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SHORT SEGMENTS OF DAP-KINASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. provisional application no. 60/159,107, filed October 13, 1999, the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to DAP-kinase fragments which are critical to the biological function of DAP-kinase as a mediator of apoptosis and can act in a dominant-negative manner when expressed ectopically.

BACKGROUND OF THE INVENTION

Death Associated Protein Kinase (DAP-kinase) is a positive mediator of apoptosis. It was isolated by a function-based gene cloning methodology, named TKO selection, which involved expression of an anti-sense cDNA library in cells, followed by selection of clones that survived in the continuous presence of an apoptotic stimulus (Deiss et al, 1991). In this system, specific inhibition of DAP-kinase protein expression by anti-sense RNA protected HeLa cells from apoptosis induced by IFN-γ (Deiss et al, 1995). Furthermore, DAP-kinase was shown to modulate cell death triggered by Fas, TNF-α (Cohen et al, 1999), and detachment from extracellular matrix (Inbal et al, 1997), indicating its general relevance to apoptosis.

DAP-kinase is a Ca²⁺/calmodulin-regulated serine/threonine kinase that is localized to the cytoskeleton, where it associates with the actin microfilament system (Cohen et al, 1997). In addition to the kinase domain, the protein carries eight ankyrin repeats, a cytoskeleton binding region and a death domain. The multi-domain structure of DAP-kinase and
its participation in a wide range of apoptotic systems imply that this protein may interact with various intracellular components to exert its function. It is, therefore, conceivable that different regions of the protein may protect from apoptosis upon ectopic expression, potentially through sequestering those putative interacting proteins. In agreement with this view, transfections with the death domain module by itself protected cells from various death-inducing signals by specifically neutralizing the function of the endogenous DAP-kinase (Cohen et al, 1999).

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**SUMMARY OF THE INVENTION**

It is an object of the present invention to provide fragments of DAP-kinase protein that are critical for its biological function as a mediator of apoptosis.

It is another object of the present invention to provide peptides which are fragments of DAP-kinase protein that can act in a dominant-negative manner when expressed ectopically or which are analogs or derivatives of such fragments.

The present invention further provides a polynucleotide encoding the peptide and a pharmaceutical composition containing the peptide.

Also provided by the present invention are a method for inhibiting apoptosis associated with DAP kinase by administering the peptide of the invention to a subject in need
thereof and a method for screening fragments of a gene product, mediating a selectable phenotype, for being able to act in a dominant negative manner when expressed ectopically.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1A illustrates a screening strategy for the isolation of fragments of DAP-kinase that confer resistance to IFN-γ-induced cell killing. Purified human DAP-kinase cDNA underwent partial DNase I digestion, and fragments were subcloned into an EBV-derived expression vector to generate a cDNA expression library of random fragments. The cDNA library was introduced into HeLa cells, and elements that conferred resistance to apoptosis were isolated and further analyzed.

Figure 1B shows a vector for generation of the DAP-kinase fragmented cDNA library. pTK01, an EBV derived vector, was modified to accommodate the cDNA library by insertion of the indicated adaptor (for details see Materials and Methods section below). The amino acid sequence of the Flag epitope (SEQ ID NO:3) encoded by the adaptor is presented above the nucleotide sequence of the adaptor (SEQ ID NOs:1 and 2).

Figure 2 lists the cell death-protective fragments of DAP-kinase isolated (passed two successive screens) by the genetic screen and shows immediately underneath a schematic representation of DAP-kinase full-length protein with the position of the library-derived protective fragments indicated.

Figures 3A-3C show present bar graphs and an immunoblot which show that DAP-kinase-derived fragments protect cells from apoptosis.

In Figure 3A, apoptosis was induced in 293 cells by transient overexpression of p55/TNF-receptor. This receptor was expressed together with an empty vector, a dominant-negative mutant of FADD or DAP-kinase fragments as indicated. Transfected
cells were identified by GFP expression, and the rate of cell
death was scored by typical morphological features. The graph
represents average values obtained from three independent
experiments, each of which included at least 300 cells.

In Figure 3B, 293 cells were induced to undergo
apoptosis upon transient overexpression of an activated mutant
of DAP-kinase (ΔCaM). The cells were transfected with this
mutant together with either an empty vector or the different
fragments as indicated. Transfectants were identified by GFP
expression and apoptosis was scored by morphology as in Figure
3A. Immediately below the bar graph in Figure 3B is an
immunoblot containing equal amounts of total cell extracts which
was reacted with anti-DAP-kinase antibodies to compare the
levels of exogenous DAP-kinase in the different transfections
(the endogenous levels are below detection levels under these
exposure conditions).

In Figure 3C, MCF7 cells were transfected with
p55/TNF-receptor together with an empty vector, or the same
vector containing the FADD death domain, or different DAP-kinase
derived fragments as indicated. Apoptosis was scored as in
Figure 3A.

Figure 4A shows is the sequence alignment of the death
domains of DAP-kinase (amino acids 1300-1398; SEQ ID NO:4) and
p75 neurotrophin receptor (amino acids 334-420; SEQ ID NO:5).
Amino acids that are included in the protective fragment (1320-
1371) are marked by light gray letters. Amino acids that form
the 6 α-helical structures are emphasized in bold letters and
brackets.

Figure 4B is a model structure of the death domain of
DAP-kinase (light gray and dark gray ribbons), constructed by
comparative modeling and overlaid on the NMR-based structure of
the p75 neurotrophin receptor (lightly shaded ribbon). The six
helices (α1 to α6) are accentuated by cylinders. In the DAP-kinase death domain model, regions that are inside and outside the protective fragment are marked with light gray and dark gray, respectively. Note the extended loops between helices α1 and α2, and α3 and α4 in DAP-kinase compared to p75. The model was generated as described in Materials and Methods section below.

Figure 4C is a model structure of the death domain of DAP-kinase presented as electrostatic potential surface. As in Figure 4B, the protective fragment is shown in light gray.

Figures 5A-5C present bar graphs and an immunoblot which illustrates that deletion of the last 17 amino acids of DAP-kinase potentiates its activity.

In Figure 5A, 293 cells were transfected with either an empty vector, or the same vector carrying the indicated versions of DAP-kinase. WT, wild type protein; Δ-tail, a truncated mutant lacking the 17 C-terminal amino acids; ΔCaM, a deletion mutant lacking the calmodulin regulatory region. Transfected cells were identified by GFP expression, and the rate of cell death was assessed 24 hours post transfection according to typical apoptotic morphology. The graph represents values obtained from three independent experiments, each including at least 300 GFP-positive cells.

In Figure 5B, protein extracts were prepared from the transfected 293 cells (see Figure 5A), and DAP-kinase protein levels were analyzed by western blotting using anti-DAP-kinase specific antibodies. The endogenous DAP-kinase is below detection levels under these exposure conditions.

In Figure 5C, an in vitro Kinase assay was performed with proteins that were immunoprecipitated from 293 cells transfected with the indicated plasmids, as described in the Materials and Methods section below. The assay included an
exogenous substrate, MLC, and the relative activity was
determined according to the rate of MLC phosphorylation, as
quantified using a phosphor-imager.

Figure 6 is a bar graph illustrating that the death
domain protein fragment of DAP-kinase protects a neuroblastoma
cell line from ceramide-induced death. BE6C cells were treated
at low cell density with 30 μM C6-ceramide at 48 hours post
transfection with the following plasmids: pcDNA3 carrying
either the DAP-kinase death domain or a non-functional mutated
form of the death domain (mutant DD). An empty vector was used
as a control, and each transfection also included a GFP plasmid
to visualize the transfectants. The number of apoptotic cells
was scored after seven hours (black bars) and 10 hours (gray
bars), respectively.

Figures 7A-7D show the uptake of rhodamine-labeled
derivative of the C-terminal peptide tail by neuronal cells.
The primary neuronal cultures were prepared by the Banker's
methodology in which the hippocampal neurons derived from 18-
day-old rat embryos were cultured at low density on cover slips
and grown on a supporting layer of glial cells. Figures 7A and
7C of the hippocampal neurons were taken under fluorescence
microscopy at one hour after adding 30 μM of the wild type
peptide into the culture medium (+peptide; Fig.7A) or DMSO alone
(-peptide; Fig.7C). Figures 7B (+peptide) and 7D (-peptide)
were taken under light microscopy. Similar results were
obtained after incubation with the labeled scrambled peptide.

Figure 8 shows the dose response curve measuring the
death protective effects of the wild type peptide in protecting
hippocampal neurons from killing by C6-ceramide. The chemically
synthesized rhodamine-labeled derivative of the C-terminal
peptide tail was applied at micromolar concentrations into the
culture medium of the primary neurons prepared as described in
Figures 7A and 7B. The peptide was administered one hour before adding 15 μM C6-ceramide. Scoring was performed after fourteen hours by the Live/Dead Viability/Cytotoxicity commercial kit.

Figure 9 is a bar graph which shows illustrates that the wild type DAP-kinase tail peptide but not the scrambled peptide protects hippocampal neurons from apoptosis. The two chemically synthesized rhodamine-labeled peptides were applied at 30 μM concentrations into the culture medium of primary neurons, one hour prior to the addition of C6-ceramide. As a control, DMSO and ethanol, the solvents of the peptides and, respectively, were added to the culture medium. Apoptosis was scored at sixteen hours. The experiments were repeated six times in quadruplicates.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention includes a genetic screen which identifies minimal fragments of DAP-kinase protein that are critical for its biological function as a mediator of apoptosis and can act in a dominant-negative manner when expressed ectopically. The approach used was based on the previously described genetic suppressor element (GSE) methodology, in which a single or multiple cDNA clones are used to generate libraries of random short cDNA fragments, cloned in both orientations in a retroviral vector (Holzmayer et al, 1992; Roninson et al, 1995; Gudkov et al, 1997). Biologically active cDNA fragments are then isolated from cells expressing the library by a positive functional selection for a specific phenotype. This concept has been previously employed to identify functional regions in several genes, including topoisomerase II (Gudkov et al, 1993), kinesin heavy chain (Gudkov et al, 1994; Axenovich et al, 1998) and p53 (Ossovskaya et al, 1996; Gallagher et al, 1997). Here,
the screen was adjusted to the conditions and principles of the TKO selection procedure (Deiss et al, 1991).

An expression library of random fragments derived from DAP-kinase cDNA, expressed in HeLa cells, was generated and 5 biologically active peptides were selected that could render cells resistant to apoptosis induced by IFN-γ. Using this unbiased method, four biologically active peptides were generated corresponding to different regions in DAP-kinase. Fragment #1 covered a part of the ankyrin repeats, and Fragment #2 mapped to a "linker" region. Fragment #3 mapped to a central core of the death domain module defining a distinct substructure with functional implications in this domain, and Fragment #4 spanned the C-terminal tail of the protein and revealed the existence of negative autoregulation exerted by this region. The last two fragments were studied in more detail since they provided novel structure/function information on the protein.

The protective fragments of DAP-kinase are shown in Figure 2 of the drawings. Fragment #1 is 48 amino acids in size and comprises the ankyrin repeats in DAP-kinase. Fragment #2 is 55 amino acids in size and comprises the "linker" region of DAP-kinase. Fragment #3 is 52 amino acids in size and comprises the death domain of DAP-kinase. Fragment #4 is 17 amino acids in size and comprises the C-terminal tail of DAP-kinase. The protein fragments, all of which protect cells from apoptosis by inhibiting the complete DAP-kinase protein, are sufficiently stable in the bloodstream so that they can be introduced to cells by conventional delivery methods, i.e., by injection, subcutaneously, etc., as well as by being expressed in vivo through genetically engineered cells.

It is particularly interesting to note that Fragment #4, which spans the C-terminal serine-rich tail of the protein, defined a new regulatory region. Ectopic expression of this
short peptide inhibited the function of DAP-kinase, whereas removal of the tail from the full-length protein enhanced its killing activity, suggesting that the C-terminal tail normally plays a negative regulatory role.

One aspect of the present invention is directed to a peptide which is capable of protecting cells from apoptosis by inhibiting DAP kinase. Preferred but non-limiting examples of such a peptide include (A) a peptide fragment having the amino acid sequence of SEQ ID NO:6 from the C-terminal tail of DAP kinase, (B) a DAP kinase peptide fragment of about 48 amino acid residues in length which comprises the amkyrin repeats in DAP kinase, (C) a DAP kinase peptide fragment of about 55 amino acid residues in length which comprises the linker region of DAP kinase, and (D) a DAP kinase peptide fragment of about 52 amino acid residues in length which comprises the death domain of DAP kinase. This aspect of the invention also encompasses shorter fragments, analogs and derivatives of the DAP kinase peptide fragment as well as an elongated peptide in which either or both the N- and C-termini of the peptide is extended by 1-4 residues, preferably Asp or Glu. Extension of the termini of the peptide with Asp or Glu residues enhances the water solubility property of the peptide.

As used herein, a "fragment" of the peptide of the present invention refers to any subset of the molecule, that is, a shorter peptide. Fragments of interest are those which are capable of protecting cells from apoptosis by inhibiting DAP kinase. Such "shorter" fragments can be generated by a systematic sequential deletion of amino acid residues from the peptide which would minimize, if possible, the size/length of the peptide without interfering with its inhibitory activity. Alternatively, systematic deletion of the DNA encoding the peptide, such as by nested deletions using Bal31 nuclease, can
be used to generate DNA which would express and produce "shorter" fragments.

An analog of the peptide according to the present invention has an amino acid sequence essentially corresponding to that of the peptide of the invention. The term "essentially corresponding to" is intended to comprehend analogs with minor changes to the sequence of the peptide which do not affect the basic characteristics thereof, particularly insofar as its ability to be inhibit DAP kinase. The type of changes which are generally considered to fall within the "essentially corresponding to" language are those which would result from conventional mutagenesis techniques of the DNA encoding the peptide of the invention, resulting in a few minor modifications, and screening for the desired activity in the manner discussed below.

Preferably, the analog is a variant of the native peptide fragment of DAP kinase which has an amino acid sequence having at least 70% identity to the native amino acid sequence and retains the DAP kinase inhibitory activity thereof. More preferably, such a sequence has at least 85% identity, at least 90% identity, or most preferably at least 95% identity to the native sequence. Variant peptides may be conveniently prepared by direct chemical synthesis of the variant peptide, using methods well known in the art. At the genetic level, these variants can ordinarily be prepared by site-directed mutagenesis (as exemplified by Adelman et al., 1983) of nucleotides in the DNA encoding the peptide molecule, thereby producing DNA encoding the variant and thereafter expressing the DNA in recombinant cell culture.

The term "sequence identity" as used herein means that the sequences are compared as follows. The sequences are aligned using Version 9 of the Genetic Computing Group's GAP
(global alignment program), using the default (BLOSUM62) matrix (values -4 to +11) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, 5 percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

Analogs in accordance with the present invention may also be determined in accordance with the following procedure. 10 Peptides encoded by any nucleic acid, such as DNA or RNA, which hybridize to the complement of the native DNA or RNA under highly stringent or moderately stringent conditions, as long as that peptide maintains the biological activity of the native sequence, are also considered to be within the scope of the present invention.

Stringency conditions are a function of the temperature used in the hybridization experiment, the molarity of the monovalent cations and the percentage of formamide in the hybridization solution. To determine the degree of stringency involved with any given set of conditions, one first uses the equation of Meinkoth et al. (1984) for determining the stability of hybrids of 100% identity expressed as melting temperature Tm of the DNA-DNA hybrid:

\[ Tm = 81.5°C + 16.6 \times \log M + 0.41 \times \%GC - 0.61 \times \%form - 500/L \]

where M is the molarity of monovalent cations, %GC is the percentage of G and C nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. For each 1°C that the Tm is reduced from that calculated for a 100% identity hybrid, the amount of mismatch permitted is increased by about 1%. Thus, if the Tm used for any given hybridization experiment at the specified salt and formamide concentrations is 10°C below
the Tm calculated for a 100% hybrid according to equation of Meinkoth, hybridization will occur even if there is up to about 10% mismatch.

As used herein, highly stringent conditions are those which are tolerant of up to about 15% sequence divergence, while moderately stringent conditions are those which are tolerant of up to about 20% sequence divergence. Without limitation, examples of highly stringent (12-15°C below the calculated Tm of the hybrid) and moderately (15-20°C below the calculated Tm of the hybrid) conditions use a wash solution of 2 X SSC (standard saline citrate) and 0.5% SDS at the appropriate temperature below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA at a temperature approximately 20° to 25°C below the Tm. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC (Ausubel, 25 1987, 1998).

"Functional derivatives" or "derivatives" as used herein covers chemical derivatives which contain additional chemical moieties not normally part of the peptide and which may be prepared from the functional groups that occur as side chains on the residues or the N- or C-terminal groups by means well known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e., they do not
destroy the DAP kinase inhibitory activity of the corresponding peptide as described herein, and do not confer toxic properties on compositions containing it.

Suitable derivatives may include aliphatic esters of the carboxyl of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives or free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl group (e.g., that of seryl or threonyl residues) formed with acyl moieties. Such derivatives may also include for example, polyethylene glycol side-chains which may mask antigenic sites and extend the residence of the complex or the portions thereof in body fluids.

Non-limiting examples of such derivatives are described below.

Cysteiny1 residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny1 residues also are derivatized by reaction with bromotrifluoroacetone, alpha-bromo-beta-(5-imidazolyl)propionic acid, chloroacetyl phosphate, B alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl-2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarboxylate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysiny1 and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization
with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; 5 chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2, 4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-10 butanedione, 1,2-cyclodexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKₐ of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-15 amino group.

The specific modification of tyrosyl residues per se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetrinitromethane. Most commonly, N-acetylimidazole and tetrinitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-[2-morpholinyl-(4-ethyl)]carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic
conditions. Either form of these residues falls within the scope of this invention.

The term "derivatives" is intended to include only those derivatives that do not change one amino acid to another of the twenty commonly-occurring natural amino acids.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the complex of the invention or analogs thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids, such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Of course, any such salts must have substantially similar biological activity to the complex of the invention or its analogs.

With regard to analogs, the 17-mer Fragment #4, which has the sequence

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Ser-Cys-Asn-Ser-Gly-Thr-Ser-Tyr-Asn-Ser-Ile-Ser-Ser-Val-Val-Ser-Arg (SEQ ID NO:6),

is used herein as an example of how an analog should retain the features of the peptide fragment, i.e., a combination of hydrophobic amino acids with amino acids which are not charged (except Arg) but capable of forming intense hydrogen bonding. Such features can be maintained in the analogs, as follows:

• Serine (S) residues in positions 1, 4, 7, 10, 12, 13 and 16 may be substituted by amino acids capable of hydrogen bond formation, i.e., threonine, or the non-
natural amino acid homoserine. Other possible substitutions are with non-charged and weakly or moderately hydrophobic residues such as Gly or Ala.

- Cys\(^2\) (C) can be replaced by natural or non-natural, non-charged, amino acids such as Gly, Ala, Val, Ser, Thr α-aminobutyric acid, and α-aminopropionic acid.

- Asn\(^3\) and Asn\(^9\) (N) can be replaced by Gln, as well as by Asp or Glu. These alterations may lead to better solubility of the peptides.

- Gly\(^5\) (G) can be replaced by different aliphatic non-charged natural or unnatural residues such as Ala, α-aminopropionic acid.

- Thr\(^6\) (T) can be replaced by aliphatic non-charged and rather moderately or weakly hydrophobic amino acids, such as Ser, Gly, Ala and the like.

- Tyr\(^8\) (Y) can be replaced by aromatic residues, natural and unnatural residues such as Phe, Trp, p-amino-Phe, methylated-Phe, halogen derivatives of Phe and Tyr, as well as by aliphatic hydrophobic residues such as Leu, Ile, Nle.

- Highly hydrophobic residues on I\(^11\), V\(^14\), V\(^15\) can be replaced by hydrophobic aliphatic or aromatic residues, natural or unnatural, such as Leu, Nor-Leu (Nle), Nor-Val (Nva), Met, Phe, methylated-Phe, Trp, Tic, Homo-Leu.

- Arg\(^17\) (R) can be substituted by positively-charged residues, natural or unnatural, such as Lys, \(N^e\)-alkylated-Lys, Orn and its alkylated derivatives, \(N^w\)-alkylated and di-alkylated derivatives of Arg.
It will be appreciated by those of skill in the art that the same type of consideration for amino acid substitutions can make for the other inhibitory peptide fragments of DAP kinase. Incorporation of modified peptide bonds, such as $\text{C}(=\text{O})-\text{N}(\text{CH}_3)-$ or $\text{A}(-\text{CH}_2-\text{N}(\text{H})-=-\text{C}(=\text{S})-\text{N}(\text{H})-)$, aimed at stabilization of the peptide toward proteolysis is also possible and beneficial especially with regard to susceptible locations, e.g., $Y^8-N^9$.

Elongation of the peptide chain by various amino acid (1-4 residues) especially related to the natural sequence (at both N- and C-terminals) may yield active derivatives. Addition of negatively charged residues, such as Glu or Asp, may enhance solubility with preservation of activity. N-terminal succinylation or glutarylation is also feasible as chemical derivatives of the peptide.

As further non-limiting examples of chemical derivatives, extension from the N-terminus of the peptide with hydrophobic aliphatic acids $\text{CH}_3(\text{CH}_2)_n\text{CO}-(\text{wherein } n = 1-16)$, or with aliphatic acids containing at least one heteroatom (e.g., S, N, or O), or with aliphatic-aromatic acids, such as phenyl-$\text{(CH}_2)_n\text{CO}-(n = 1-6)$, may enhance incorporation of the peptide into cells. These modifications can be performed on water-soluble (i.e., peptides extended at either terminals by addition of Asp or Glu residues [1-4]) peptides. Addition of polyethylene glycol chains (PEG) (MW = 2000-10,000) may lead to most efficient membrane-crossing derivatives. The association between the peptide chain and the extension may be through an amide (CO-NH) formation or via bonding such as $\text{A}(-\text{CH}_2-\text{N}(\text{H})- or via terminal SH moiety, e.g., $\text{A}(-\text{CH}_2-\text{Cys-peptide } (-\text{CH}_2-S-)$.

Biological assessments for the DAP kinase inhibitory activity of the peptides, and analogs and derivatives thereof, are quite readily performed in the well-calibrated system of
glutamate-treated hippocampal neurons. The inhibitory activity of the peptides, and analogs and derivatives thereof, can also be assayed with neuronal cell lines that undergo apoptosis in response to various insults including deprivation of growth factors, ceramides or killing cytokines (the latter being perhaps a more practical screening system).

The response of retinal ganglion cells to two defined injuries, glutamate eye injections and crush injury of the optic nerve, in test animals is preferably used as in vivo model systems for testing the efficacy of peptides, and analogs and derivatives thereof, in protecting neuronal cells from apoptosis. The reasons being:

a) The relevance of DAP-kinase to the death of retinal ganglion cells has been recently established in the DAP-kinase KO mice. The laboratory of the present inventors found that in DAP-kinase deficient mice the number of retinal ganglion cells which survived after intravitreal glutamate injection or the crush injury of the optic nerve was higher in a statistically significant extent. This implies that DAP-kinase is a central participant in the death of retinal ganglion cells and therefore a functional peptide should be effective.

b) The eye bulb is considered as an isolated box where compounds can be introduced by intravitreal injections, and be effective without being diluted by excess of fluid. Thus peptide injections may be maximally effective.

c) The experimental system is a model for different human pathologies such as glaucoma. The best peptide derivatives will be injected into the vitreal body of a wild-type mice at different time points before and after the crush injury of the optic nerve or glutamate administration. The number of survival cells in the retina will be assessed by counting. The effects will be compared to the protective
effects obtained by the deletion of DAP-kinase in the KO mice in order to evaluate the efficacy of the peptide.

The peptides, and analogs and derivatives thereof, of the present invention can be combined with a pharmaceutically acceptable carrier, and optionally other therapeutic and/or prophylactic ingredients. The carriers must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

The peptide can be administered in the form of a combination of the peptide with a pharmaceutically acceptable carrier. The compositions of the present invention thus include at least one peptide analog or derivative thereof along with a pharmaceutically acceptable carrier.

Pharmaceutical formulations suitable for oral administration wherein the carrier is a solid are most preferably presented as unit dose formulations, such as boluses, capsules, and the like, as well as sachets or tablets, each containing a predetermined amount of the active ingredient. A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active conjugate in a free-flowing form, such as a powder or granules optionally mixed with a binder, lubricant, interdiluent, lubricating, surface active or dispersing agent. Molded tablets may be made by molding the active conjugate with an inert liquid diluent. Tablets may be optionally coated and, if uncoated, may optionally be scored. Capsules may be prepared by filling the active conjugate, either alone or in admixture with one or more accessory ingredients, into the capsule cases and then sealing them in the usual manner. Cachets are analogous to capsules wherein the active conjugate together with any optional accessory ingredient is sealed in a rice paper envelope.
Pharmaceutical formulations suitable for oral administration in which the carrier is a liquid may conveniently be presented as a solution in an aqueous liquid or a non-aqueous liquid, or an oil-in-water or water-in-oil liquid emulsion.

Pharmaceutical formulations suitable for parenteral administration are conveniently presented in unit dose or multi-dose container which are sealed after introduction of the formulation until required for use.

It should be understood that in addition to the aforementioned carrier ingredients the pharmaceutical formulations described above may include, as appropriate, one or more additional carrier ingredients, such as diluents, buffers, flavoring agents, binders, surface active agents, thickeners, lubricants, preservative (including anti-oxidants) and the like, and substances included for the purpose of rendering the formulation isotonic with the blood of the intended recipient.

The pharmaceutical formulations may be any formulation in which the active compound may be administered and include those suitable for oral or parenteral (including intramuscular and intravenous) administration. The formulations may, where appropriate, be conveniently presented in discrete dosage units and may be prepared by any of the methods well known in the art of pharmacy. All of the methods include the step of bringing into association the active compound with liquid carriers or finely divided solid carriers of both and then, if necessary, shaping the product into the desired formulation.

In order to inhibit apoptosis associated with DAP kinase activity in cells, a patient in need thereof is treated with a therapeutically effective amount of at least one of the peptides of the present invention. Because the peptide according to the present invention are relatively stable in the bloodstream, they can be administered by any conventional means
of administering peptides. Alternatively, the peptide can be administered by causing expression of the peptide in vivo.

The term "effective amount" with respect to the active agent, wherein the active agent is a peptide or a fragment, analog or derivative thereof, refers to an amount of the active agent which is capable of inducing a therapeutic alteration in the physiological state of the patient receiving the active agent as a result of inhibiting DAP kinase activity. Such an amount can be empirically determined by those of skill in the art.

Further provided by the present invention is a method of treatment comprising administering the active agent to an individual. Similarly, as with the pharmaceutical composition, depending on the nature of the active agent, the method is practical in inhibiting cell apoptosis associated with DAP kinase activity. Thus, this invention provides a method for treating a disease or a disorder associated with apoptosis comprising introducing into the cells of an affected individual a therapeutically effective amount of the active agent, thereby preventing apoptosis of the cells.

Another aspect of the present invention is a method for generally screening fragments of a gene product, mediating a selectable phenotype, for capability of acting in a dominant negative manner when expressed ectopically. The preferred embodiment of this method is applied to DAP kinase as the gene product. This method involves:

(A) Fragmenting a cDNA encoding a gene product, which mediates a selectable phenotype, to obtain random cDNA fragments.

(B) Inserting the random cDNA fragments into an EBV-based episomal shuttle vector capable of propagation in bacterial and mammalian cells to generate a library of random
cDNA fragments, wherein the random cDNA fragments are operably linked to a promoter for expressing the random cDNA fragments and the EBV-based episomal shuttle vector has a selectable marker and an interferon responsive enhancer element which stimulates the expression of the random cDNA fragment from the operably-linked promoter.

(C) Transforming mammalian host cells with the library of random cDNA fragments in the EBV-based episomal shuttle vector to obtain transformed host cells.

(D) Selecting for transformed host cells that act in a dominant negative manner to the selectable phenotype of the gene product.

(E) Isolating a cDNA fragment which encodes a peptide fragment of the gene product and which acts in a dominant negative manner to the gene product. In the preferred embodiment of this method, peptide fragments of DAP kinase, which are in a dominant negative manner to DAP kinase, are isolated.

In the preferred embodiment of this method, peptide fragments of DAP kinase, which act in a dominant negative manner to DAP kinase, are isolated.

The term "operably linked" is intended to refer to an "operative linkage" in which the regulatory DNA sequences (i.e., promoter) and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region as well as the DNA sequence which, when transcribed into RNA, will signal the initiation of protein synthesis.

Having now generally described the invention, the same will be more readily understood through reference to the
following example which is provided by way of illustration and is not intended to be limiting of the present invention.

EXAMPLE

MATERIALS AND METHODS

Construction of DAP-Kinase cDNA Library

Full length DAP-kinase cDNA, a 5 Kb fragment, was excised from a Bluescript plasmid and purified. Five µg of this cDNA were subjected to partial DNase I (Sigma) digestion as described (Gudkov et al, 1997). The reaction was stopped by addition of EDTA to a final concentration of 25 mM, at different time points, to obtain fragment preparations of various average lengths (between 50 bp and 2 Kb). The vector in which the library was constructed, was based on pTK01 that was previously developed in the laboratory of the present inventors (Deiss et al, 1991). First, a synthetic adaptor containing a Flag epitope, a blunt cloning site and three stop codons, was inserted into pTK01 under an SV-40 promoter, as detailed in Figure 1B. cDNA fragments were blunted with T4 DNA polymerase and Klenow (NEB), and directly ligated with an excess of vector. The cassette was flanked by unique restriction sites on both sides to allow directional re-subcloning of the inserts. The amount of DNA used for ligation was adjusted so that more than 100,000 colonies were obtained, a complexity that maximized the potential representation of fragments from different regions of the molecule, and at a large variety of sizes. Plasmid DNA was prepared directly from pooled bacterial colonies to avoid an amplification step, and PCR analysis of the resulting library confirmed that the insert size range remained unchanged.
Cell Culture and Transfection Procedures

293, MCF7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Biolab), 2 mM glutamine, and 100 U/ml penicillin and streptomycin (Gibco). Human interferon-γ (Rephrogen) was added to the culture media at a 1000 U/ml concentration. Transfections were performed by the standard CaPO₄ precipitation method. For the apoptosis assays, 293 cells were plated one day before transfection (6 x 10⁵ cells/9 cm plate). A transfection mix for each plate included 1 μg of EGFP encoding plasmid (Clontech), 3 μg of the "killing" plasmid (pcDNA3 carrying either p55-TNF-receptor, or DAP-kinase ΔCaM), and 9 μg of either empty vector or a plasmid carrying the library fragments. To compare killing potency of different forms of DAP-kinase, 1 μg pEGFP and 10 μg of pcDNA3 were used carrying each of the indicated forms. Apoptosis was assessed 24 hours after transfection under a fluorescent microscope and according to morphological features.

Selection of Functional Elements in Cells

Six plates of HeLa cells (5 x 10⁵ cells/9 cm plate) were each transfected with 10 μg of the library DNA. Forty-eight hours post transfection cells were trypsinized and plated on 20 plates (6 x 10⁵ cells/15 cm plate) in medium containing 200 μg/ml hygromycin B (Calbiochem) and 1000 U/ml interferon-γ. After 3 weeks of selection, cells from surviving colonies were collected, and episomes were extracted by Hirt's method (Deiss et al, 1991).
Protein Analysis

Cell pellets were lysed in PLB buffer, separated by SDS-PAGE and blotted onto nitrocellulose membranes as described (Cohen et al, 1997). Filters were blocked in PBS plus 0.1% 5 Tween 20 and 10% w/v skim dry milk, and then incubated with anti-DAP-kinase monoclonal antibodies (Sigma) diluted 1:1000 for 1 hour. The filters were washed in PBS-Tween, and then incubated with horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson). Antibodies were visualized by enhanced 10 chemiluminescence according to manufacturer's instructions (Supersignal, Pierce).

Molecular Modeling

The sequence alignment presented in Figure 4A was obtained using the Smith-Waterman algorithm (Smith et al, 1981) and is almost identical to the previously published alignment of the present inventors (Feinstein et al, 1995), with the difference being a one amino acid shift in the position of helix 3. The 3D model was constructed using the Homology module of InsightII (MSI/Biosym Inc., San Diego, CA) and was based on the NMR structure of the closely related p75 neurotrophin receptor (Liepinsh et al, 1997), that was recently deposited in the Protein Data Bank (Bernstein et al, 1977). The initial model was energy minimized by the Encad program (Levitt, 1983). In this minimization, the C atoms were restrained to their initial positions so that the overall structure was not disrupted. The energy minimized structure was verified by calculating the 3D-1D compatibility scores (Luthy et al, 1992). These identified only a single potentially misfolded region, confined to the 12 amino acid loop between helices 3 and 4, for which an accurate 30 structure could not be reliably constructed.
In Vitro Kinase Assay

293 cells were plated one day before transfection (10⁶ cells/9 cm plate) and transfected by the CaPO₄ procedure with 1 μg pEGFP and 10 μg of pcDNA3 carrying each of the indicated 5 forms of DAP-kinase, all tagged with an HA epitope at their N-terminus. Cell pellets were lysed in PLB buffer as described (Cohen et al, 1997), and immunoprecipitation of recombinant DAP-kinase protein from 1 mg total extract was performed with 3 μl of anti-HA monoclonal antibodies in 500 μl of PLB supplemented 10 with protease and phosphatase inhibitors for two hours at 4°C. The immunoprecipitates were washed three times with PLB, and once with reaction buffer (50 mM Hepes pH 7.5, 8 mM MgCl₂ and 0.1 mg/ml BSA). The proteins bound to the beads were incubated for 10 minutes at 30°C in 50 μl of reaction buffer containing 15 15 μCi[α-³²P] ATP (3 pmole), 50 μM ATP, 2 μg MLC (Sigma), CaCl₂ (0.1 mM) and bovine calmodulin (1 μM, Sigma). Protein sample buffer was added to terminate the reaction, and after boiling the proteins were analyzed on 12% SDS-PAGE. The gel was blotted onto a nitrocellulose membrane, ³²P-labeled proteins were 20 visualized by autoradiography, and the rate of relative MLC phosphorylation was measured using a phosphor-imager. DAP-kinase levels were determined by immunoblotting with monoclonal anti DAP-kinase antibodies.

RESULTS

25 Functional Dissection of DAP-kinase by a Genetic Screen.

To isolate biologically active peptides of DAP-kinase, an expression library of its randomly fragmented cDNA was generated and a positive functional selection in HeLa cells treated with IFN-γ (Figure 1A) was carried out. This is the
system from which DAP-kinase was originally isolated (Deiss et al, 1995).

The library was constructed in pTKO1 (Deiss et al, 1991), an EBV-based episomal vector, that carries an IFN-5 responsive enhancer element (ISRE), which stimulates the expression of the library inserts during selection, and was previously shown to be very effective in this procedure (Deiss et al, 1991). Into this vector a suitable expression cassette was first introduced, which provided an initiator methionine in a favorable translation initiation context within a Flag epitope, followed by a cloning site and stop codons in all three reading frames (Figure 1B). DAP-kinase cDNA fragments were generated by incomplete DNase I digestion, and ligated into the vector. Since the fragmentation and subcloning direction were both random, it was assumed that half of the fragments were inserted in a sense orientation, and that one-third of these (i.e., about 16% of the total inserts) would be expressed in the correct reading frame. The choice of an episomal shuttle vector provided three major advantages: (1) relatively high efficiency of stable transfection not requiring chromosomal integration; (2) reduced appearance of false positive clones that could result from insertional mutagenesis; and (3) direct recovery of plasmids from cells that survived the selection, and immediate propagation of these plasmids in either bacterial or mammalian cells.

The DAP-kinase cDNA library was introduced into HeLa cells by transfection, and the cells were then subjected to double selection with hygromycin B and IFN-γ for three weeks. Cell colonies that survived this prolonged selection were pooled, and episomes were isolated by Hirt's extraction and used to transform bacteria. The cDNA inserts of plasmids from 70 randomly chosen bacterial colonies were amplified by PCR and
sequenced. Thirteen fragments turned out to be inserted in a sense orientation, and out of the sense fragments, 18 clones encoded peptides in the authentic reading frame of DAP-kinase. Of the 18 clones, four fragments appeared only once, and the five rest appeared multiple times corresponding altogether to nine different fragments. Since the aim was to study the function of different structural motifs of the protein, attention was particularly concentrated on those sense fragments in the correct frame. To distinguish between functionally active and false positive peptides, the biological function of individual fragments was tested as detailed below.

A Secondary Screen Identified Functional Peptides that Inhibit DAP-kinase Function in TNF-Induced Apoptosis

The HeLa/IFN-γ system, from which DAP-kinase was isolated, was suitable for the first round of functional selection, since in this system a single genetic change was sufficient to yield a weak, yet selectable phenotype of increased survival in a population of cells subjected to a long-term selection (Deiss et al, 1991). However, as this characteristic of the system may also lead to a significant level of non-specific background, fragments obtained in this selection were individually tested by a secondary screen.

For the second assay, apoptosis induced by high levels of p55 TNF receptor was used. This is a system in which an essential role for DAP-kinase has been previously established (Cohen et al, 1999). In this assay system one could rapidly examine the ability of the isolated fragments to inhibit DAP-kinase function and, thus, protect cells from apoptosis. To this aim, the p55 TNF-receptor was transfected into human kidney epithelial 293 cells together with either an empty pTK01 vector or with a vector carrying individual fragments from the first
selection, and the level of apoptosis was assessed 24 hours later. Transfected cells were identified by GFP expression and the rate of apoptosis was scored microscopically according to typical morphological features. A peptide was defined positive if its co-introduction into cells reduced the extent of TNF-receptor-induced apoptosis by more than 50%.

Using this criterion, the ability of four out of the nine different DAP-kinase-derived peptides to confer resistance to apoptosis was established (Figure 2). One of them (Fragment #3) appeared six times, and each of the other three appeared twice. The remaining fragments were scored as false positive in these assays. The extent of protection by the positive clones ranged between 60-70% (Figure 3A). It resembled the extent of protection obtained by the previously established inhibitory fragment of DAP-kinase—the 99 amino acids of the death domain (Figure 3A; see also Cohen et al, 1999). As previously discussed, this partial, yet significant, extent of protection is characteristic of targets which function at a downstream position along apoptotic pathways. In contrast, and as expected, the dominant negative mutant of FADD which functions much earlier in the system, i.e., at the receptor proximal level (Chinnaiyan et al, 1996), completely blocked the p55 TNF receptor-induced apoptosis (Figure 3A). This screen was first performed with the rescued fragments in the original pTK01 vector (Figure 3A), and then repeated after sub-cloning the fragments into pcDNA3 vector, yielding similar results (not shown).

The detailed mapping of the functionally active fragments to several regions in the protein is shown in Figure 2. Three of them span short regions (48-55 amino acids in size) and map to the ankyrin repeats, the “linker region” (that does not carry any known protein motives), and the death domain. The
fourth is a stretch of 17 amino acids at the very C-terminus of the protein. The isolation of an element residing within the death domain was notable, since it confirmed the reliability of the selection strategy.

To verify that the effects of these fragments resulted from specific inhibition of DAP-kinase function, two lines of experiments were carried out. First, it was shown that the different fragments could protect 293 cells from death induced by overexpression of DAP-kinase itself. In this assay, the present inventors utilized an activated mutant of DAP-kinase, in which a deletion of the calmodulin binding and regulatory regions rendered the kinase domain constitutively active, thereby generating a potent death-inducing protein (Cohen et al, 1997). The activated DAP-kinase (designated ΔCaM) was transfected into 293 cells together with either an empty vector, or the same vector carrying the different selected cDNA fragments. As shown in Figure 3B, expression of the various peptides reduced the extent of apoptosis induced by the activated DAP-kinase, without affecting its protein expression levels. Since the levels of the ectopically expressed activated DAP-kinase exceed by far those of the endogenous kinase, it was not surprising that the extent of protection in this experimental setting was lower than in the TNF-based system. In other words, the high levels of the transfected DAP-kinase could not be neutralized by the peptides as efficiently as in the previous experiment (Figure 3A) where only the endogenous DAP-kinase was present.

To further examine the specificity of these fragments, their function was tested in MCF7 breast carcinoma cells that do not contain DAP-kinase. The present inventors have previously shown that in these cells, killing by TNF-α did not require DAP-kinase (Cohen et al, 1999). In this system, co-transfection of
DAP-kinase-derived fragments failed to protect the cells from apoptosis induced by transfection with p55 TNF receptors (Figure 3C). These results indicate that the function of these fragments in protecting cells from apoptosis is DAP-kinase-5 dependent, as in the absence of DAP-kinase these elements lack any anti-apoptotic activity.

The work described here yielded four protective fragments. Obviously, the extent of this screen has not been exhaustive, and more peptides are expected to be isolated should a more comprehensive screen be performed. Yet, from the fragments that were so far isolated, the two that map to the death domain and the tail seemed particularly interesting, and further analysis focused on these fragments.

**Molecular Modeling of the Death Domain Reveals a Distinct 3D Sub-Structure for Fragment #3**

Fragment (#3) was derived from the death domain, a conserved module whose critical role in DAP-kinase-mediated apoptosis has been established (Cohen et al, 1999). To elucidate the structural basis for the activity of the rescued fragment, a three-dimensional (3D) model structure of the death domain was constructed. The molecular modeling was based on the sequence similarity between DAP-kinase death domain and the closely related intracellular domain of the p75 neurotrophin receptor, for which an NMR structure was recently published (Liepinsh et al, 1997).

The predicted structure of the DAP-kinase death domain consists of 99 amino acid residues that fold to form 6 α-helices (Figure 4B). As in p75, helices α1, α5 and α6 lie parallel to each other and are perpendicular to helices α2, α3 and α4. One notable difference between the two death domains is the presence of longer loops extending between helices α1 and α2 and helices
α3 and α4 of the DAP-kinase death domain. Since these loops are unique to DAP-kinase, they may contribute to the specificity of interaction with other proteins. The region marked in light gray (Figures 4A, 4B and 4C) represents Fragment #3, which was isolated as an effective inhibitor of DAP-kinase. This region, whose exact composition is marked in the sequence alignment (Figure 4A), completely spans helices α2, α3 and α4, which, as revealed by the molecular model and emphasized in Figure 4C, are clustered to form a structurally distinct element. This substructure is stabilized by hydrophobic interactions, particularly between helices α2 and α4, that are likely to direct correct folding of the peptide even when expressed separately, and hence allow binding of this distinct module to its putative target protein(s). Of note, the addition of an N-terminal Flag epitope (see Figure 1) is not expected to interfere with the folding of the peptide. First because the Flag tag is composed mostly of hydrophilic residues, and second, since it is connected to a 9-amino acid long stretch of the loop between helices α1 and α2, which physically separates the tag from the core of the module.

Thus, according to this model structure, Fragment #3 constitutes a defined substructure in the death domain, a feature that may underlie its effective biological function.

**An Autoinhibitory Function of DAP-Kinase C-Terminal Tail**

Fragment #4 was particularly interesting. It mapped to the very last 17 amino acids in the short C-terminal region that immediately follows the death domain. The amino acid sequence of this peptide (SCNSGTSYNSSVSVSR) is rich in serines, a feature that is typical for many death-domain-containing proteins (Feinstein et al, 1995). In one case, the Fas receptor, it has been shown that the C-terminal tail negatively
regulates signal transduction (Itoh et al, 1993). The fact that ectopic expression of the C-terminal tail could inhibit DAP-kinase function, indicated that the tail could have a defined regulatory role in the context of the complete molecule as well. However, it was not readily determined a priori if the tail is normally essential for activation of DAP-kinase, execution of its function, or for negative autoregulation.

To further explore the nature of regulation which is mediated by the C-terminal tail, this fragment was deleted from the full-length protein, and the rate of apoptosis induced by WT DAP-kinase (DAPk-WT) and truncated DAP-kinase (DAPk-tail) was compared. In case of a positive regulation by the tail, or of a direct role in execution of protein's function, a deletion is expected to cause a reduction in the apoptotic function.

Conversely, if the tail is involved in negative regulation of DAP-kinase, then a deletion should result in potentiation of the death-promoting function of the protein. A tail-truncated version of the protein was generated by introducing a stop codon at position 1415, thus, removing the last 17 amino acids of the protein (tail mutant). Quantitative apoptosis assays were then used to compare the potency of this deletion mutant to that of the wild type protein. The constitutively active kinase mutant (ΔCaM) was used in these assays as a control for gain of function. It was found that truncation of the last 17 amino acids greatly potentiated the ability of DAP-kinase to induce apoptosis in 293 cells (Figure 5A). The extent of apoptosis induced by the truncated mutant was significantly higher than that induced by the WT protein (58% vs 25%) and comparable to the ΔCaM mutant (63%), while expression levels were equal for all forms (Figure 5B). To test if this enhancement of killing potency was due to an increase in the catalytic activity of the protein, an in vitro kinase assay was performed, in which
recombinant proteins from transfected cells were immunoprecipitated, and then incubated with myosin light chain (MLC) as an external substrate (Cohen et al, 1997). In this assay, the truncated tail mutant displayed kinase activity that was indistinguishable from that of the WT protein, whereas the ΔCaM mutant, carrying an activated kinase domain, clearly showed an increased kinase activity (Figure 5C). Thus, the enhancement of killing potency could not be attributed to a higher kinase activity. These results imply that the tail does not directly affect the kinase domain, but rather acts at another level of controlling the pro-apoptotic activity of DAP-kinase, conceivably through modulating interactions of other domains in the protein with critical targets.

Dominant-Negative Mutants of DAP-Kinase Protect Neuronal Cells from Apoptosis

a. The First Validation Test for Using DAP-Kinase Peptides in Neurodegenerative Pathologies

The DAP-kinase “death domain” and the C-terminal peptide were used in order to assess the participation of DAP-kinase in neuronal cell death. These specific inhibitory protein domains were first tested in neuronal cell lines subjected to undergo apoptosis by ceramides and then in primary neurons aiming at elaborating conditions in which the delivery into primary hippocampal neurons will be as simple as possible.

In the first preliminary step, an immortalized human neuroblastoma cell line (BE6C) was transfected with pcDNA3 expression vector carrying either the death domain (CAPk-DD), a non-functional mutated form of the death domain (CAPk-mDD—cf. Cohen et al, 1999, for details on the mutation), or with an empty vector. The vectors were co-transfected together with GFP to visualize the transfectants. FuGENE was used, which yields
high transfection efficiencies and low cell toxicity. The chosen apoptotic trigger was ceramide, a second messenger produced by sphingomyelin hydrolysis mediating a large spectrum of apoptotic signals. The C6-ceramide derivative was used; this derivative penetrates into cultured cells and turns on apoptotic pathways. A non-functional analog, dihydro-ceramide, was used as a negative control. The titration curves indicated that in neuroblastoma cells, 30 mM of C6-ceramide initiated a synchronous type of apoptotic cell death, reaching about 60% cell death in 7-10 hours. The effect was cell density-dependent. It displayed the classical hallmarks of apoptosis such as PARP cleavage. The cells were exposed to C6-ceramide at 48 hours post transfection. The percent of apoptotic cells among the green cells was assessed under the fluorescence microscope. It was found that expression of the death domain protected the cells by about 45-65% from ceramide-induced cell death, as shown in Figure 6. The mutant death domain of DAP-kinase had no death-protective effects and was indistinguishable from the empty vector, as shown in Figure 6.

b. Synthesis of Death-Protective Peptides Capable of Penetrating into Neuronal Cells with no Further Manipulations

This preliminary result led to attempting the main goal, i.e., checking the importance of DAP-kinase in apoptosis of primary hippocampal neurons and developing for this purpose DAP-kinase synthetic peptides that easily penetrate into neurons. A peptide was chemically synthesized which corresponds to the last 17 amino acids of the tail, Fragment #4, and several approaches were used in attempts to obtain a derivative which can enter into cells and can be relatively stable. Examination of the amino acid sequence, as shown in Table 1, suggested that
the binding of a hydrophobic residue to the peptide may facilitate cellular entry.

**TABLE 1**

**THE AMINO ACID SEQUENCE OF THE CHEMICALLY SYNTHESIZED PEPTIDES**

Wild type: SCNSGTYSNSISSVSVSR (SEQ ID NO:6)
Scrambled: SCSRVSGSNSYTVNIS (SEQ ID NO:7)

Tetramethylrhodamine isothiocyanate was covalently linked to the N-terminus of the peptide. This technique increased the hydrophobicity of the peptide and made it possible to follow its entry into cells by fluorescence microscopy. When applied at micromolar concentrations into the culture medium of HeLa cells and primary hippocampal neurons, the cells were rapidly labeled, i.e., within an hour, and if removed by washing after the first hour, the label persisted in the cells for a few additional hours, decaying gradually. This rhodamine-coupled peptide was used in the experiments shown in Figures 7-9, where it effectively protected hippocampal neurons from ceramide-induced cell death.

Another rhodamine-labeled peptide containing the same amino acid composition in a randomized order, designated scrambled peptide, was similarly synthesized, as shown in Table 1. This scrambled peptide penetrated into the primary neurons at the same efficiency as the wild type peptide and, therefore, was used as an internal control for assessing specificity.

The chemically synthesized rhodamine-labeled derivatives of the C-terminal peptide tail, when applied to hippocampal neurons at day three of culturing, resulted in
almost 100% efficiency of intracellular delivery. Within one hour of peptide administration into the medium, all of the neurons showed strong rhodamine staining, which was evenly distributed in the cell bodies, as well as in axons and dendrites, as shown in Figures 7A and 7B. The tail peptide very efficiently protected the hippocampal neurons from ceramide-induced death. A dose response curve of the peptide is shown in Figure 8.

The average protection displayed by 30 μM peptide measured at fourteen hours post 15 μM C6-ceramide was 58% (within P < 8.7 x 10^-5). This is shown in Figure 9. The scrambled peptide had no effects on death by ceramide, thus suggesting specificity towards the amino acid sequence of the DAP-kinase tail (cf. Figure 9).

Interestingly, the peptide also protected the primary neurons from glutamate-induced cell death, indicating that different insults kill hippocampal neurons through DAP-kinase. The foregoing provides the first direct evidence that neutralization of DAP-kinase is effective against apoptosis.

DISCUSSION

Identification of functional regions in complex proteins is key to understanding their mechanism of action, and is commonly directed by conserved structural motifs. Here, a random, unbiased approach was used to identify regions in DAP-kinase that are critical for its ability to participate in apoptotic processes. Functional domains of DAP-kinase were identified by expression and selection of dominant negative peptides that inhibited the activity of the protein and, thus, prevented cells from undergoing apoptosis. Altogether, four functional fragments were identified, two of which (fragments #3 and #4) were studied here in more detail. The mere rescue of
these biologically active fragments highlighted critical functional domains of DAP-kinase and defined minimal portions of the molecule that are capable of interfering with its death-inducing activities when artificially expressed in cells. When tested in MCF7 cells that lack endogenous DAP-kinase, these fragments did not inhibit apoptosis, indicating that their function is DAP-kinase dependent. Ectopic expression of each of these elements could protect cells from TNF-receptor-induced apoptosis to approximately the same extent suggesting that each of these domains is necessary for mediating the apoptotic effects. These data are consistent with a model according to which several independent regions are simultaneously involved in executing the pro-apoptotic function of DAP-kinase.

Interestingly, Fragment #3 which appeared at multiple independent copies in the selection resides at the core of the death domain module. It has previously been shown that the death domain was essential for the execution of DAP-kinase function, since deletion of the entire domain from the full-length protein reduced its ability to kill cells without affecting the kinase activity (Cohen et al, 1999). Death domains in general either homodimerize or heterodimerize with other death-domain-containing proteins. The death domain of DAP-kinase did not homodimerize, nor was it capable of interacting with some of the known death domains, which suggested that it interacts with yet unidentified proteins (Cohen et al, 1999). The isolation of Fragment #3 in this screen attributed for the first time a functional role to a precise substructure in this domain. The molecular modeling of the death domain of DAP-kinase shown here, suggested that it contains 6 α-helical structures and that Fragment #3 encompasses the central three helices, implying a critical role for these helices in mediating protein-protein interactions. According to
the model-structure, the position of helices a2, a3 and a4 with respect to each other is stabilized by hydrophobic interactions among them. This observation lends support to the possibility that this 3D structure may also form when these three helices are expressed as a separate fragment, enabling it to recognize and physically associate with proteins that normally bind DAP-kinase death domain, a hypothesis that is reinforced by the specific biological activity of this fragment.

The data obtained herein, therefore, suggest that within the death domain, the central core is sufficient for critical interactions. This is consistent with mutational analysis and recent molecular modeling of the death domains of Fas and FADD which proposed that helices a2 and a3 from the two proteins are directly involved in forming the contact between them (Huang et al, 1996; Jeong et al, 1999).

Besides the death domain, the other three elements that were isolated by this screen map to regions in DAP-kinase that had not been previously shown to be necessary for the induction of cell death. Among those, the major emphasis was put in this work on the C-terminal tail. The possible unique function of the selected segments from the region encompassing the ankyrin repeats and the “linker” should be further studied in the future.

Since DAP-kinase is expressed in growing cells, its ability to induce cell death should be tightly regulated and activated only upon apoptotic triggers. One level of regulation relates to the catalytic activity, that is enhanced by binding of Ca\(^2\)/Calmodulin (Cohen et al, 1997). A second regulatory mechanism was revealed in this study and engages the C-terminal amino acid tail that immediately follows the death domain. Fragment #4, consisting of the very last 17 amino acids of DAP-kinase, can inhibit in trans the function of the complete
protein. The biological activity of this tail could hypothetically be modeled in one of two ways: (1) the tail is normally required for execution of DAP-kinase function by interaction with another protein, and excess of the peptide sequesters a cellular downstream effector; or (2) the tail is involved in negative regulation of DAP-kinase, probably through intramolecular folding that prevents interaction of other regions in DAP-kinase with downstream effectors and, therefore, excess peptide can inhibit the effect of DAP-kinase on its targets. The actual mechanism was revealed when the last 17 amino acids of the protein were deleted, and it was found that this truncation greatly potentiated the death-inducing potency of DAP-kinase when overexpressed in cells, without affecting its catalytic activity. These results are consistent with the second proposed model, according to which the tail normally serves an autoinhibitory function.

Remarkably, the presence of such serine-rich tails (although not their exact sequence) is conserved among other death domain-containing proteins (Feinstein et al, 1995). A similar regulatory function was previously described for Fas, where deletion of 15 amino acids from the C-terminus enhanced the killing activity of the receptor (Itoh et al, 1993). More recent studies proposed at least two mechanisms by which the C-terminal tail of Fas may regulate its signal transduction. One observation was that deletion of the tail enhanced the interaction between the death domains of Fas and FADD (Chinnaiyan et al, 1995). In addition, a protein phosphatase capable of binding the Fas C-terminus has been isolated (Sato et al, 1995). Although the exact role that Fas-associated phosphatase (FAP-1) plays in signal transduction is unclear (Cuppen et al, 1997; Yanagisawa et al, 1997), its association with Fas inhibited death induction in certain cell systems. In
this latter case, excess peptide which titrated out the inhibitor stimulated, rather than inhibited, apoptosis, a scenario which does not apply for DAP-kinase tail. Notably, the apparent difference in electrophoretic mobility between the WT and the tail-deleted proteins is larger than predicted by size differences alone, suggesting that the tail may undergo a post translational modification, perhaps phosphorylation, to regulate its activity.

The study described here identified functional regions in DAP-kinase and established the tools to perform a more comprehensive screen. In addition to defining key regions in DAP-kinase, the peptides which have been already identified can serve as a basis for isolation of proteins that interact with these regions in DAP-kinase, and for generating specific low molecular mass inhibitors for this protein, that can be used to modulate apoptotic processes.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.
All references cited herein, including journal articles or abstracts, published or corresponding U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by references.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.
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Chinnaiyan et al, "FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced Apoptosis", J Biol Chem 271(9):4961-4965 (1996)

Cohen et al, "DAP-kinase is a Ca2+/calmodulin-dependent, cytoskeletal-associated protein kinase, with cell death-inducing functions that depend on its catalytic activity", EMBO J 16(5):998-1008 (1997)


Liepinsh et al, "NMR structure of the death domain of the p75 neurotrophin receptor", EMBO J 16(16):4999-5005 (1997)


SEQUENCE LISTING

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        BERISSI, Hanna
        RAVEH, Tal
        FRIDKIN, Matityahu

<120> SHORT SEGMENTS OF DAP-KINASE

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Ala Met Asn Leu Gly Leu Pro Asp Leu Val Ala Lys Tyr Asn Thr Asn
  35   40   45
Asn Gly Ala Pro Lys Asp Phe Leu Pro Ser Pro Leu His Ala Leu Leu
  50   55   60
Arg Glu Trp Thr Thr Tyr Pro Glu Ser Thr Val Gly Thr Leu Met Ser
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 35 40 45
Arg Ala Leu Leu Ala Ser Trp Gly Ala Gln Asp Ser Ala Thr Leu Asp
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<223> Description of Artificial Sequence: Synthetic

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 1  5  10  15
Ser
WHAT IS CLAIMED IS:

1. An isolated peptide capable of protecting cells from apoptosis by inhibiting DAP-kinase and selected from the group consisting of:

(A) a peptide consisting of the amino acid sequence of SEQ ID NO:6 from the C-terminal tail of DAP-kinase;
(B) a DAP-kinase peptide fragment of about 48 amino acid residues in length which comprises the ankyrin repeats in DAP-kinase;
(C) a DAP-kinase peptide fragment of about 55 amino acid residues in length which comprises the linker region of DAP-kinase;
(D) a DAP-kinase peptide fragment of about 52 amino acid residues in length which comprises the death domain of DAP-15 kinase;
(E) a fragment of (A), (B), (C) or (D) which is capable of protecting cells from apoptosis by inhibiting DAP-kinase;
(F) an analog of (A), (B), (C), (D) or (E) which is capable of protecting cells from apoptosis by inhibiting DAP-kinase;
(G) a peptide of (A), (B), (C), (D), (E) or (F) which is extended at one or both of its termini by one to four amino acid residues;
(H) a peptide of (A), (B), (C), (D), (E) or (F) which is extended at one or both of its termini with Asp or Glu residues; and
(I) a derivative of (A), (B), (C), (D), (E), (F), (G) or (H) which is capable of protecting cells from apoptosis;

2. The isolated peptide of claim 1, which is a peptide consisting of the amino acid sequence of SEQ ID NO:6.
3. The isolated peptide of claim 1, which is a DAP-kinase peptide fragment of about 48 amino acid residues in length which comprises the ankyrin repeats in DAP-kinase;

4. The isolated peptide of claim 1, which is a DAP-5 kinase peptide fragment of about 55 amino acid residues in length which comprises the linker region of DAP-kinase;

5. The isolated peptide of claim 1, which is a DAP-kinase peptide fragment of about 52 amino acid residues in length which comprises the death domain of DAP-kinase;

6. The isolated peptide of claim 1, which is a fragment of (A), (B), (C) or (D) which is capable of protecting cells from apoptosis by inhibiting DAP-kinase;

7. The isolated peptide of claim 1, which is an analog of (A), (B), (C), (D) or (E) which is capable of protecting cells from apoptosis by inhibiting DAP-kinase; and

8. The isolated peptide of claim 1, which is a peptide of (A), (B), (C), (D), (E) or (F) which is extended at one or both of its termini by one to four amino acid residues;

9. The isolated peptide of claim 1, which is a peptide of (A), (B), (C), (D), (E) or (F) which is extended at one or both of its termini with Asp or Glu residues;

10. The isolated peptide of claim 1, which is a derivative of (A), (B), (C), (D), (E), (F), (G) or (H) which is capable of protecting cells from apoptosis;

11. A pharmaceutical composition for protecting cells from the apoptotic activity of DAP-kinase, comprising an effective amount of a peptide according to any one of claims 1 to 10 and a pharmaceutically acceptable carrier.

12. Use of the peptide of any one of claims 1 to 10 in the manufacture of a medicament for inhibiting the apoptotic activity of DAP-kinase.
13. A method for inhibiting apoptosis associated with the activity of DAP kinase, comprising administering to a subject in need thereof an effective amount of the peptide of any one of claims 1 to 10 to neutralize the effect of DAP kinase and to inhibit apoptosis associated with DAP kinase.

14. A polynucleotide encoding the peptide of any one of claims 2 to 9.

15. A vector comprising the polynucleotide of claim 14.

16. A host cell transformed with the vector of claim 15.

17. A method for screening fragments of a gene product, mediating a selectable phenotype, for capability of acting in a dominant negative manner when expressed ectopically, comprising:

   fragmenting a cDNA encoding a gene product, which mediates a selectable phenotype, to obtain random cDNA fragments;

   inserting the random cDNA fragments into an EBV-based episomal shuttle vector capable of propagation in bacterial and mammalian cells to generate a library of random cDNA fragments, wherein the random cDNA fragments are operably linked to a promoter for expressing the random cDNA fragments and the EBV-based episomal shuttle vector has a selectable marker and an interferon responsive enhancer element which stimulates the expression of the random cDNA fragment from the operably-linked promoter;

   transforming mammalian host cells with the library of random cDNA fragments in the EBV-based episomal shuttle vector to obtain transformed host cells;
selecting for transformed host cells that act in a dominant negative manner to the selectable phenotype of the gene product; and

isolating a cDNA fragment which encodes a peptide 5 fragment of the gene product which acts in a dominant negative manner to the gene product.

18. The method of claim 17, wherein the mammalian host cells are human cells.

19. A peptide fragment encoded by the cDNA fragment isolated according to the method of claim 17.

20. The invention as shown and described in the drawings and the disclosure.
Protective fragments of DAP-kinase

<table>
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<tr>
<th>Fragment</th>
<th>Size (amino acids)</th>
<th>Position in DAP-K</th>
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<tr>
<td>#1</td>
<td>48 (451-498)</td>
<td>Ankyrin repeats</td>
</tr>
<tr>
<td>#2</td>
<td>55 (1142-1196)</td>
<td>'Linker' region</td>
</tr>
<tr>
<td>#3</td>
<td>52 (1320-1371)</td>
<td>Death domain</td>
</tr>
<tr>
<td>#4</td>
<td>17 (1415-1431)</td>
<td>C-terminal tail</td>
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</table>

![Diagram showing the position of fragments](image-url)

**FIGURE 2**
FIGURE 3A

TNF-induced apoptosis in 293 cells

FIGURE 3B

DAP-kinase-induced apoptosis in 293 cells

FIGURE 3C

TNF-induced apoptosis in MCF7 cells
FIG. 4A

DAP-kinase: DIHASDLNLLETRKLSRRLLDPDDPLGDWCLLAMNLGL-PDLVAKYNTNN
p75: GSNYYSLPLTKKEEVEKLLN--------GDWRHLAGEGQPEHIDSPTEA

DAP-kinase: GAPKDFLFSPLEHALLRWVTYESTVQILSNKLPGLGRRDAADLLLKASS
p75: ---------CPVRALLASGQAQDSATDLALLAALRRIQRADIVESLCESE

FIG. 4B
FIG. 4C
DAP-kinase death domain protects from C6-Ceramide

% Apoptosis

1  pcDNA3
2  Death Domain
3  mutant DD

FIGURE 6
15 micro M Ceramide

Peptide (micro M)

FIGURE 8
Toxicity induced by incubation with C6-Ceramide (15uM, 16h) and blocked by preincubation with DAPK pep (30uM, 1h).

FIGURE 9