences unique to the MLK7-encoding polynucleotide sequence, and therefore hybridize under highly stringent or moderately stringent conditions only (i.e., "specifically") to polynucleotides encoding MLK7 (or fragments thereof). Polynucleotide fragments of genomic sequences of the invention comprise not only sequences unique to the coding region, but also include fragments of the full-length sequence derived from introns, regulatory regions, and/or other non-translated sequences. Polynucleotides of the invention can be labeled in a manner that permits their detection, including radioactive, fluorescent, and enzymatic labeling.

Fragment polynucleotides are particularly useful as probes for detection of full-length or fragment MLK7 polynucleotides. One or more polynucleotides can be included in kits that are used to detect the presence of a polynucleotide encoding MLK7, or used to detect variations in a polynucleotide sequence encoding MLK7.

The invention is also directed to polynucleotides encoding MLK7 polypeptides that hybridize under moderately stringent or high stringency conditions to the non-coding strand, or complement, of the polynucleotide in SEQ ID NO:1. Exemplary highly stringent hybridization conditions are as follows: hybridization at 42C in a hybridization solution comprising 50% formamide, 1% SDS, 1 M NaCl, 10% Dextran sulfate, and washing twice for 30 minutes at 60C in a wash solution comprising 0.1 X SSC and 1% SDS. It is understood in the art that conditions of equivalent stringency can be achieved through variation of temperature and buffer, or salt concentration as described Ausubel, et al. (Eds.), *Protocols in Molecular Biology*, John Wiley & Sons (1994), pp. 6.0.3 to 6.4.10. Modifications in hybridization conditions can be empirically determined or precisely calculated based on the length and the percentage of guanosine/cytosine (GC) base pairing of the probe. The hybridization conditions can be calculated as described in Sambrook, et al., (Eds.), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (1989), pp. 9.47 to 9.51, which is incorporated herein by reference in its entirety.

Recombinant expression constructs such as plasmids and viral DNA vectors incorporating polynucleotides of the invention are also provided. Preferably, MLK7-

encoding polynucleotides are operatively linked to an endogenous or exogenous promoters, enhancers, operators, or regulatory element binding sites, or any combination thereof. Expression constructs of the invention can also include sequences encoding one or more selectable markers that permit identification of host cells bearing the construct.

5 Expression constructs can also include sequences that facilitate, and preferably promote, homologous recombination in a host cell. Preferred constructs of the invention also include sequences necessary for replication in a host cell. Expression constructs are preferably utilized for production of an encoded protein, but can also be utilized to amplify a MLK7-encoding polynucleotide sequence.

10 In another embodiment of the invention, host cells are provided, including prokaryotic and eukaryotic cells, comprising a polynucleotide of the invention (or vector of the invention) in a manner which permits expression of the encoded MLK7 polypeptide. Polynucleotides of the invention can be introduced into the host cell as part of a plasmid, or as linear DNA comprising an isolated protein coding region or a viral
15 vector. Methods for introducing DNA into a host cell are well known and routinely practiced in the art and include transformation, transfection, electroporation, nuclear injection, or fusion with carriers such as liposomes, micelles, ghost cells, and protoplasts. Expression systems of the invention include bacterial, yeast, fungal, plant, insect, invertebrate, vertebrate, and mammalian cells systems.

20 Host cells of the invention can provide immunogens based on the MLK7 polypeptide for development of antibodies that are specifically immunoreactive with MLK7. Host cells of the invention are also useful in methods for the large-scale production of MLK7 polypeptide wherein the cells are grown in a suitable culture medium and the desired polypeptide product is isolated from the cells, or from the
25 medium in which the cells are grown, by purification methods known in the art, e.g., conventional chromatographic methods including immunoaffinity chromatography, receptor affinity chromatography, hydrophobic interaction chromatography, lectin affinity chromatography, size exclusion filtration, cation or anion exchange chromatography, high pressure liquid chromatography (HPLC), reverse phase HPLC, and the like. Still other
30 methods of purification include those methods wherein the desired protein is expressed and purified as a fusion protein having a specific tag, label, or chelating moiety that is recognized by a specific binding partner or agent. The purified protein can be cleaved to

yield the desired protein, or can be left as an intact fusion protein. Cleavage of the fusion component may produce a form of the desired protein having additional amino acid residues as a result of the cleavage process.

The present invention is also directed to antisense polynucleotides that recognize
5 and hybridize to polynucleotides encoding MLK7. Full-length and fragment antisense polynucleotides are provided. Fragment antisense molecules of the invention include those that specifically recognize and hybridize to MLK7 RNA or DNA. Antisense polynucleotides are particularly relevant to regulating expression of MLK7 by those cells expressing MLK7 mRNA. Antisense polynucleotides (preferably 10 to 20 base-pair
10 oligonucleotides) capable of specifically binding to MLK7 expression control sequences or MLK7 RNA are introduced into cells (e.g., by a viral vector or colloidal dispersion system such as a liposome). Phosphorothioate and methylphosphonate antisense oligonucleotides are specifically contemplated for therapeutic use by the invention. Suppression of MLK7 expression at either the transcriptional or translational level is
15 useful to generate cellular or animal models for diseases/conditions characterized by aberrant MLK7 expression.

The invention also provides purified and isolated mammalian MLK7 polypeptides encoded by a polynucleotide of the invention. Preferably, the MLK7 polypeptide comprises the amino acid sequence set out in SEQ ID NO:2. The present
20 invention is also directed to polypeptides that have at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55% or at least 50% identity and/or homology to the preferred polypeptide of the invention. Percent amino acid sequence "identity" with respect to the preferred polypeptide of the invention is defined herein as the percentage of amino acid residues in
25 the candidate sequence that are identical with the residues in the MLK7 sequence after aligning both sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Percent sequence "homology" with respect to the preferred polypeptide of the invention is defined herein as the percentage of amino acid residues in
30 the candidate sequence that are identical with the residues in the MLK7 sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum

percent sequence identity, and also considering any conservative substitutions as part of the sequence identity.

The polypeptide of the invention can be isolated from natural cell sources or can be chemically synthesized, but is preferably produced by recombinant procedures involving host cells of the invention. Use of mammalian host cells is expected to provide for such post-translational modifications (e.g., glycosylation, truncation, lipidation, and phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention. Glycosylated and non-glycosylated forms of MLK7 polypeptides are also provided.

The present invention is also directed to MLK7 polypeptide variants or analogs. In one example, insertion variants are provided wherein one or more amino acid residues supplement an MLK7 amino acid sequence. Insertions can be located at either or both termini of the protein, or can be positioned within internal regions of the MLK7 amino acid sequence. Insertion variants with additional residues at either or both termini can include, for example, fusion proteins and proteins including amino acid tags or labels. Insertion variants include MLK7 polypeptides wherein one or more amino acid residues are added to a MLK7 acid sequence, or to a biologically active fragment thereof. Variant products of the invention also include mature MLK7 polypeptides wherein leader or signal sequences are removed, with additional amino terminal residues. The additional amino terminal residues can be derived from another protein, or can include one or more residues that are not identifiable as being derived from specific proteins.

The present invention also provides MLK7 variants having additional amino acid residues that result from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as part of a glutathione-S-transferase (GST) fusion product provides the desired polypeptide having an additional glycine residue at position -1 after cleavage of the GST component from the desired polypeptide. Variants that result from expression in other vector systems are also contemplated. Insertional variants also include fusion proteins wherein the amino terminus and/or the carboxy terminus of MLK7 is/are fused to another polypeptide. In addition, other fusion proteins comprising MLK7 or fragments thereof are contemplated by the invention. Numerous fusion partner proteins are well known to the skilled artisan.

In another aspect, the invention provides deletion variants wherein one or more amino acid residues in a MLK7 polypeptide are removed. Deletions can be effected at one or both termini of the MLK7 polypeptide, or with removal of one or more non-terminal amino acid residues of MLK7. Deletion variants, therefore, include all fragments of an
5 MLK7 polypeptide.

The invention also embraces polypeptide fragments of SEQ ID NO:2 wherein the fragments maintain biological (e.g., ligand binding and/or kinase activity) or immunological properties of an MLK7 polypeptide. Fragments comprising at least 5, 10, 15, 20, 25, 30, 35, 40, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650,
10 700, 750, 800, 850, 900, 950, or 1000 consecutive amino acids of any of the polypeptides described herein are contemplated by the present invention. Preferred polypeptide fragments have antigenic properties unique to, or specific for, human MLK7 and its allelic and species homologs. Fragments of the invention having the desired biological and immunological properties can be prepared by any of the methods well known and
15 routinely practiced in the art. Additional preferred fragments include the kinase domain of MLK7 (MLK7_{KD}), the leucine zipper region of MLK7 (MLK7_{LZ}), and the kinase domain linked to the leucine zipper region of MLK7 (MLK7_{KD/LZ}).

In another aspect of the present invention, substitution variants of MLK7 polypeptides are provided. Substitution variants include those polypeptides wherein one
20 or more amino acid residues of a MLK7 polypeptide are removed and replaced with alternative residues. In one aspect, the substitutions are conservative in nature. The present invention, however, also includes substitutions that are also non-conservative. Conservative substitutions for this purpose can be defined as set out below. Variant polypeptides include those wherein conservative substitutions have been introduced by
25 modification of polynucleotides encoding polypeptides of the invention. A conservative substitution is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions include, but are not limited to, non-polar (Gly, Ala, Pro, Ile, Leu, and Val), polar-uncharged (Cys, Ser, Thr, Met, Asn, and Gln), polar-charged (Asp, Glu, Lys, and Arg) aromatic (His, Phe, Trp, and
30 Tyr), and other (Asn, Gln, Asp and Glu). Alternatively, conservative amino acids can be grouped as described in Lehninger, (Biochemistry, Second Edition; Worth Publishers, Inc. NY, NY (1975), pp.71-77) wherein conservative substitutions include, but are not

limited to, non-polar (hydrophobic) 1) aliphatic: Ala, Leu, Ile, Val, and Pro; 2) aromatic: Phe and Trp 3) sulfur-containing: Met; 4) borderline: Gly; or uncharged-polar 1) hydroxyl: Ser, Thr, and Tyr; 2) amides: Asn and Gln; 3) sulfhydryl: Cys; 4) borderline: Gly; or positively charged (basic): Lys, Arg, and His; or negatively charged (acidic): Asp and Glu. As still another alternative, exemplary conservative substitutions include, but are not limited to, Ala (Val, Leu, Ile), Arg (Lys, Gln, Asn), Asn (Gln, His, Lys, Arg), Asp (Glu), Cys (Ser), Gln (Asn), Glu (Asp), His (Asn, Gln, Lys, Arg), Ile (Leu, Val, Met, Ala, Phe), Leu (Ile, Val, Met, Ala, Phe), Lys (Arg, Gln, Asn), Met (Leu, Phe, Ile), Phe (Leu, Val, Ile, Ala), Pro (Gly), Ser (Thr), Thr (Ser), Trp (Tyr), Tyr (Trp, Phe, Thr, Ser), and Val (Ile, Leu, Met, Phe, Ala). It should be understood that the definition of polypeptides of the invention is intended to include polypeptides bearing modifications other than insertion, deletion, or substitution of amino acid residues. By way of example, the modifications may be covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic, and inorganic moieties.

15 In a related embodiment, the present invention provides compositions comprising purified polypeptides of the invention. Preferred compositions comprise, in addition to the polypeptide of the invention, a liquid, semisolid, or solid diluent that serves as a vehicle, excipient, carrier or medium. The compositions can also comprise, in addition to the polypeptide of the invention, a pharmaceutically acceptable (i.e., sterile and non-toxic) liquid, semisolid, or solid diluent that serves as a vehicle, excipient, carrier or medium. Any diluent known in the art can be used including, but are not limited to, water, saline solutions, polyoxyethylene sorbitan monolaurate, magnesium stearate, methyl- and propylhydroxybenzoate, talc, alginates, starches, lactose, sucrose, dextrose, sorbitol, mannitol, glycerol, calcium phosphate, mineral oil, and cocoa butter.

25 A polynucleotide comprising any of the MLK7 nucleotide sequences described above can be synthesized by PCR, with the PCR oligonucleotide primers produced from the nucleotide sequences provided herein. See U.S. Patents 4,683,195 and 4,683,202. In addition, numerous cloning and *in vitro* amplification methodologies are well known to those skilled in the art. Examples of these techniques are found in, for example, Berger *et al.*, *Guide to Molecular Cloning Techniques*, Methods in Enzymology, 152, Academic Press, Inc., San Diego, CA (Berger), which is incorporated herein by reference in its entirety.

The polynucleotides of the present invention, and fragments derived therefrom, are useful for screening for restriction fragment length polymorphism (RFLP) associated with particular disorders, as well as for genetic mapping.

Antisense oligonucleotides, or fragments of a nucleotide sequence set forth in
5 SEQ ID NO:1, or sequences complementary or homologous thereto, derived from the nucleotide sequences of the present invention encoding MLK7 are useful as diagnostic tools for probing gene expression in various tissues. For example, tissue can be probed *in situ* with oligonucleotide probes carrying detectable groups by conventional autoradiography techniques to determine native expression of the polynucleotide or
10 pathological conditions relating thereto. Antisense oligonucleotides are preferably directed to regulatory regions of a nucleotide sequence set forth in SEQ ID NO:1, or mRNA corresponding thereto, including, but not limited to, the initiation codon, TATA box, enhancer sequences, other regulatory sequences, and the like.

Automated sequencing methods can be used to obtain or verify the nucleotide
15 sequence of an MLK7 polynucleotide. The MLK7 polynucleotide sequences of the present invention are believed to be 100% accurate. However, as is known in the art, nucleotide sequence obtained by automated methods can contain some errors. Nucleotide sequences determined by automation are typically at least about 90%, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of a
20 given nucleic acid molecule. The actual sequence can be more precisely determined using manual sequencing methods, which are well known in the art.

Another aspect of the present invention is directed to vectors, or recombinant expression vectors, comprising any of the nucleic acid molecules described above. Vectors are used herein either to amplify DNA or RNA encoding MLK7 and/or to express
25 DNA which encodes MLK7. Preferred vectors include, but are not limited to, plasmids, phages, cosmids, episomes, viral particles or viruses, and integratable DNA fragments. Preferred viral particles include, but are not limited to, adenoviruses, baculoviruses, parvoviruses, herpesviruses, poxviruses, adeno-associated viruses, Semliki Forest viruses, vaccinia viruses, and retroviruses. Preferred expression vectors include, but are not
30 limited to, pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). Other expression vectors include, but are not limited to, pSPORT vectors, pGEM vectors (Promega),

pPROEXvectors (LTI, Bethesda, MD), Bluescript vectors (Stratagene), pQE vectors (Qiagen), pSE420 (Invitrogen), and pYES2 (Invitrogen).

Preferred expression vectors are replicable polynucleotide constructs in which a polynucleotide sequence encoding MLK7 is operably linked or connected to suitable control sequences capable of effecting the expression of an MLK7 in a suitable host. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding, and sequences which control the termination of transcription and translation. Preferred vectors preferably contain a promoter that is recognized by the host organism. The promoter sequences of the present invention can be prokaryotic, eukaryotic or viral. Examples of suitable prokaryotic sequences include the PR and PL promoters of bacteriophage lambda (The bacteriophage Lambda, Hershey, A. D., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1973), which is incorporated herein by reference in its entirety; Lambda II, Hendrix, R. W., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1980), which is incorporated herein by reference in its entirety); the trp, recA, heat shock, and lacZ promoters of *E. coli* and the SV40 early promoter (Benoist *et al.*, *Nature*, **1981**, 290, 304-310, which is incorporated herein by reference in its entirety). Additional promoters include, but are not limited to, mouse mammary tumor virus, long terminal repeat of human immunodeficiency virus, maloney virus, cytomegalovirus immediate early promoter, Epstein Barr virus, rous sarcoma virus, human actin, human myosin, human hemoglobin, human muscle creatine, and human metallothionein.

Additional regulatory sequences can also be included in preferred vectors. Preferred examples of suitable regulatory sequences are represented by the Shine-Dalgarno of the replicase gene of the phage MS-2 and of the gene cII of bacteriophage lambda. The Shine-Dalgarno sequence can be directly followed by DNA encoding MLK7 and result in the expression of the mature MLK7. Moreover, suitable expression vectors can include an appropriate marker that allows the screening of the transformed host cells. An origin of replication can also be provided either by construction of the vector to include an exogenous origin or can be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter may be sufficient. Alternatively, rather than using vectors which contain viral origins of replication, one skilled in the art can transform mammalian cells by the method of co-

transformation with a selectable marker and MLK7 DNA. An example of a suitable marker is dihydrofolate reductase (DHFR) or thymidine kinase (tk) (see, U.S. Patent 4,399,216). Methods for construction of mammalian expression vectors are disclosed in, for example, Okayama *et al.*, *Mol. Cell. Biol.*, **1983**, 3, 280, Cosman *et al.*, *Mol.*
5 *Immunol.*, **1986**, 23, 935, Cosman *et al.*, *Nature*, **1984**, 312, 768, EP-A-0367566, and international publication WO 91/18982, each of which is incorporated herein by reference in its entirety.

Another aspect of the present invention is directed to transformed host cells having an expression vector comprising any of the nucleic acid molecules described
10 above. Expression of the nucleotide sequence occurs when the expression vector is introduced into an appropriate host cell. Suitable host cells for expression of the polypeptides of the invention include, but are not limited to, prokaryotes, yeast, and eukaryotes. If a prokaryotic expression vector is employed, then the appropriate host cell can be any prokaryotic cell capable of expressing the cloned sequences. Suitable
15 prokaryotic cells include, but are not limited to, bacteria of the genera *Escherichia*, *Bacillus*, *Salmonella*, *Pseudomonas*, *Streptomyces*, and *Staphylococcus*.

If a eukaryotic expression vector is employed, then the appropriate host cell can be any eukaryotic cell capable of expressing the cloned sequence. Preferably, eukaryotic cells are cells of higher eukaryotes. Suitable eukaryotic cells include, but are not limited
20 to, non-human mammalian tissue culture cells and human tissue culture cells. Preferred host cells include, but are not limited to, insect cells, HeLa cells, Chinese hamster ovary cells (CHO cells), African green monkey kidney cells (COS cells), human 293 cells, and murine 3T3 fibroblasts. Propagation of such cells in cell culture has become a routine procedure (see, *Tissue Culture*, Academic Press, Kruse and Patterson, eds. (1973), which
25 is incorporated herein by reference in its entirety).

In addition, a yeast host may be employed as a host cell. Preferred yeast cells include, but are not limited to, the genera *Saccharomyces*, *Pichia*, and *Kluveromyces*. Preferred yeast hosts are *S. cerevisiae* and *P. pastoris*. Preferred yeast vectors can contain an origin of replication sequence from a 2T yeast plasmid, an autonomously replication
30 sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Shuttle vectors for replication in both yeast and *E. coli* are also included herein.

Alternatively, insect cells may be used as host cells. In a preferred embodiment, the polypeptides of the invention are expressed using a baculovirus expression system (see, Luckow *et al.*, *Bio/Technology*, **1988**, 6, 47, Baculovirus Expression Vectors: A Laboratory Manual, O'Rielly *et al.* (Eds.), W.H. Freeman and Company, New York, 5 1992, and U.S. Patent 4,879,236, each of which is incorporated herein by reference in its entirety). In addition, the MAXBAC™ complete baculovirus expression system (Invitrogen) and the BAC-TO-BAC™ System (Life Technologies) can, for example, be used for production in insect cells.

Also comprehended by the present invention are antibodies (e.g., monoclonal 10 and polyclonal antibodies, single chain antibodies, chimeric antibodies, bifunctional/bispecific antibodies, humanized antibodies, human antibodies, and complementary determining region (CDR)-grafted antibodies, including compounds that include CDR sequences which specifically recognize a polypeptide of the invention) which bind to MLK7, preferably specific for MLK7, or fragments thereof. Antibody 15 fragments, including Fab, Fab', F(ab')₂, and Fv, are also provided by the invention. The term "specific for," when used to describe antibodies of the invention, indicates that the variable regions of the antibodies of the invention recognize and bind MLK7 polypeptides exclusively (i.e., are able to distinguish MLK7 polypeptide from other known MLK polypeptides by virtue of measurable differences in binding affinity, despite the possible 20 existence of localized sequence identity, homology, or similarity between MLK7 and such polypeptides). It will be understood that specific antibodies may also interact with other proteins (for example, *S. aureus* protein A or other antibodies in ELISA techniques) through interactions with sequences outside the variable region of the antibodies, and, in particular, in the constant region of the molecule. Screening assays to determine binding 25 specificity of an antibody of the invention are well known and routinely practiced in the art (see Harlow *et al.* (Eds.), *Antibodies A Laboratory Manual*; Cold Spring Harbor Laboratory; Cold Spring Harbor, NY (1988), Chapter 6).

Antibodies of the invention are useful for therapeutic purposes (by modulating activity of MLK7), diagnostic purposes to detect or quantitate MLK7, and purification of 30 MLK7. Kits comprising an antibody of the invention for any of the purposes described herein are also contemplated. In general, a kit of the invention also includes a control antigen for which the antibody is immunospecific.

It is contemplated that in other human disease states or abnormal conditions, preventing the expression of, or inhibiting the activity of, MLK7 will be useful in treating disease states or abnormal conditions. It is contemplated that antisense therapy or gene therapy may be applied to regulate the expression or activity of an MLK7 polypeptide.

5 The present invention is also directed to kits, including pharmaceutical kits. The kits can comprise any of the polynucleotides described above, any of the polypeptides described above, or any antibody which binds to a polypeptide of the invention as described above, as well as a negative control. The kit preferably comprises additional components, such as, for example, instructions, solid support, reagents helpful for
10 quantification, and the like.

Another aspect of the present invention is directed to compositions, including pharmaceutical compositions, comprising any of the polynucleotides or recombinant expression vectors described above and an acceptable carrier or diluent. Preferably, the carrier or diluent is pharmaceutically acceptable. Suitable carriers are described in the
15 most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field, which is incorporated herein by reference in its entirety. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils can also be used. The formulations are
20 sterilized by commonly used techniques.

Specific binding compounds, including natural ligands and synthetic compounds, can be identified or developed using isolated or recombinant MLK7 products, MLK7 variants, or preferably, cells expressing such products. Binding compounds are useful for purifying MLK7 products and detection or quantification of MLK7 products in fluid and
25 tissue samples using known immunological procedures. Binding compounds are also manifestly useful in modulating (i.e., blocking, inhibiting or stimulating) biological activities of MLK7, especially those activities involved in the JNK stress-activated kinase pathway. Thus, compounds that bind MLK7 polynucleotides or polypeptides can be identified.

30 The polynucleotide and polypeptide sequence information provided by the present invention also makes possible identification of binding compounds with which an MLK7 polypeptide or polynucleotide will interact. Methods to identify binding

compounds include solution assays, *in vitro* assays wherein an MLK7 polypeptide is immobilized, and cell-based assays. Identification of binding compounds of MLK7 polypeptides provides candidates for therapeutic or prophylactic intervention in pathologies associated with normal and aberrant MLK7 biological activity.

5 The invention includes several assay systems for identifying MLK7 binding compounds. In solution assays, methods of the invention comprise the steps of i) contacting an MLK7 polypeptide with one or more candidate binding compounds and ii) identifying the compounds that bind to the MLK7 polypeptide. Identification of the compounds that bind the MLK7 polypeptide can be achieved by isolating the MLK7
10 polypeptide/binding compound complex, and separating the binding compound from the MLK7 polypeptide. An additional step of characterizing the physical, biological, and/or biochemical properties of the binding compound is also contemplated in other embodiments of the invention. In one aspect, the MLK7 polypeptide/binding compound complex can be isolated using an antibody immunospecific for either the MLK7
15 polypeptide or the candidate binding compound.

 In still other embodiments, either the MLK7 polypeptide or the candidate binding compound comprises a label or tag that facilitates its isolation, and methods of the invention to identify binding compounds include a step of isolating the MLK7 polypeptide/binding compound complex through interaction with the label or tag. An
20 exemplary tag of this type is a poly-histidine sequence, generally around six histidine residues, that permits isolation of a compound so labeled using nickel chelation. Other labels and tags, such as the FLAG® tag (Eastman Kodak, Rochester, NY), well known and routinely used in the art, are embraced by the invention.

 In one variation of an *in vitro* assay, the invention provides a method comprising
25 the steps of i) contacting an immobilized MLK7 polypeptide with a candidate binding compound and ii) detecting binding of the candidate compound to the MLK7 polypeptide. In an alternative embodiment, the candidate binding compound is immobilized and binding of MLK7 is detected. Immobilization can be accomplished using any of the methods well known in the art, including covalent bonding to a support, a bead, or a
30 chromatographic resin, as well as non-covalent, high affinity interactions such as antibody binding, or use of streptavidin/biotin binding wherein the immobilized compound includes a biotin moiety. Detection of binding can be accomplished i) using a radioactive

label on the compound that is not immobilized, ii) using of a fluorescent label on the non-immobilized compound, iii) using an antibody immunospecific for the non-immobilized compound, or iv) using a label on the non-immobilized compound that excites a fluorescent support to which the immobilized compound is attached, as well as other
5 techniques well known and routinely practiced in the art.

The invention also provides cell-based assays to identify binding compounds of a MLK7 polypeptide. In one embodiment, the invention provides a method comprising the steps of contacting an MLK7 polypeptide expressed on the surface of a cell with a candidate binding compound and detecting binding of the candidate binding compound to
10 the MLK7 polypeptide. In a preferred embodiment, the detection comprises detecting a calcium flux or other physiological event in the cell caused by the binding of the compound.

Agents that modulate (i.e., increase, decrease, or block) MLK7 activity or expression can be identified by incubating a putative modulator with a cell containing a
15 MLK7 polypeptide or polynucleotide and determining the effect of the putative modulator on MLK7 activity or expression. The selectivity of a compound that modulates the activity of MLK7 can be evaluated by comparing its effects on MLK7 to its effect on other MLK compounds. Selective modulators can include, for example, antibodies and other proteins, peptides, or organic molecules which specifically bind to an MLK7
20 polypeptide or an MLK7-encoding polynucleotide. Modulators of MLK7 activity will be therapeutically useful in treatment of diseases and physiological conditions in which normal or aberrant MLK7 activity is involved. Compounds identified as a result of the methods described herein (binding compounds and/or modulators of MLK7 polynucleotides and/or polypeptides) can be used in the treatment of diseases and
25 conditions related to the JNK stress-activated kinase pathway, including, but not limited to, neurodegenerative diseases or conditions and inflammatory conditions or diseases. Representative diseases or conditions include, but are not limited to, Alzheimer's disease (Zhu *et al.*, *J. Neurochem.*, **2001**, 76, 435-441), Huntington's disease (Yasuda *et al.*, *Genes Cells*, **1999**, 4, 743-756; Liu, *J. Biol. Chem.*, **1998**, 273, 28873-28877), multiple sclerosis
30 (Bonetti *et al.*, *Am. J. Pathol.*, **1999**, 155, 1433-1438), neuropathic pain (Rausch *et al.*, *Neuroscience*, **2000**, 101, 767-777), Parkinson's disease (Saporito *et al.*, *J. Neurochem.*, **2000**, 75, 1200-1208), traumatic brain injury (Raghupathi *et al.*, *J. Neurotrauma*, **2000**,

17, 927-938), rheumatoid arthritis (Schett *et al.*, *Arthritis Rheum.*, **2000**, 43, 2501-2512), ethanol exposure during neural development (McAlhany *et al.*, *Brain Res. Dev.*, **2000**, 119, 209-216), pancreatitis (Hofken *et al.*, *Biochem. Biophys. Res. Commun.*, **2000**, 276, 680-685), polycystic kidney disease (Arnould *et al.*, *J. Biol. Chem.*, **1998**, 273, 6013-6018), cardiovascular ischaemia (Force *et al.*, *Circ. Res.*, **1996**, 78, 947-953), hypertension (Xu *et al.*, *J. Clin. Invest.*, **1996**, 97, 508-514), chemotherapy-induced endothelial apoptosis, glioblastoma (Potapova *et al.*, *J. Biol. Chem.*, **2000**, 275, 24767-24775), tamoxifen-resistant breast tumors (Schiff *et al.*, *J. Natl. Cancer Inst.*, **2000**, 92, 1926-1934), endothelial cell effects resulting from high glucose levels in diabetes mellitus (Ho *et al.*, *Circulation*, **2000**, 101, 2618-2624), and the like. MLK7 polynucleotides and polypeptides, as well as MLK7 modulators, can also be used in diagnostic assays for such diseases or conditions.

Another aspect of the present invention is directed to methods of identifying compounds that bind to either MLK7 or polynucleotides encoding MLK7, comprising contacting MLK7, or a polynucleotide encoding the same, with a compound, and determining whether the compound binds MLK7, or a nucleic acid molecule encoding the same. Binding can be determined by binding assays which are well known to the skilled artisan, including, but not limited to, gel-shift assays, Western blots, radiolabeled competition assay, phage-based expression cloning, co-fractionation by chromatography, co-precipitation, cross linking, interaction trap/two-hybrid analysis, southwestern analysis, ELISA, and the like, which are described in, for example, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, NY, which is incorporated herein by reference in its entirety. The compounds to be screened include (which may include compounds which are suspected to bind MLK7, or a nucleic acid molecule encoding the same), but are not limited to, extracellular, intracellular, biologic or chemical origin. The MLK7 polypeptide or polynucleotide employed in such a test may either be free in solution, attached to a solid support, borne on a cell surface or located intracellularly or associated with a portion of a cell. One skilled in the art can, for example, measure the formation of complexes between MLK7 and the compound being tested. Alternatively, one skilled in the art can examine the diminution in complex formation between MLK7 and its substrate caused by the compound being tested.

Another aspect of the present invention is directed to methods of identifying compounds that modulate (i.e., increase or decrease) activity of MLK7 comprising contacting MLK7 with a compound, and determining whether the compound modifies activity of MLK7. The activity in the presence of the test compound is measured to the activity in the absence of the test compound. The activity of MLK7 polypeptides of the invention can be determined by, for example, examining the kinase activity or ability to phosphorylate particular substrates including, but not limited to, MKK4 and MKK7.

In preferred embodiments of the invention, methods of screening for compounds that modulate MLK7 activity comprise contacting test compounds with MLK7 and assaying for the presence of a complex between the compound and MLK7. In such assays, the ligand is typically labeled. After suitable incubation, free ligand is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of the particular compound to bind to MLK7.

Candidate modulators contemplated by the invention include compounds selected from libraries of either potential activators or potential inhibitors. There are a number of different libraries used for the identification of small molecule modulators, including: i) chemical libraries, ii) natural product libraries, and iii) combinatorial libraries comprised of random peptides, oligonucleotides or small organic molecules. Chemical libraries consist of random chemical structures, some of which are analogs of known compounds or analogs of compounds that have been identified as "hits" or "leads" in other drug discovery screens, some of which are derived from natural products, and some of which arise from non-directed synthetic organic chemistry. Natural product libraries are collections of microorganisms, animals, plants, or marine organisms which are used to create mixtures for screening by: i) fermentation and extraction of broths from soil, plant or marine microorganisms or ii) extraction of plants or marine organisms. Natural product libraries include polyketides, non-ribosomal peptides, and variants (non-naturally occurring) thereof. Combinatorial libraries are composed of large numbers of peptides, oligonucleotides, or organic compounds as a mixture. These libraries are relatively easy to prepare by traditional automated synthesis methods, PCR, cloning, or proprietary synthetic methods. Of particular interest are non-peptide combinatorial libraries. Other libraries of interest include peptide, protein, peptidomimetic, multiparallel synthetic collection, recombinatorial, and polypeptide libraries. Identification of

modulators through use of the various libraries described herein permits modification of the candidate “hit” (or “lead”) to optimize the capacity of the “hit” to modulate activity.

Other assays can be used to identify compounds that bind to or modulate the activity of an MLK7 polypeptide, including assays that identify ligands of the target protein through measuring direct binding of test ligands to the target protein, as well as assays that identify ligands of target proteins through affinity ultrafiltration with ion spray mass spectroscopy/HPLC methods or other physical and analytical methods. Alternatively, such binding interactions are evaluated indirectly using the yeast two-hybrid system described in Fields *et al.*, *Nature*, **1989**, 340, 245-246, and Fields *et al.*, *Trends in Genetics*, **1994**, 10, 286-292, both of which are incorporated herein by reference in their entirety.

In a particular embodiment, the novel compounds identified by the screening methods according to the invention are low molecular weight organic molecules, in which case a composition or pharmaceutical composition can be prepared thereof for oral intake, such as in tablets. The compositions, or pharmaceutical compositions, comprising the nucleic acid molecules, vectors, polypeptides, antibodies and compounds identified by the screening methods described herein, can be prepared for any route of administration including, but not limited to, oral, intravenous, cutaneous, subcutaneous, nasal, intramuscular or intraperitoneal. The nature of the carrier or other ingredients will depend on the specific route of administration and particular embodiment of the invention to be administered. Examples of techniques and protocols that are useful in this context are, inter alia, found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A (ed.), 1980, which is incorporated herein by reference in its entirety.

The dosage of these low molecular weight compounds will depend on the disease state or condition to be treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. For treating human or animals, between approximately 0.5 mg/kg of body weight to 500 mg/kg of body weight, between approximately 1.0 mg/kg of body weight to 100 mg/kg of body weight, or between approximately 10 mg/kg of body weight to 50 mg/kg of body weight of the compound can be administered. Therapy is typically administered at lower dosages and is continued until the desired therapeutic outcome is observed. The proper dosage depends on various factors such as the type of disease being treated, the particular

composition being used and the size and physiological condition of the patient. Therapeutically effective doses for the compounds described herein can be estimated initially from cell culture and animal models. For example, a dose can be formulated in animal models to achieve a circulating concentration range that initially takes into account
5 the IC_{50} as determined in cell culture assays. The animal model data can be used to more accurately determine useful doses in humans.

Another aspect of the present invention is directed to heterodimers containing MLK7 and any of the MLK proteins. There have been reports of leucine zipper-dependent heterodimers between family members. For example, MLK3 can co-immunoprecipitate
10 co-expressed MLK2 (Leung *et al.*, *J. Biol. Chem.*, **1998**, 273, 32408-32415). Co-expression in the same cell can result in heterodimers as well as homodimers of these proteins. Heterodimers could have a unique activity compared to the homodimers and represent an additional target for screening in the methods described above.

In order that the invention disclosed herein may be more efficiently understood,
15 examples are provided below. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting to the invention in any manner. Throughout these examples, molecular cloning reactions, and other standard recombinant DNA techniques, were carried out according to methods described in
20 *Maniatis et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press (1989) (hereinafter, "*Maniatis et al.*"), using commercially available enzymes, except where otherwise noted. Examples 1 and 2 below are actual. Example 3 is prophetic.

EXAMPLES

25 **Example 1: Isolation of Polynucleotide Encoding Human MLK7**

A. Isolation of cDNA Encoding Human MLK7

A survey of recently added human genomic DNA sequences revealed a putative seventh member of the mixed lineage kinase family. This human kinase, designated MLK7, was found in Genbank Accession No. AL133380, a completed genomic sequence
30 mapping to q42.2-q43 of chromosome 1. Alignment of the putative kinase domain places this protein in the previously defined MLK1-3 subfamily, with 76% homology to its closest relatives, MLKs 1 and 3. The putative leucine zipper region shown in Figure 2

would also place this protein in that subfamily, having four putative alpha helical repeats separated by only a 6 amino acid stretch. The full-length protein has the highest homology with MLK1 (44% identity), which was sufficient to allow tentative identification of both the putative N- and C-termini of the protein. Only two recently-deposited MLK7 ESTs
5 were present in Genbank (Accession No. AW813675 from stomach and AW408639 from lymph tissue (germinal center B cells)), requiring additional proof that this genomic region was expressed and not a pseudogene. Clones containing part of the cDNA encoding MLK7 were obtained using PCR screening of pooled human adrenal gland, adult brain, kidney, liver, lymph node, pancreas, prostate, and stomach cDNA libraries
10 (Edge Biosystems). The PCR primers used to screen the libraries were 5'-ATGAAAGAATGCTGGCAACAAGACCCTC-3' (SEQ ID NO:4) and 5'-AGGTAAACTGATTCGATGTCCATCTTTG-3' (SEQ ID NO:5). One partial cDNA was isolated repeatedly, encoding a middle portion of the cDNA. Other clones contained genomic sequence or were unrelated. These data are consistent with this cDNA being of
15 very low abundance in these libraries. This sequence was then extended on the 5' and 3' ends by PCR based on homology with MLK1. Extension by 5' RACE was also attempted, but was unsuccessful due to the low abundance and exceptionally high GC content of this cDNA.

The full length human MLK7 cDNA was assembled into the pcDNA6-V5His
20 plasmid (Invitrogen). The plasmid contains the full-length human cDNA (without the stop codon) in frame with a C-terminal epitope and affinity purification fusion partner and is identified as pcDNA6-hMLK7. This plasmid, pcDNA6-hMLK7, was submitted for deposit with American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209, on 24 May 2001 under Accession No. ATCC. The full length cDNA and
25 deduced amino acid sequences of human MLK7 are provided in SEQ ID NO:1 and 2, respectively.

B. Structural Comparison to Members of the Mixed Lineage Kinase Family

The kinase domains of the six members of the mixed lineage kinase family are
30 shown aligned to the putative kinase domain of MLK7 (Figure 1). Amino acids that are conserved in tyrosine kinases, serine/threonine kinases or both are shown as designated (consensus amino acids from Hanks *et al.*, *Meth. Enzymol.*, **1991**, 200, 38-62). MLK7,

like other members of the mixed lineage kinase family, shares homology throughout most of the kinase domain with tyrosine kinases. The presence of lysine in the sequence HRDLKSRN (SEQ ID NO:3) in the catalytic domain of subdomain VIb suggests that, like other members of the MLK family, this would catalyze serine/threonine phosphorylation. Alignment of these kinase domains using the Clustal method (with PAM250 residue weight table) in the MegAlign program (Lasergene DNASTar) predicts MLK7 to be a member of the MLK1-3 subfamily (Figure 2). The percent homology among the kinase domains of the MLK family is shown in Table 1.

Table 1

Kinase Domain	MLK1	MLK2	MLK3	MLK7	DLK	LZK	MLK6
MLK1	-----	75.4%	77.7%	75.4%	42.9%	41.7%	42.0%
MLK2	-----	-----	76.5%	73.1%	44.6%	42.5%	41.6%
MLK3	-----	-----	-----	76.2%	42.5%	42.5%	40.0%
MLK7	-----	-----	-----	-----	38.3%	39.2%	40.4%
DLK	-----	-----	-----	-----	-----	87.1%	37.1%
LZK	-----	-----	-----	-----	-----	-----	35.4%
MLK6	-----	-----	-----	-----	-----	-----	-----

The full-length MLK7 protein shows weaker homology (using the same analysis as described above) to the family than that of the kinase domain (Table 2).

Table 2

Full Length	MLK1	MLK2	MLK3	MLK7	DLK	LZK	MLK6α
MLK1	-----	49.0%	43.6%	43.1%	22.2%	19.4%	20.1%
MLK2	-----	-----	44.3%	41.8%	23.6%	19.7%	19.6%
MLK3	-----	-----	-----	44.9%	22.7%	20.3%	18.8%
MLK7	-----	-----	-----	-----	19.1%	18.6%	18.5%
DLK	-----	-----	-----	-----	-----	50.5%	18.8%
LZK	-----	-----	-----	-----	-----	-----	17.6%
MLK6α	-----	-----	-----	-----	-----	-----	-----

There is a putative SH3 domain N-terminal to the kinase domain. There is also a putative dual leucine zipper region C-terminal to the kinase domain, followed by a small region comprised of a preponderance of basic amino acids similar to the nuclear localization signals from several proteins. The putative leucine zipper region (Figure 4) also places MLK7 in the MLK1-3 subfamily, having four putative alpha-helical repeats separated by

only a six amino acid stretch. The full-length protein has high homology with MLK1 (43.1%), which was sufficient to allow identification of both the putative N- and C-termini of the protein. This homology was used to clone the full-length MLK7 cDNA. The domain structure of MLK7 is shown in Figure 3. The sequence of the leucine zipper region of MLK7 is shown and compared to other MLK proteins in Figure 4.

C. Expression Profile of hMLK7

The tissue distribution of hMLK7 was evaluated using the Rapid Scan™ gene expression panel (Origene). Rapid Scan™ is a PCR based approach for using first strand cDNAs derived from 24 different tissues to generate an expression profile in a 96 well plate format. These 24 cDNAs are arrayed in 4-log dilutions enabling amplification and visualization within the linear range of PCR. The protocol requires PCR reactions to be carried out in a 96-well thermal cycler, using gene specific primer pairs and visualizing the amplification on agarose gels. Table 3 shows qualitative data obtained from duplicate screening of the panel.

Table 3

	<u>Tissue</u>	<u>Edge Primer Pair</u>
	brain	++
	heart	+
20	kidney	++
	spleen	-
	liver	++
	colon	++
	lung	++
25	small intestine	+
	muscle	+
	stomach	++
	testis	++
	placenta	-
30	salivary gland	++
	thyroid gland	+
	adrenal gland	++
	pancreas	++
	ovary	+
35	uterus	-
	prostate	+
	skin	-
	peripheral blood leukocytes	+
	bone marrow	+
40	fetal brain	+
	fetal liver	+

These experiments utilized the primer pair ATGAAAGAATGCTGGCAACAAGACC CTC (SEQ ID NO:4) and AGGTAAACTGATTCGATGTCCATCTTTG (SEQ ID NO:5). A score of ++ in this MLK7 analysis indicated a visible PCR product at the two highest cDNA concentrations. More than half of the tissue screen gave little or no detectable product, which suggested that MLK7 mRNA is not in high abundance in any tissue.

Example 2: Recombinant Expression of Biologically Active MLK7

A. JNK Pathway Activation by Full-Length MLK7 Expression in Mammalian Cells

MLK family members overexpressed in mammalian cells activate the cJun-NH2-terminal kinase (JNK) pathway (Hirai *et al.*, 1997, *J. Biol. Chem.*, 272, 15167; Merritt *et al.*, 1999, *J. Biol. Chem.*, 274, 10195). To determine whether full-length MLK7 does the same, CHO cells were co-transfected with plasmids that drive constitutive expression of MLK7 and constitutive expression of JNK1, determining the JNK activity in lysates by ELISA.

The coding region for full-length MLK7 was inserted into the mammalian expression vector pcDNA6V5/HisA (Invitrogen) in-frame with the C-terminal V5 epitope and His₆ affinity purification fusion partner. Each plasmid was co-transfected with a JNK1 expression vector into CHO cells plated in six-well dishes, using the reagent LipofectaminePLUS (Life Technologies) according to the manufacturer's recommendations. Duplicate cultures were transfected for each condition. As a positive control, an expression vector for MLK3 was substituted for MLK7; as a negative control, an empty vector (no protein coding region) was substituted. Two days after transfection, the cells were treated for six hours with CEP11004 at three concentrations (250 nM, 1 μM, and 5 μM), or with DMSO vehicle control, in reduced serum medium (DMEM plus 0.5% fetal bovine serum). At the end of this treatment the cells were washed twice with ice-cold phosphate-buffered saline and lysed with 0.1 ml per well of 1% (v/v) Triton X-100, 20 mM Tris pH 7.6, 50 mM NaCl, protease inhibitor cocktail (1 mM Pefabloc SC, 10 μM E-64, 10 μM leupeptin, 10 μM pepstatin A, 1 mM EDTA), and phosphatase inhibitor cocktail (25 mM β-glycerophosphate, 2 mM activated sodium orthovanadate). The JNK activity in transfected cell lysates was measured using an ELISA-based format as described for receptor tyrosine kinases (Angeles *et al.*, 1996, *Anal. Biochem.*, 236, 49-55). The NH2-terminus of the JNK substrate cJun (residues 1-79) was expressed in *E. coli*

as a GST fusion protein and purified by standard glutathione affinity methods. Smith *et al.*, *Gene*, **1988**, 67, 31-40. A 96-well microtiter plate (FluoroNUNC Maxisorp) was coated with substrate solution (10 µg/ml GST-cJun in Tris-buffered saline), then washed several times with wash buffer (0.05% Tween-20 in Tris-buffered saline). The plate was
5 blocked with block buffer (3% BSA and 0.2% Tween-20 in Tris-buffered saline) for one hour at 37C, then washed several times with wash buffer. Concentrated assay buffer was then added to each well, followed by lysate corresponding to equal protein for each sample. The kinase reaction was initiated by adding ATP to 50 µM and incubating at 37C for 15 minutes. The final assay mixture (100 µl per well) contained 20 mM HEPES, pH
10 7.4, 0.02% BSA, 20 mM MgCl₂, 2 mM DTT, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM activated sodium orthovanadate, and 50 µM ATP. The kinase reaction was terminated by addition of EDTA to 83 mM, and the plate was washed several times with wash buffer. The phosphorylated product was detected by incubation with anti-phospho-cJun (Ser73) antibody (NEB 9164, 1:5000 in block buffer), washing, incubation with goat
15 anti-rabbit IgG-alkaline phosphatase conjugate (Southern Biotechnology Associates #4050-04, 1:2500 dilution in block buffer), washing, and reaction with the fluorogenic alkaline phosphatase substrate 4-methylumbelliferyl phosphate (0.02 mg/ml in 1.0 M diethanolamine, pH 9.6, 5 mM MgCl₂) for 30 minutes at 37C. The reaction was stopped with 1.0 M dibasic sodium phosphate and the product measured in a microplate reader
20 (Cytofluor, 360 nm excitation, 460 nm emission). A preliminary ELISA was performed to determine the maximum amount of lysate protein that yielded a linear signal. In this experiment, the MLK3 was the limiting sample, maintaining linearity only up to 80 ng of lysate protein. Assays were then performed on 80 ng of each lysate.

Expression of MLK7 was confirmed by probing Western blots of cell lysates
25 with antibody to the V5 epitope tag (data not shown). MLK7 appeared at an apparent mobility corresponding to 120 kDa (expected 117.4 kDa). Expression of MLK7 elevated JNK activity over the vector control (Figure 5). Treatment of MLK7-transfected cells with CEP11004 partially reduced JNK activity, just as this treatment does to MLK3-transfected cells. These data confirm that MLK7 activates the JNK pathway in
30 mammalian cells, just like other MLK family members, and just as predicted by the *in vitro* data.

B. *In Vitro* Activity of Recombinant GST-MLK7_{KD} and GST-MLK7_{KD/LZ}

A portion of the human MLK7 protein encoding either the kinase domain (amino acids 93-416) or the kinase domain/leucine zipper (amino acids 93-489) were expressed with an NH₂-terminal GST fusion partner in Sf21 insect cells using standard methods (e.g. Meyer *et al.*, *J. Neurochem.*, **1994**, 62, 825-833) and purified using glutathione affinity purification methods (Smith *et al.*, *Gene*, **1988**, 67, 31-40). The kinase activities of baculoviral human GST-MLK7_{KD} and GST-MLK7_{KD/LZ} were demonstrated using three different substrates, kinase-dead forms of GST-MKK4 and GST-MKK7, and myelin basic protein (MBP). Additionally, activation of the JNK pathway activation by the two GST-MLK7 forms was shown *in vitro*. Both GST-MLK7_{KD} and GST-MLK7_{KD/LZ} were able to phosphorylate inactivated MKK4 and, as a consequence of this phosphorylation event, MKK4 was activated and was able to phosphorylate its protein substrate GST-JNK1 β 1(K55A). Similar results were obtained when inactivated MKK7 was used as the downstream target of MLK7.

C. *Phosphorylation of Myelin Basic Protein*

The kinase activity of baculoviral GST-MLK7_{KD} and GST-MLK7_{KD/LZ} were assessed separately using the Millipore Multiscreen TCA "in-plate" format as described for protein kinase C (Pitt *et al.*, *J. Biomol. Screening*, **1996**, 1: 47-51). Briefly, each 50- μ l assay mixture contained 20 mM Hepes, pH 7.2, 5 mM EGTA, 15 mM MgCl₂, 1 mM DTT, 25 mM β -glycerophosphate, 100 μ M ATP, 0.25 μ Ci [γ -³²P]ATP, 500 μ g/ml myelin basic protein (UBI #13-104), and 0.1-5 μ g/ml of baculoviral GST-MLK7_{KD} (or GST-MLK7_{KD/LZ}). Samples were incubated for 15 minutes at 37C. The reaction was stopped by adding ice cold 50% TCA and the proteins were allowed to precipitate for 30 minutes at 4C. The plates were then washed with ice cold 25% TCA. Supermix scintillation cocktail was added, and the plates were allowed to equilibrate for 1-2 hours prior to counting using the Wallac MicroBeta 1450 PLUS scintillation counter.

Both GST-MLK7_{KD} and GST-MLK7_{KD/LZ} were able to phosphorylate myelin basic protein, a generic protein kinase substrate (Figure 6). The amount of phosphorylated product formed (reported as radioactive counts) was dependent on the amount of enzyme used in the assay. Baculoviral GST-MLK3_{KD} was used as the control enzyme in this analysis to show phosphorylation of myelin basic protein by a member of the mixed

lineage kinase family.

D. Phosphorylation of kinase-inactive GST-MKK4 and GST-MKK7

Kinase-inactive versions of the two known MAPKK proteins in the JNK
5 pathway, MKK4 and MKK7, are expressed as full-length proteins with an N-terminal
GST fusion partner in bacteria and purified using standard glutathione affinity
purification protocols. Smith *et al.*, *Gene*, **1988**, 67, 31-40. These are used to assess the
activity of the MLK7 kinase domain.

Assessment of the kinase activities of baculoviral human GST-MLK7_{KD} and
10 GST-MLK7_{KD/LZ} was carried out using a modification of the ELISA-based assay
described for receptor tyrosine kinases (Angeles *et al.*, *Anal. Biochem.*, **1996**, 236, 49-55).
The 96-well microtiter plate (FluoroNUNC Maxisorp) was coated overnight at 4C with
20 µg/ml substrate solution (GST-MKK4 (K113A) or GST-MKK7β1(K149A) in Tris-
buffered saline). The plate was then blocked with block buffer (3% BSA in Tris-buffered
15 saline containing 0.05% Tween-20) for 1 hour at 37C. The 100-µl assay mixture
containing 20 mM Hepes, pH 7.2, 50 µM ATP, 15 mM MgCl₂, 1 mM DTT, 5 mM
EGTA, and 25 mM β-glycerophosphate was then added to the plate. The kinase reaction
was initiated by adding 10-100 ng/ml of recombinant human baculoviral GST-MLK7_{KD}
or GST-MLK7_{KD/LZ} and the plate was allowed to incubate at 37C for 15 minutes. The
20 phosphorylated product was detected by adding a phospho-threonine antibody (NEB #
226-1) at a dilution of 1:5000 in block buffer. After a 1-hour incubation at 37C, 100 µl of
europium-N1 labeled anti-rabbit antibody (Wallac # AD0105) at 1:20000 dilution in
block buffer was added, and the plate was incubated at 37C for another hour. A low pH
enhancement solution (Wallac # 1244-105) was then added and the plate was gently
25 agitated for 5 minutes at room temperature. The fluorescence of the resulting solution was
measured using the Victor 2 Multilabel Counter (Model # 1420-018).

Members of the mixed lineage kinase subfamily of serine/threonine protein
kinases have been shown to phosphorylate and activate MKK4 (Rana *et al.*, *J. Biol.*
Chem., **1996**, 271, 19025-19028; Hira *et al.*, *J. Biol. Chem.*, **1997**, 272, 15167-15173) as
30 well as MKK7 (Hira *et al.*, *J. Biol. Chem.*, **1998**, 273, 7406-7412; Merritt *et. al.*, *J. Biol.*
Chem., **1999**, 274, 10195-10202). These two MKK proteins were, therefore, utilized to
examine the enzymatic activities of baculoviral GST-MLK7_{KD} and GST-MLK7_{KD/LZ}. As

shown in Figure 7, both forms of baculoviral GST-MLK7 catalyzed the phosphorylation of the two MKK protein substrates, GST-MKK4(K113A) and GST-MKK7(K149A). Product formation (reported as fluorescence units) was proportional to the concentration of enzyme used. In terms of specific activity, both GST-MLK7 forms were less active
5 than GST-MLK3KD (control enzyme), with GST-MLK7_{KD} displaying about 2x higher activity than GST-MLK7_{KD/LZ}. Consistent with other MLK reactions, the activities of both GST-MLK7_{KD} and GST-MLK7_{KD/LZ} were inhibited by EDTA, conferring the requirement for metal ions (Mg⁺²) (data not shown). The ELISA-based assay described here could potentially be used for identifying inhibitors of MLK7.

10

E. In Vitro Activation of JNK Pathway by GST-MLK7_{KD} and GST-MLK7_{KD/LZ}

Since both forms of MLK7 were able to phosphorylate MKK4 and MKK7 *in vitro* (Figure 7), MLK7, like the other members of the MLK family, can activate the JNK/SAPK pathway (Tibbles *et al.*, *EMBO J.*, **1996**, 15, 7026-7035; Hira *et al.*, *J. Biol. Chem.*, **1997**, 272, 15167-15173; Hira *et al.*, *J. Biol. Chem.*, **1998**, 273, 7406-7412). To
15 demonstrate that MLK7 is a direct activator of the MKKs, an *in vitro* kinase cascade reaction was performed. This system utilized recombinant GST-MLK7, inactivated MKK4/7, and kinase-inactive GST-JNK1 β 1(K55A) and coupled the MLK7-catalyzed phosphorylation/ activation of MKK4/7 to the phosphorylation of inactive GST-JNK1 by
20 activated MKK4/7.

The assay was performed by first incubating inactivated MKK4/7 with GST-MLK7_{KD} or GST-MLK7_{KD/LZ} in a kinase reaction mixture containing 20 mM Hepes, pH 7.2, 50 μ M ATP, 15 mM MgCl₂, 1 mM DTT, 5 mM EGTA, and 25 mM β -glycerophosphate, for 15 minutes at 37C. GST-MLK3_{KD} was utilized as the control
25 enzyme. An aliquot of this mixture (preactivated MKK4/7; Product 1) was added to a polypropylene, non-treated 96-well microtiter plate containing the MKK4/7 assay mixture. The MKK4/7 reaction utilized the same assay buffer as the GST-MLK7 reaction except for the addition of kinase-dead GST-JNK1 β 1(K55A). Reaction was allowed to proceed for 15 minutes at 37C then stopped with 100 mM EDTA (Product 2). Analysis of
30 phosphorylated JNK1 was performed by transferring Product 2 into a FluoroNUNC Maxisorp 96-well microtiter plate. Following a 1 hour incubation at 37C, the plate was blocked with 3% BSA in TBST. The detection antibody, phospho-specific JNK antibody

(Promega # V7931/2), was added and the plate was incubated for an hour at 37C. Addition of the secondary antibody, Eu-N1 labeled anti-rabbit antibody (Wallac # AD0105) immediately followed, and the plate was incubated at 37C for another hour. A low pH enhancement solution (Wallac # 1244-105) was then added, and the fluorescence
5 of the resulting solution was measured using the Victor 2 Multilabel Counter (Model # 1420-018).

As shown in Figure 8 (bars 7 and 8), both GST-MLK7_{KD} and GST-MLK7_{KD/LZ} were able to phosphorylate MKK4 and as a consequence of this phosphorylation event, MKK4 was activated and was able to phosphorylate its protein substrate GST-
10 JNK1 β 1(K55A). Similar results were observed with MKK7 as the downstream target of MLK7 (Figure 8, bars 10 and 11). Relative to MLK3 (control enzyme; see inset in Figure 8), the MLK7_{KD}- and MLK7_{KD/LZ}-driven JNK activation signals were weaker, reflecting the observed lower specific activities of these enzyme preparations (Figures 6 and 7). Overall, the *in vitro* data indicate that MLK7 can phosphorylate and activate MKK4/7,
15 thereby acting as a MAPKK kinase in the JNK/SAPK pathway.

Example 3: Feasibility of using GST-MLK7_{KD} enzyme assays to screen for inhibitors

The MLK enzymatic activity is further evaluated in the MBP phosphorylation assay and apparent Michaelis constants (K_m) for ATP and MBP can be determined.

20 The apparent K_m values for ATP and MBP are expected to be about 103 μ M and 21 μ M, respectively. These values can be compared to values obtained for MLK1-3. Those proteins are also expressed as truncations of either the kinase domain or kinase and leucine zipper domains with N-terminal GST fusion partners.

The initial evaluation of MLK7 activity is performed using the radioactive MBP
25 phosphorylation assay. An ELISA assay is adopted in order to obtain a sufficient signal with lower enzyme concentrations in order to conduct kinetic and inhibition studies.

ELISA Assay Protocol: Assays are performed in 96 well FluoroNunc Maxisorp ELISA plates coated with 10 μ g/ml GST-MKK4 (kinase dead mutant) diluted in Tris buffered saline (TBS). Coating is achieved by allowing the MKK4 substrate to stand in
30 the wells for 16 hours at 4C in a humidified chamber. After coating, excess buffer is aspirated, plates are washed 3 times with TBS containing 0.05% Tween 20 (v/v), and blocked with 3% BSA (w/v) in TBS-T (200 μ l/well) for 1 hour at 37C. All subsequent

incubations are carried out using 100 μ l/well volumes for one hour at 37C in a humidified chamber. After blocking, plates are washed 3 times in TBS-T and TBS, respectively. The kinase reaction is performed in 20 mM HEPES, pH 7.4, 30 μ M ATP, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4 , 5 mM EGTA, and 25 mM β -glycerophosphate (90 μ l/well) and 50 ng/ml GST-MLK7_{KD} (10 μ l/well) for 30 minutes at 37C. As a negative control, 0.5 M EDTA (30 μ l/well) is added to the reaction prior to incubation. Plates are washed 3 times in TBS-T and polyclonal antibody 226.1 (New England Biolabs), which detects phosphorylated MKK4, is added to wells at a 1/5000 dilution in blocking buffer. After incubation, plates are washed and alkaline phosphatase conjugated goat anti-rabbit secondary antibody is added at a 1/2500 dilution in blocking buffer. Following a 1-hour incubation and plate washing, 4-methyl umbelliferyl phosphate (0.2 mg/ml final concentration) is added to wells and allowed to develop for 45 minutes. The reaction is terminated with 0.5 M Na_2HPO_4 and read on a fluorescence plate reader at 360 nm excitation wavelength and 460 nm emission wavelength.

Optimization of ELISA: The ELISA is optimized with regard to substrate preference and coating concentration, primary antibody dilution, and development time for the fluorogenic substrate. Magnesium ion requirements, sensitivity to reductants and DMSO can also be evaluated. Both MKK4 and MKK7 kinase dead mutants are suitable substrates for phosphorylation by GST-MLK7_{KD} and are expected to demonstrate a dose-dependent increase in signal strength with increasing substrate concentration. Subsequent experiments are routinely performed using 20 μ g/ml MKK4 as the substrate. Primary antibody 226.1 (New England BioLabs) dilution is assayed, and under conditions employed for the ELISA, a 1/5000 dilution was determined to be optimal. Two reference kinase inhibitors, K252a and staurosporine, can be examined for their ability to inhibit GST-MLK7_{KD} activity.

The disclosures of each patent, patent application and publication cited or described in this document are hereby incorporated herein by reference, in their entirety.

Various modification of the invention, in addition to those described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Polynucleotide sequence (SEQ ID NO:1)

5	ATG	GCT	TTG	CGG	GGC	GCC	GCG	GGA	GCG	ACC	GAC	ACC	CCG	GTG	42
	Met	Ala	Leu	Arg	Gly	Ala	Ala	Gly	Ala	Thr	Asp	Thr	Pro	Val	
10	TCC	TCG	GCC	GGG	GGA	GCC	CCC	GGC	GGC	TCA	GCG	TCC	TCG	TCG	84
	Ser	Ser	Ala	Gly	Gly	Ala	Pro	Gly	Gly	Ser	Ala	Ser	Ser	Ser	
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	Trp	Ala	Ala	Leu	Tyr	Asp	Tyr	Glu	Ala	Arg	Gly	Glu	Asp	Glu	
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	Asp	Ala	Ala	Val	Ser	Gly	Asp	Glu	Gly	Trp	Trp	Ala	Gly	Gln	
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	Val	Gln	Arg	Arg	Leu	Gly	Ile	Phe	Pro	Ala	Asn	Tyr	Val	Ala	
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	Ala	Thr	Trp	Gln	Gly	Gln	Glu	Val	Ala	Val	Lys	Ala	Ala	Arg	
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	Arg	Arg	Glu	Ala	Arg	Leu	Phe	Ala	Met	Leu	Arg	His	Pro	Asn	
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	Ile	Ile	Glu	Leu	Arg	Gly	Val	Cys	Leu	Gln	Gln	Pro	His	Leu	
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	Cys	Leu	Val	Leu	Glu	Phe	Ala	Arg	Gly	Gly	Ala	Leu	Asn	Arg	
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	Ala	Leu	Ala	Ala	Ala	Asn	Ala	Ala	Pro	Asp	Pro	Arg	Ala	Pro	
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Polypeptide sequence (SEQ ID NO:2)

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SEQUENCE LISTING

<110> Cephalon, Inc.
 Angeles, Thelma S.
 Durkin, John T.
 Holskin, Beverly P.
 Meyer, Sheryl L.
 Spais, Chrysanthé M.

<120> Novel Mixed Lineage Kinase 7 (MILK7) Polypeptide, Polynucleotides Encoding The Same, And Methods Of Use Thereof

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Gln	Pro	His	Leu	Cys	Leu	Val	Leu	Glu	Phe	Ala	Arg	Gly	Gly	Ala	Leu	
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Asn	Arg	Ala	Leu	Ala	Ala	Ala	Asn	Ala	Ala	Pro	Asp	Pro	Arg	Ala	Pro	
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ggc	ccc	cgc	cgc	gcg	cgc	cgc	atc	cct	ccg	cac	gtg	ctg	gtc	aac	tgg	720
Gly	Pro	Arg	Arg	Ala	Arg	Arg	Ile	Pro	Pro	His	Val	Leu	Val	Asn	Trp	
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Ala	Val	Gln	Ile	Ala	Arg	Gly	Met	Leu	Tyr	Leu	His	Glu	Glu	Ala	Phe	
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gtg	ccc	atc	ctg	cac	cgg	gac	ctc	aag	tcc	agc	aac	att	ttg	cta	ctt	816
Val	Pro	Ile	Leu	His	Arg	Asp	Leu	Lys	Ser	Ser	Asn	Ile	Leu	Leu	Leu	
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Trp	Glu	Glu	Ala	Ala	Ser	Ala	Asn	Ala	Ala	Thr	Val	Ser	Ile	Glu	Met	
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gct tct tca aaa cat aga ccg tca cat cac aga cgg acc atg tct gat Ala Ser Ser Lys His Arg Pro Ser His His Arg Arg Thr Met Ser Asp 885 890 895	2688
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Glu Lys Ile Glu His Asp Asp Ile Cys Asn Lys Thr Leu Lys Ile Thr
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Gln Gln Leu Ala Glu Arg Glu Ile Asp Val Leu Glu Arg Glu Leu Asn
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Lys Gly Lys Phe Lys Arg Ser Arg Leu Lys Leu Lys Asp Gly His Arg

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Ser Glu Asp Pro Leu Val Asp Ser Ala Pro Val Thr Cys Asp Ser Glu
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Pro Ala Leu Met Pro Arg Leu Asp Thr Asp Cys Ser Val Ser Arg Asn
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Leu Pro Ser Ser Phe Leu Gln Gln Thr Cys Gly Asn Val Pro Tyr Cys
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28

What is claimed is:

1. An isolated polynucleotide comprising SEQ ID NO:1.
2. An isolated polynucleotide comprising a sequence that encodes a polypeptide
5 comprising SEQ ID NO:2.
3. A polynucleotide comprising a nucleotide sequence complementary to at least a portion of SEQ ID NO:1.
- 10 4. An expression vector comprising the polynucleotide of claim 1.
5. The expression vector of claim 4 wherein said vector is a plasmid or viral particle.
6. A host cell transformed with a vector of claim 4.
- 15 7. The transformed host cell of claim 6 wherein said cell is a bacterial cell, yeast cell, insect cell, or mammalian cell.
8. A method of producing a polypeptide comprising SEQ ID NO:2 comprising the
20 steps of:
 - a) introducing a recombinant expression vector of claim 4 into a compatible host cell;
 - b) growing said host cell under conditions for expression of said polypeptide; and
 - c) recovering said polypeptide.
- 25 9. A composition comprising a polynucleotide of claim 1 and a carrier or diluent.
10. A composition comprising a recombinant expression vector of claim 4 and a carrier or diluent.
- 30 11. An isolated polypeptide encoded by a polynucleotide of claim 1.

12. An isolated polypeptide comprising SEQ ID NO:2.
13. The polypeptide of claim 11 wherein said polypeptide comprises SEQ ID NO:2.
- 5 14. A composition comprising a polypeptide of claim 11 and a carrier or diluent.
15. A composition comprising a polypeptide of claim 12 and a carrier or diluent.
16. An isolated antibody that binds to an epitope on a polypeptide of claim 12.
- 10 17. The antibody of claim 16 wherein said antibody is a monoclonal antibody.
18. A composition comprising an antibody of claim 16 and a carrier or diluent.
- 15 19. A kit comprising an antibody that binds to a polypeptide of claim 12.
20. A kit comprising a polynucleotide of claim 1.
21. A kit comprising a polypeptide of claim 12.
- 20 22. A method of identifying a compound that binds a polypeptide of claim 12 comprising the steps of:
- a) contacting said polypeptide with a compound; and
- b) determining whether said compound binds to said polypeptide.
- 25 23. The method of claim 20 wherein binding of said compound to said polypeptide is determined by a protein binding assay.
24. The method of claim 23 wherein said protein binding assay is selected from the
- 30 group consisting of a gel-shift assay, Western blot, radiolabeled competition assay, phage-based expression cloning, co-fractionation by chromatography, co-precipitation, cross linking, interaction trap/two-hybrid analysis, southwestern analysis, and ELISA.

25. A method of identifying a compound that binds a polynucleotide of claim 1 comprising the steps of:

- a) contacting said polynucleotide with a compound; and
- b) determining whether said compound binds said polynucleotide.

5

26. The method of claim 24 wherein binding is determined by a gel-shift assay.

27. A method for identifying a compound that modulates the activity of a polypeptide of claim 12 comprising the steps of:

- 10 a) contacting said polypeptide with a compound; and
- b) determining whether said polypeptide activity has been modulated.

28. The method of claim 27 wherein said activity is phosphorylation of a substrate.

15 29. The method of claim 28 wherein said activity is JNK activation.

30. A compound identified by the method of claim 22.

31. A compound identified by the method of claim 25.

20

32. A compound identified by the method of claim 27.

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I		II	
A. Phosphate Anchor		ATP Binding	
MLK1	144	LTLEEEIIGIGGFGKVYRAFWIG-----	DEVAVKAAARHDPDEDISQ-----
MLK2	98	LQLEEEIIGVGGFGKVYRALWRG-----	EEVAVKAAARLDPEKDPAV-----
PTK1	117	LRLEEVIGIGGFGKVYRGSWRG-----	ELVAVKAAARQDPDEDISV-----
MLK7	124	LELKELIGAGGFGQVYRATWQG-----	QEVAVKAAARQDPEQDAAA-----
DLK	125	ILDLQWVGSGAQQGAVFLGRFHG-----	EEVAVKKVRDLKETDI-----
LZK	168	ISELQWLGSGAQQGAVFLGKFRA-----	EEVAIKKVREQNETDI-----
MLK6	16	LQFFENC GGSGFGSVYRAKWIS-----	QDKEVAVKKLLK-----
III		IV	
MLK1		TIENVROEAKLFAML-----	KHPNIIALRGVCLKE-----
MLK2		TAEQVCQEARLFGAL-----	QHPNIIALRGACLP-----
MLK3		TAESVROEARLFGAL-----	AHPNIIALKAVCLEE-----
MLK7		AAESVRRERARLFGAL-----	RHPNIIELRGVCLQQ-----
DLK		-----KHLRKL-----	KHPNIIITFKGVCTQA-----
LZK		-----KHLRKL-----	KHPNIIAFKGVCTQA-----
MLK6		----IEKEAEILSVL-----	SHRNIIQFYGVILEP-----
V		VIa	
MLK1		GPLNRVL-SG-----	KRIPPDILVNWAVQIARGMNYLHDEAIV
MLK2		GALSRVL-AG-----	RRVPPHV--LVNWAVQVARGMNYLHNDAPV
MLK3		GPLSRAL-AG-----	RRVPPHV--LVNWAVQIARGMNYLHCEALV
MLK7		GALSNRALAA-----	AANAAPDPRAPGPRRA-----
DLK		GQLYEVLRLAG-----	RPVTPSL--LVDWSMGIAGGMNYLH---LH
LZK		GQLYEVLRLAG-----	RKITPRL--LVDWSTGIASGMNYLH---LH
MLK6		GSLYDYINSN-----	RSEEMDMDHIMTWATDVAKGMNYLHMEAPV
VIb		VII	
Catalytic Domain		Activation Loop	
a			
MLK1		-----PIIHRDLKSSNILILQKVENGDLSENK-ILKITDFGLAREW-HRT-----	TKMSAA
MLK2		-----PIIHRDLKSSNILILEAIENHNLDAT-VLKITDFGLAREW-HKT-----	TKMSAA
MLK3		-----PVIHRDLKSSNILLLOPIESDDMEHK-TLKITDFGLAREY-HKT-----	TQMSAA
MLK7		-----PILHRDLKSSNILLLEKIEHDDICNK-TLKITDFGLAREW-HRT-----	TKMSTA
DLK		-----KIIHRDLKSPNMLITY-----	DD-VVKISDFGTSKELSDKS-----
LZK		-----KIIHRDLKSPNVLVTH-----	TD-AVKISDFGTSKELSDKS-----
MLK6		-----KVIHRDLKSRNVVIA-----	ADG-VLKICDFGASR-FHNHT-----
VIII		IX	
P+1 Loop			
P			
MLK1		-----GTAWMAPEVI-----	RASMFSK-GSDVWSYGV-LLWELLT-GEVPFRGI-----
MLK2		-----GTAWMAPEVI-----	RLSLFSK-SSDVWSFGV-LLWELLT-GEVPYREI-----
MLK3		-----GTAWMAPEVI-----	KASTFSK-GSDVWSFGV-LLWELLT-GEVPYRGI-----
MLK7		-----GTAWMAPEVI-----	KSSLFSK-GSDIWSYGV-LLWELLT-GEVPYRGI-----
DLK		-----GTVAWMAPEVI-----	RNEPVSE-KVDIWSFGV-VLWELLT-GEIPYKDV-----
LZK		-----GTVAWMAPEVI-----	RNEPVSE-KVDIWSFGV-VLWELLT-GEIPYKDV-----
MLK6		-----GTFFWMAPEVI-----	QSLPVSE-TCDTYSYGV-VLWEMLT-REVPFKGL-----
X		XI	
G			
MLK1		DGLRVAYGVAMNKLALPIPST-----	CPEPFAK-LMEDCWNPDPHSRPSFTNI-----
MLK2		DALAVAYGVAMNKLTLPIPST-----	CPEPFAR-LLEECWDPDPHGRPDFGSI-----
MLK3		DCLAVAYGVAVNKLTLPIPST-----	CPEPFAQ-LMADCWAQDPHRPDPFASI-----
MLK7		DGLAVAYGVAVNKLTLPIPST-----	CPEPFAK-LMKECWQDQPHIRPSFALI-----
DLK		DSSAIIWGVGSNSLHLPVPSS-----	CPDGFKI-LLRQCWNKPRNRPSFRQI-----
LZK		DSSAIIWGVGSNSLHLPVPST-----	CPDGFKI-LMKQTWQSKPRNRPSFRQT-----
MLK6		EGLQVAWLVEKNERLTIPSS-----	CPRSAE-LLHQCWADAKKRPSFKQI-----

Figure 1

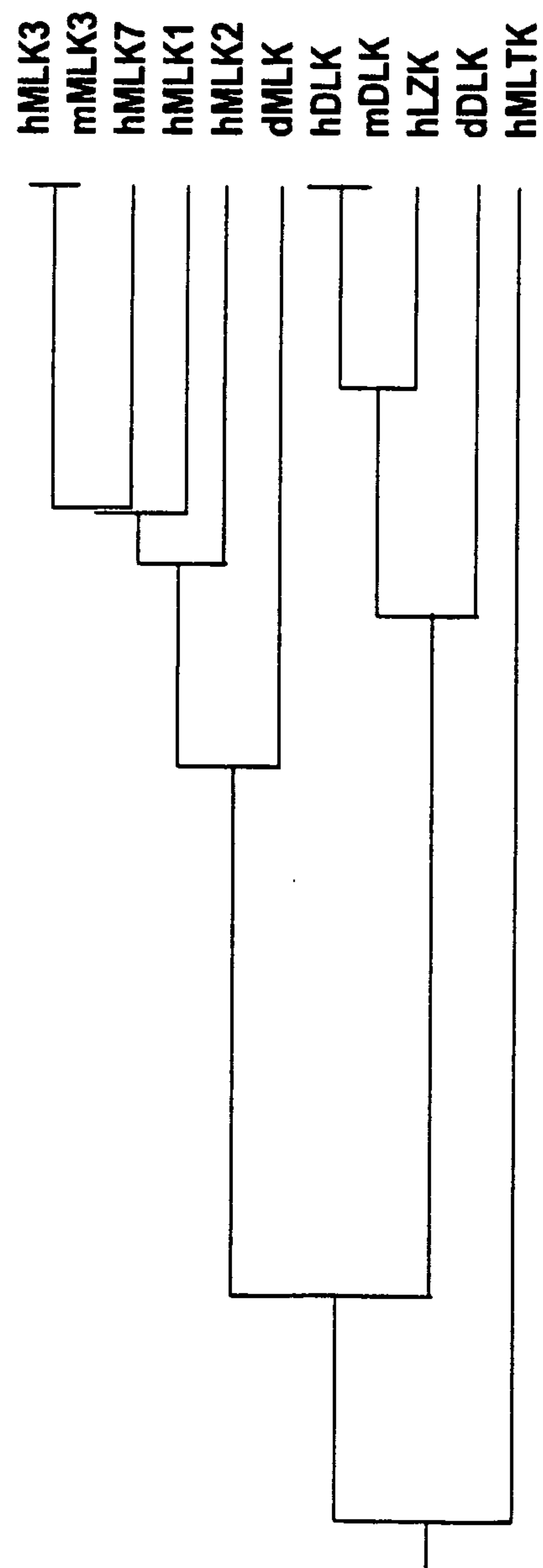
Figure 2

Figure 3

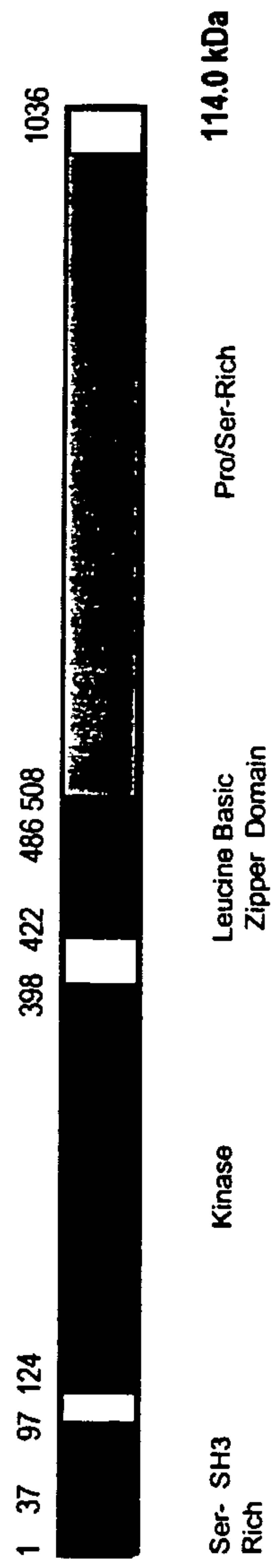
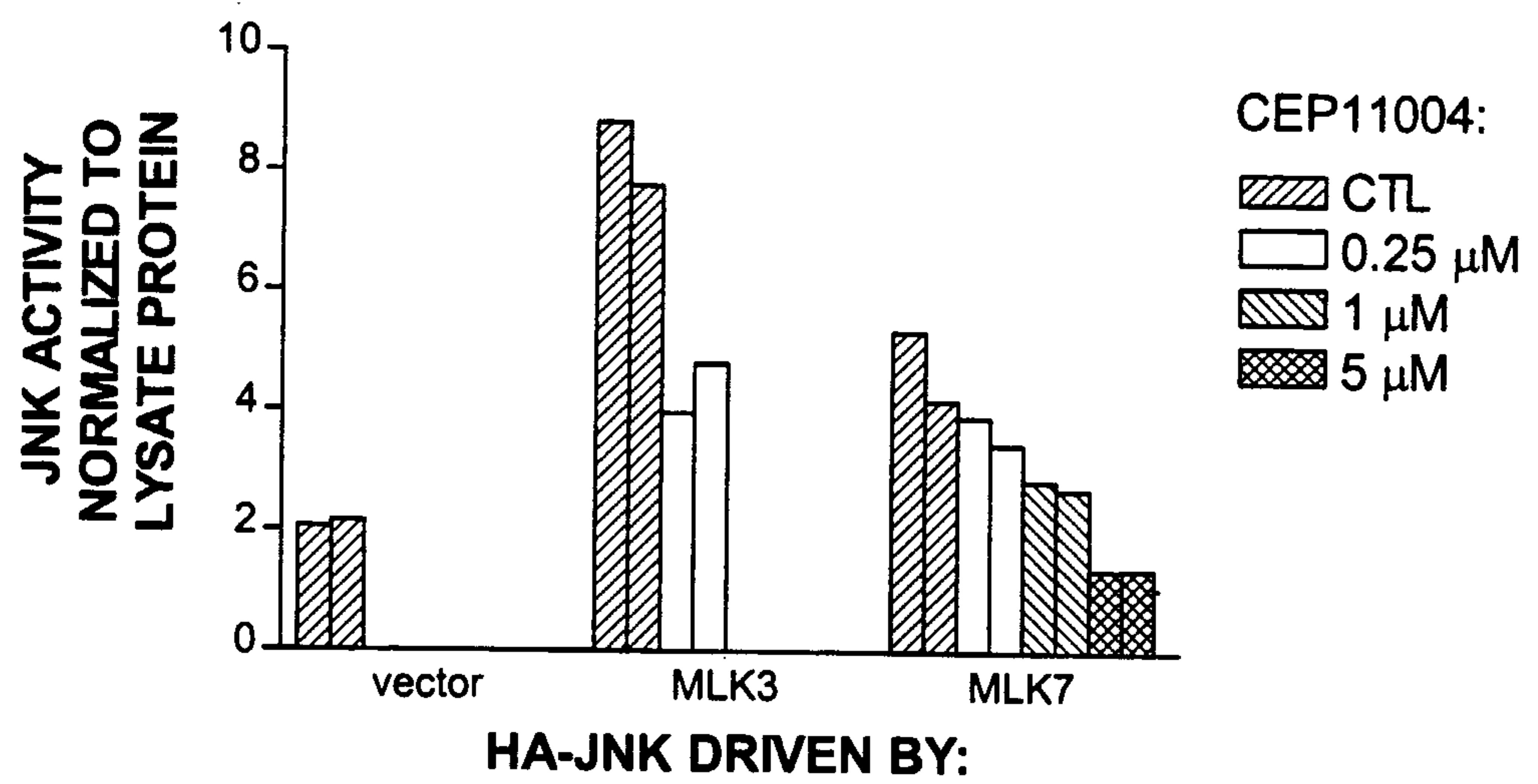


Figure 4

MLK1	427	kHEIQEMfDQ	LRAKeKE	LRTWeEE	LTRAALQ	-----QKNQeEL	LRRReQE	LAEReID	ILEReLN	II	IHQL
MLK2	381	kLEIQHMFDD	LRTKeKE	LRSReEE	LLRAAQE	-----QRFQeEQ	LRRReQE	LAEReMD	IVEReLH	L	LLMCQL
MLK3	400	kREIQGLfDE	LRAKeKE	LLSReEE	LTRAARE	-----QRSQaEQ	LRRReHL	LAQWeLE	VFEReLT	L	LLQVD
MLK7	422	kLEIQQMfDE	LRTKeKE	LRSReEE	LTRAALQ	-----QKSQeEL	LKRReQQ	LAEReID	VLEReLN	IL	IFQL
DLK	387	rEEVKLHfEK	IKSEgTC	IHRLeEE	LVMRRRE/18aa	/ANNLyME	LNALmLQ	LELKeRE	LLRReQA	L	ERRCP
LZK	430	rEEVKKHfEK	IKSEgTC	IHRLeEE	LIRRRRE/18aa	/ANNLyME	LSAImLQ	LEMReKE	L	IKReQA	VEKKYP
MLK6	284	rCEIEATLER	LKKLeRD	LSFKeQE	LKEReRR	LKMWeQK	L	TEQSNTP			

small letters = “a” position; non-polar preferred

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Figure 5

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Figure 6

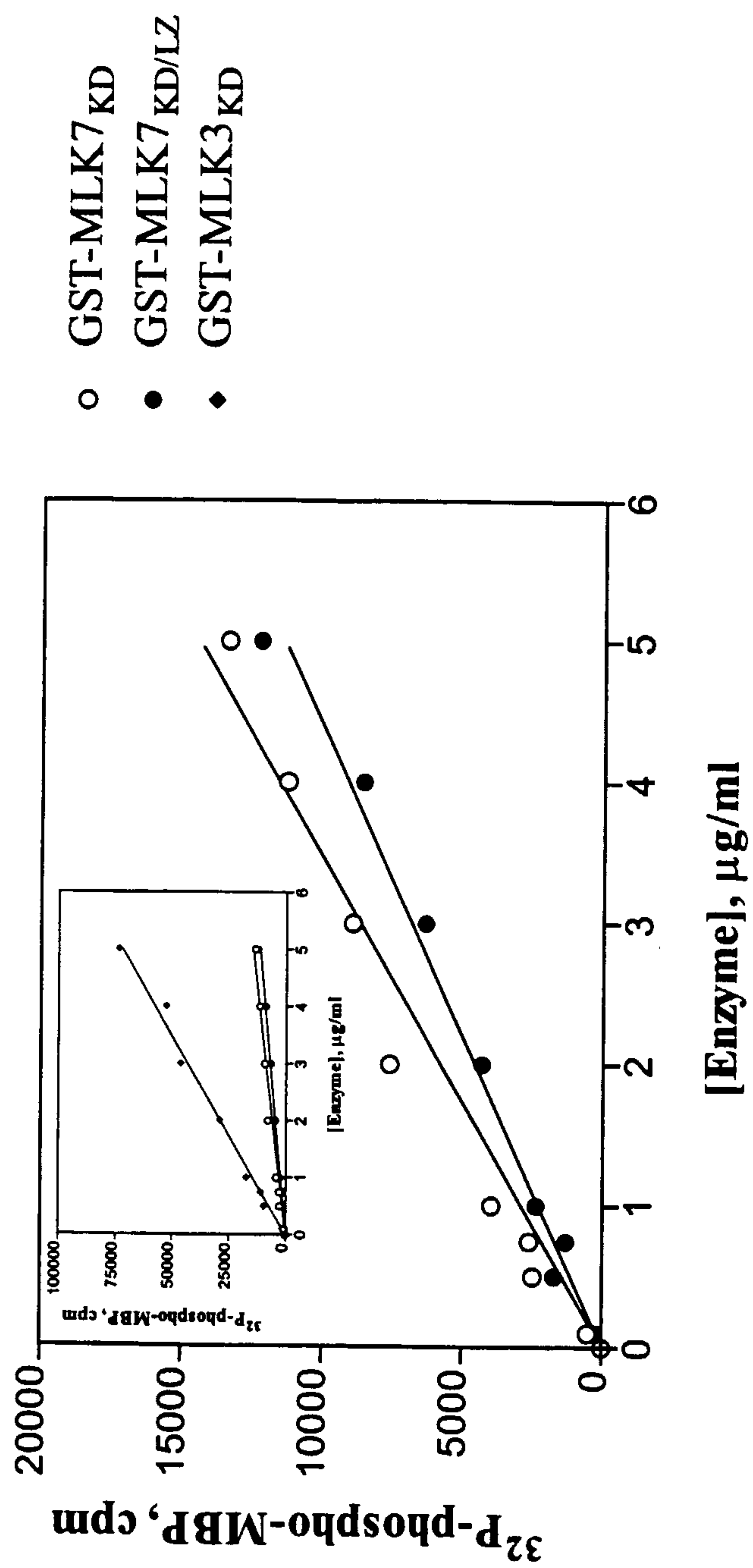
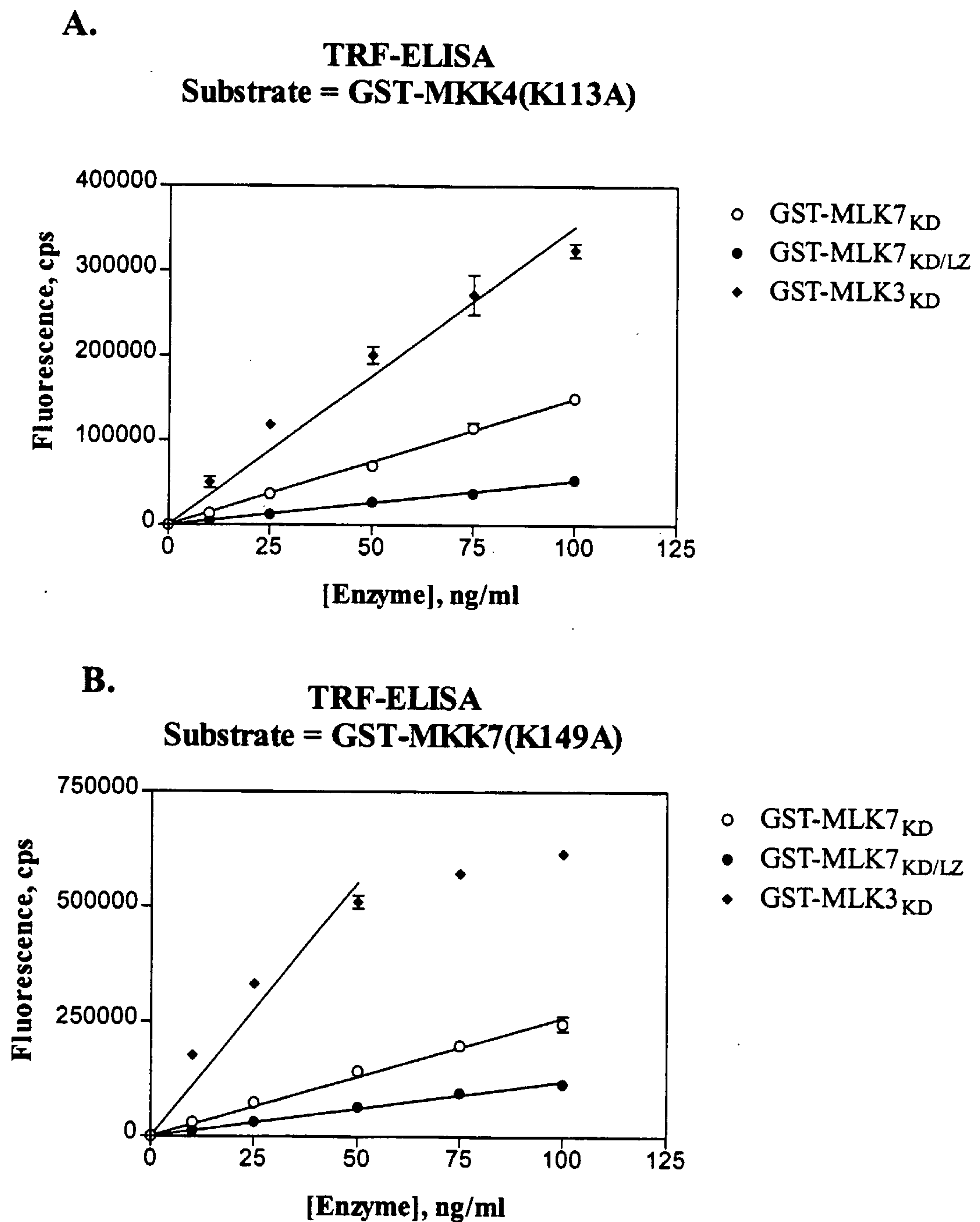


Figure 7

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Figure 8

