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(54) Title: A NOVEL Bak BINDING PROTEIN, DNA ENCODING THE PROTEIN, AND METHODS OF USE THEREOF

(57) Abstract

The present invention provides polynucleotide sequences (bp) encoding a Bak Binding Protein (BBP) and fragments thereof that bind to Bak. The invention also provides a BBP which binds to Bak. The invention also provides recombinant host cells containing polynucleotides encoding BBP. The invention further provides antibodies that specifically bind to BBP. The invention further provides methods for detecting agents such as drugs that alter the binding of a BBP with a Bak protein. The invention further provides methods for detecting the presence of bbp or BBP in a biological sample, and further provides methods for modulating the levels of BBP in a cell. This invention additionally encompasses novel peptides, designated the "BBP Binding Domains" and the respective polynucleotides, designated "bpbbd-1" and "bpbbd-2" which are involved in the interaction between Bak and BBP.
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A NOVEL Bak BINDING PROTEIN, DNA ENCODING THE PROTEIN, AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Provisional Applications Numbers 60/041,328, filed March 20, 1997 and 60/071,097, filed January 9, 1998.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

(Not Applicable)

FIELD OF THE INVENTION

The present invention relates to DNA encoding a protein that binds to a protein involved in apoptosis. The invention further relates to methods for identifying agents that modulate activity levels of a protein binding to a protein involved in apoptosis. The invention additionally encompasses novel peptides, designated the “BBP Binding Domains” which are involved in the interaction between a protein involved in apoptosis and a protein that binds to it.
SUMMARY OF THE INVENTION

Substantially purified DNA encoding a novel Bak binding protein, termed Bak Binding Protein, or BBP, is provided. The substantially purified BBP protein and compositions thereof are also provided. Diagnostic and therapeutic methods utilizing the DNA and proteins are also provided. Methods of screening for pharmaceutical agents that modify Bak and BBP activity levels are also provided. The invention additionally encompasses novel peptides, designated the “BBP Binding Domains” and novel nucleotides, designated “bbpdb-1” and “bbpdb-2” encoding the peptides, which are involved in the interaction between a protein involved in apoptosis and a protein that binds to it.

The present invention encompasses an isolated polypeptide comprising SEQ ID NO:7, isolated polypeptides comprising a linear sequence of six or more amino acids of SEQ. ID NO:7, isolated polypeptides having at least one of the biological functions of the polypeptide of SEQ ID NO:7, isolated polypeptides comprising a fragment of SEQ ID NO:7, wherein said fragment binds to Bak protein under appropriate conditions and fusion polypeptides comprising the polypeptide of SEQ ID NO:7 or fragments thereof.

The present invention also encompasses isolated polynucleotides comprising SEQ. ID NO:6 and polynucleotide sequences complementary thereto, isolated polynucleotides comprising a fragment of at least 18 consecutive nucleotides of SEQ ID NO:6, isolated polynucleotides encoding the polypeptide of SEQ ID NO:7, isolated polynucleotides comprising a sequence that encodes a polypeptide having at least one of the biological functions of the polypeptide of SEQ ID NO:7 and a polynucleotide complementary thereto, isolated polynucleotides comprising a fragment of SEQ ID NO:6, wherein said fragment encodes a polypeptide that binds to Bak protein under appropriate conditions, and any of the aforementioned isolated polynucleotide, which are operably linked to control sequences for expression.
Also encompassed by the present invention are recombinant vectors comprising any of the aforementioned polynucleotides, as well as recombinant host cells modified to contain the polynucleotides, wherein the recombinant host cells specifically can be bacterial or eukaryotic.

Also encompassed by the present invention are methods for screening potential therapeutic agents that modulate the interaction between Bak and BBP comprising the steps of: (a) combining a Bak and a BBP under conditions in which they interact, to form a test sample; (b) exposing the test sample to a potential therapeutic agent and; (c) monitoring the interaction of the Bak and the BBP, wherein a potential therapeutic agent is selected for further study when it modifies the interaction compared to a control test sample to which no potential therapeutic agent has been added. In one embodiment, the potential therapeutic agent is selected from the group consisting of a pharmaceutical agent, a cytokine, a small molecule drug, a cell-permeable small molecule drug, a hormone, a combination of interleukins, a lectin, a stimulating agent, a bispecific antibody, a peptide mimetic, and an antisense oligonucleotide. In another embodiment, the Bak is selected from the group consisting of Bak, a fragment of Bak sufficient to effect binding to a BBP, and a fusion protein comprising a portion of Bak sufficient to effect binding to a BBP. The fusion protein can comprise epitope-tagged Bak. In one embodiment, the BBP is selected from the group consisting of epitope-tagged BBP and proteins homologous to SEQ ID NO:7. In one embodiment of the present invention, the monitoring step is selected from the group consisting of co-precipitation, protein interactive trapping and ELISA.

The present invention also encompasses compositions comprising a monoclonal or polyclonal antibody or an antigen-binding fragment thereof which forms a complex with a BBP but is substantially unreactive with dissimilar proteins.
The present invention further encompasses a method of detecting the presence of a BBP protein in a biological sample comprising the steps of: a) obtaining a cell sample; b) exposing the contents of the cells to antibodies; c) adding anti-BBP-specific antibodies to the cell sample; d) maintaining the cell sample under conditions that allow the antibodies to complex with the BBP; and e) detecting the antibody-BBP complexes formed.

In one embodiment, a method is provided for detecting the expression of a bbp gene in a biological sample comprising the steps of identifying the presence of RNA encoding the bbp. In one embodiment, identification comprises Northern blotting.

Also encompassed by the present invention are methods identifying bbp mRNA comprising the steps of: (a) obtaining a cell sample; (b) obtaining RNA from the cell sample; (c) performing a polymerase chain reaction on the RNA using primers corresponding to unique regions of bbp; and (d) detecting the presence of products of the polymerase chain reaction.

The present invention also provides methods of modulating apoptosis-induced cell death comprising modulating the endogenous levels of BBP. In a specific embodiment, the BBP levels are increased or decreased by modulating expression of an endogenous bbp gene. In one embodiment, the BBP is encoded by an endogenous gene. Alternatively, the BBP is encoded by a recombinant gene, wherein in a specific embodiment, expression of the recombinant gene is under the control of an inducible promoter. In one embodiment, the recombinant gene is transfected into cells ex vivo and further comprising the steps of reintroducing the transfected cells into an animal. Alternatively, the recombinant gene is transfected into cells in vivo.

The present invention also encompasses methods of inducing apoptosis in a patient in need thereof comprising administering a therapeutically effective amount of the BBP.
The present invention further encompasses isolated polypeptides comprising amino acids 103-126 of SEQ ID No: 2, or derivatives thereof.

Also encompassed are isolated and purified peptides comprising a BBP Binding Domain, isolated polypeptides comprising a linear sequence of six or more amino acids of a BBP Binding Domain, isolated polypeptides having at least one of the biological functions of a BBP Binding Domain, or isolated polypeptides comprising a fragment of a BBP Binding Domain wherein said fragment binds to BBP protein under appropriate conditions. Also encompassed are fusion polypeptides comprising a BBP Binding Domain or fragments thereof.

The present invention further encompasses isolated polynucleotides comprising nucleotides 507-578 of SEQ. ID NO:1, and polynucleotide sequences complementary thereto, isolated polynucleotides comprising a fragment of at least 18 consecutive nucleotides of bbpdb-1, isolated polynucleotides comprising nucleotides 611-668 of SEQ. ID NO:1, and polynucleotide sequences complementary thereto, isolated polynucleotides comprising a fragment of at least 18 consecutive nucleotides of bbpdb-2, isolated polynucleotides encoding a BBP Binding Domain, isolated polynucleotide comprising a sequence that encodes a polypeptide having at least one of the biological functions of a BBP Binding Domain and a polynucleotide complementary thereto and any of these isolated polynucleotides which is operably linked to control sequences for expression.

In one embodiment, a recombinant vector comprises these polynucleotides. In a specific embodiment, recombinant host cells are modified to contain the polynucleotides. In a specific embodiment, these host cells are bacterial or eukaryotic.

Also encompassed by the present invention are methods of modulating apoptosis-induced cell death comprising modulating the endogenous levels of a BBP Binding Domain, wherein the BBP Binding Domain levels can be increased or decreased by modulating expression of an endogenous bak gene. In alternative embodiments, the BBP Binding Domain is encoded by an endogenous gene or a recombinant gene. In one embodiment, the expression of the recombinant gene is
under the control of an inducible promoter. In alternative embodiments, the
recombinant gene is transfected into cells \textit{ex vivo} and further comprising the steps
of reintroducing the transfected cells into an animal, or the recombinant gene is
transfected into cells \textit{in vivo}.

Also encompassed by the present invention are methods of modulating
apoptosis in a patient in need thereof comprising administering a therapeutically
effective amount of a BBP Binding Domain.

The present invention additionally encompasses isolated polypeptides
comprising amino acids 138-156 of SEQ ID No: 2, or derivatives thereof.

\section*{BACKGROUND}

Apoptosis, or programmed cell death, is a normal physiologic process that
leads to individual cell death. This process of programmed cell death is involved
in a variety of normal and pathogenic biological events and can be induced by a
number of unrelated stimuli. Changes in the biological regulation of apoptosis
also occur during aging and are responsible for many of the conditions and
diseases related to aging. Recent studies of apoptosis have implied that a common
metabolic pathway leading to cell death can be initiated by a wide variety of
signals, including hormones, serum growth factor deprivation, chemotherapeutic
agents, ionizing radiation and infection by human immunodeficiency virus (HIV).
Today} 12:102; and Sheppard and Ascher (1992) \textit{J. AIDS} 5:143. Agents that
modulate the biological control of apoptosis thus have therapeutic utility in a wide
variety of conditions.

While apoptosis is a normal cellular event, it can also be induced by pathological conditions and a variety of injuries. Apoptosis is involved in a wide variety of conditions including but not limited to, cardiovascular disease; cancer regression; immune disorders, including but not limited to systemic lupus erythematosus; viral diseases; anemia; neurological disorders; diabetes; hair loss; rejection of organ transplants; prostate hypertrophy; obesity; ocular disorders; stress; aging; and gastrointestinal disorders, including but not limited to, diarrhea and dysentery. In the myocardium, apoptotic cell death follows ischemia and reperfusion.

In Alzheimer's disease, Parkinson's disease, Huntington's chorea, epilepsy, amyotrophic lateral sclerosis, stroke, ischemic heart disease, spinal cord injury and many viral infections, for example, abnormally high levels of cell death occur. In at least some of these diseases, there is evidence that the excessive cell death occurs through mechanisms consistent with apoptosis. Among these are 1) spinal cord injury, where the severing of axons deprives neurons of neurotrophic factors necessary to sustain cellular viability; 2) stroke, where after an initial phase of necrotic cell death due to ischemia, the rupture of dead cells releases excitatory neurotransmitters such as glutamate and oxygen free radicals that stimulate apoptosis in neighboring healthy neurons; and 3) Human Immunodeficiency Virus (HIV) infection, which induces apoptosis of T-lymphocytes.

In contrast, the level of apoptosis is decreased in cancer cells, which allows the cancer cells to survive longer than their normal cell counterparts. As a result of the increased number of surviving cancer cells, the mass of a tumor can increase even if the doubling time of the cancer cells does not increase. Furthermore, the high level of expression in a cancer cell of the bcl-2 gene, which is involved in regulating apoptosis and, in some cases, necrotic cell death, renders
the cancer cell relatively resistant to chemotherapeutic agents and to radiation therapy.

In recent years, a family of proteins has been discovered that controls apoptosis. The prototype of this family is Bcl-2, a protein that inhibits most types of apoptotic cell death and is thought to function by regulating an antioxidant pathway at sites of free radical generation. Hockenbery et al. (1993) Cell 75:241-251. More recent data suggests that Bcl-2 can also function as a channel protein and as an adaptor/docking protein. Reed, et al. (1997) Nature 387:773-776. Together, the Bcl-2 family of proteins are important intracellular modulators of apoptosis and can be divided into two groups based on their effect on apoptosis. Thus, in a general sense, Bcl-2, Bcl-xL, Mcl-1, BHRF-1 and E1B19K are cell death inhibitors (anti-apoptotic), while Bak, Bax and Bcl-xS accelerate cell death (pro-apoptotic).

Bcl-2 family members are generally localized to the outer mitochondrial membrane, the nuclear membrane and the endoplasmic reticulum, where they associate with membranes by virtue of their C-terminal hydrophobic tail. All members of the family have two highly conserved regions, called BH1 and BH2, that permit specific interactions between two members to form stable dimers. Their mechanism of action is presently unclear; however, it is known that the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family members in a cell is critical to the cell’s survival following initiation of an apoptotic signal.

Proteins that interact with and alter the activity of Bcl-2 have been described. For example, BAG-1 binds Bcl-2, enhances the anti-apoptotic effect of Bcl-2 and furthermore activates Raf-1. Wang et al. (1996) Proc. Natl. Acad. Sci. USA 93:7063-7068; Takayama et al. (1995) Cell 80:279-284. Other proteins with bcl-2 binding activity include the ras-related R-ras p23, BAP and Bad. Fernandez-Sarabia and Bischoff (1993) Nature 366:274-275; U.S. Patent No. 5,539,085; U.S. Patent No. 5,539,094; PCT Application WO 96/13614. Identification of such proteins is of great importance, since an understanding of the protein-protein interactions in which apoptosis-related proteins are involved can not only provide
insights into the mechanisms of action of these proteins, but can also provide a focal point toward which apoptosis-modulating therapies can be designed. For example, disruption of a protein-protein interaction between an apoptosis-related protein and a protein which enhances its function can decrease the level of apoptosis in a cell. This may be a desired effect in a tissue which displays an inappropriately high level of apoptosis.

Bak is a member of the Bcl-2 family and is expressed in heart and other tissues. Bak protein is capable of either killing cells, or actively protecting cells from cell death, depending on how this protein interacts with other cellular proteins. Bcl-2 family members are extremely important in determining the fate of a cell following an apoptotic signal, and Bak may be the most important in the major organs such as heart. In the treatment of heart disease, viral infection and cancer, modulation of the interactions between proteins that control apoptosis is a major focal point.

Accordingly, there is a need to identify the proteins that operate to control cell death, and to develop therapeutic reagents that modify the actions of those proteins. The present invention relates to a novel Bak binding protein (BBP), the gene encoding the novel protein, methods for detecting substances that alter the specific binding between Bak and BBP, as well as diagnostic and therapeutic methods utilizing BBP. The invention additionally encompasses novel peptides, designated the “BBP Binding Domains” and novel nucleotides, designated “bbpdb-1” and “bbpdb-2” (collectively “bbpdb”) encoding the peptides, which are involved in the interaction between a protein involved in apoptosis and a protein that binds to it.

All references cited herein are hereby incorporated by reference in their entirety.
BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the strategy used to isolate BBP clones.

Figure 2 depicts the cDNA sequence (SEQ ID NO:1 and 3) of bak and the translated amino sequence of Bak (SEQ ID NO:2).

Figure 3 depicts the Bak (SEQ ID NO:2) derivative proteins BakΔ2-TM (SEQ ID NO:4) and BakΔ3 (SEQ ID NO:5). The N-terminal residues are indicated by rightward arrows. The C-terminus of BakΔ2-TM is indicated by the leftward arrow.

Figure 4 depicts the cDNA sequence (SEQ ID NO:6) of bbp and the translated amino acid sequence (SEQ ID NO:7) of BBP.

Figure 5 depicts the results of a Northern blot analysis of multiple tissues with probes specific for BBP.

Figure 6 summarizes the interactions of BBP with other proteins.

Figure 7 shows the results of Western blot analysis of the interactions between BBP, BBP derivatives, BHRF-1, E1B19K and Bak.

Figure 8 shows the results of Western blot analysis of the interactions between BBP-Loop1, BBP-Loop2 and the Bak derivatives Bak 1-50 (Figure 8A) and BakΔ3 (Figure 8B).

Figure 9 depicts the amino acid sequence of Bak and the regions of Bak that interact with BBP loop 1. Each horizontal line above the amino acid sequence shown indicate locations on the Bak protein where BBP loop1 interacted with a 15 amino acid peptide having the sequence shown directly below the respective line.

MODES OF CARRYING OUT THE INVENTION

The present invention encompasses substantially purified nucleotide sequences encoding the novel Bak Binding Protein (BBP); the protein encoded thereby; methods of screening potential therapeutic agents that modify the
interaction between BBP and proteins that bind thereto; and methods for detecting substances that alter the specific binding between Bak and BBP. The invention additionally encompasses novel peptides, designated the "BBP Binding Domains" which are involved in the interaction between a protein involved in apoptosis and a protein that binds to it.

The following definitions are for the purpose of clarifying the terms used herein, and are not meant to be limiting. Where definitions or techniques are specified with reference to BBP or bbp, the general teachings provided apply also to BBP Binding Domains or bbpbd, respectively.

The present invention encompasses the polypeptide BBP and modifications of BBP that retain at least one of the biological functions of BBP. The present invention also encompasses the polypeptides BBP Binding Domains and modifications of BBP Binding Domains that retain at least one of the biological functions of BBP Binding Domains. The terms "polypeptide", "peptide" or "protein" are used interchangeably herein. By "polypeptide" is meant a linear sequence of ten or more amino acids.

By "biologically effective amount" and "therapeutically effective amount" is meant a concentration of the component able to improve or ameliorate a condition related to apoptosis.

The term "BBP" encompasses polypeptide and polypeptide fragments of BBP (SEQ ID NO:7) containing at least a portion of BBP that binds to Bak protein or a fragment thereof, or has any other biological or other useful function characteristic of BBP. The term "BBP Binding Domains" encompasses polypeptides and polypeptide fragments of Bak containing at least a portion of Bak protein that binds to BBP or a fragment thereof, or has any other biological or other useful function characteristic of BBP Binding Domains. A BBP is defined primarily by its ability to associate in vitro and in vivo with a Bak protein. A BBP Binding Domain is defined primarily by its ability to associate in vitro and in vivo with BBP. As used herein, "associate" or "interact" or "bind" means that a BBP and a Bak protein have a binding affinity for each other such that the BBP and a
Bak protein or BBP Binding Domain form a bound complex. The affinity of binding of a BBP and a Bak protein is sufficiently specific such that the bound complex can form in vivo in a cell and can form in vitro under appropriate conditions, as described herein. The formation or dissociation of a bound complex can be identified as described in the Examples or using other well known methods.

The size of the BBP or BBP Binding Domain polypeptide fragments can be only the minimum size required to provide a desired function. It can optionally comprise additional amino acid sequence, either native to BBP or BBP Binding Domain or from a heterologous source, as desired. BBP or BBP Binding Domain fragments may contain only ten consecutive amino acids from a BBP or BBP Binding Domain amino acid sequence. Polypeptides comprising ten amino acids, more preferably about 15 amino acids, more preferably about 25 amino acids, more preferably about 50 amino acids from the sequence are also included. Even more preferred are polypeptides comprising the entire BBP or BBP Binding Domain amino acid sequence.

The term “BBP” further encompasses a substantially purified preparation of BBP (SEQ ID NO:7), or a fragment of BBP that is an antigenic polypeptide containing from six to 151 amino acid residues of BBP (preferably at least six, more preferably at least 12, and most preferably at least 18), which polypeptide fragment contains an epitope of BBP such that an antibody raised against the fragment (or against a conjugate of the polypeptide and a carrier, including, but not limited to, keyhole limpet hemocyanin) forms an immune complex with BBP itself.

The term “BBP Binding Domain” further encompasses a substantially purified preparation of BBP Binding Domain, or a fragment of BBP that is an antigenic polypeptide containing from six to 23 amino acid residues of a BBP Binding Domain (preferably at least six, more preferably at least 12, and most preferably at least 18), which polypeptide fragment contains an epitope of a BBP Binding Domain such that an antibody raised against the fragment (or against a
conjugate of the polypeptide and a carrier, including, but not limited to, keyhole limpet hemocyanin) forms an immune complex with a BBP Binding Domain itself.

Such antibodies can be either monoclonal or polyclonal, and are generated by standard methods including the step of immunizing an animal with an antigen containing an antigenic portion of BBP or BBP Binding Domain.

The terms "BBP" and "BBP Binding Domains" also include modified polypeptides that are functionally equivalent to BBP or BBP Binding Domain, respectively, or fragments thereof. Modifications include addition, insertion or deletion of amino acids. Other examples of modified polypeptides include polypeptides with conservative substitutions in comparison with the prototype BBP or BBP Binding Domain sequence. Substitutions can include changing or modifying one or more amino acids, and insertion of heterologous amino acid sequences. Amino acid substitutions, if present, include conservative substitutions that do not deleteriously affect folding or functional properties of the peptide. Groups of functionally related amino acids within which conservative substitutions can be made are glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tryptophan/tyrosine. Polypeptides of this invention can be modified post-translationally (e.g., acetylation or phosphorylation) or can be modified synthetically (e.g., the attachment of a labeling group).

Some fragments of BBP or BBP Binding Domain or the modified amino acids encompassed by the present invention may lack some biological activities characteristic of native proteins (e.g., binding to Bak or BBP, respectively) but may have activities, and therefore be useful for other purposes, such as for raising antibodies to BBP or BBP Binding Domain epitopes, as an immunological reagent to detect and/or purify BBP or BBP Binding Domain-binding antibodies by affinity chromatography, or as a competitive or noncompetitive agonist,
antagonist, or partial agonist of native BBP or BBP Binding Domain protein function.

The invention also encompasses BBP or BBP Binding Domain conjugated to a label capable of producing a detectable signal or other functional moieties. Suitable labels include, but are not limited to, radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent dyes, chemiluminescent dyes, bioluminescent compounds and magnetic particles. See, for examples of patents teaching the use of such labels, U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

The invention further comprises fusion proteins comprising (1) BBP or BBP Binding Domain or fragments thereof, covalently attached to (2) a second polypeptide. Such fusion polypeptides can be made by any of a number of standard techniques well known to those of ordinary skill, including recombinant methods, in which case the covalent attachment is a peptide bond, or chemical conjugation, in which case the covalent attachment is another type of bond, such as a disulfide bond. A BBP or BBP Binding Domain fusion polypeptide can be prepared, for example, by chemical synthesis, or by creating and translating a polynucleotide in which the peptide regions are encoded in the desired relationship. Useful heterologous sequences for inclusion in a fusion polypeptide include sequences that provide for secretion from a host cell, sequences that enhance immunological reactivity relative to BBP or the BBP fragment, or BBP Binding Domain or the respective fragment, sequences that facilitate the coupling of the polypeptide to an immunoassay support or a vaccine carrier, portions of an immunoglobulin molecule, immunological tags, or sequences that mediate specific binding to a cell surface or intracellular receptor.

The polypeptides described above can be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding the desired peptide sequence. Alternatively, polypeptides can be synthesized by chemical methods.
Purification or isolation of BBP or BBP Binding Domain expressed either
by recombinant DNA or from biological sources such as tissues can be
accomplished by any method known in the art. Protein purification methods are
known in the art. Generally, the terms “substantially purified” or “substantially
isolated” or “isolated” are used interchangeably to indicate proteins which are free
of other, contaminating cellular substances, particularly proteins. Preferably, the
purified BBP or BBP Binding Domain is more than eighty percent pure and most
preferably more than ninety-five percent pure.

Suitable methods of protein purification are known in the art and include,
but are not limited to, affinity chromatography, immunoaffinity chromatography,
size exclusion chromatography, HPLC and FPLC. Any purification scheme that
does not result in substantial degradation of the protein is suitable for use in the
present invention.

The invention further encompasses isolated polynucleotides (or nucleic
acids) encoding polypeptides substantially identical to BBP or BBP Binding
Domain or portions thereof. The term polynucleotide as used herein, can be DNA
or RNA, either coding or noncoding strands. Similarly “bbp” includes bbp cDNA,
genomic DNA and synthetic or semi-synthetic DNAs and RNAs and are
additional embodiments of the present invention.

Also included are isolated polynucleotides encoding polypeptides
substantially identical to allelic variants of SEQ ID NO:7 or to homologs of BBP
or BBP Binding Domain from species other than man. The isolated
polynucleotide preferably contains a DNA sequence that hybridizes under
stringent conditions (as defined below) with the DNA sequence of SEQ ID NO:6,
or the complement thereof, and can contain the sequence of SEQ ID NO:6. It is
preferably incorporated into a vector (a virus, phage, or plasmid) which can be
introduced by transfection or infection into a cell. The vector preferably includes
one or more expression control sequences, in which case the cell transfected by
the vector is capable of expressing the polypeptide.
By “isolated nucleotide” is meant a single- or double-stranded nucleotide that is free of the genes which, in the naturally-occurring genome of the animal from which the isolated nucleotide is derived, flank the bbp gene or the bbpbδ sequence. The term therefore includes, for example, either or both strands of a cDNA encoding the nucleic acid of interest, or an allelic variant thereof; a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryotic or eukaryotic cell; or a genomic DNA fragment (e.g., produced by PCR (polymerase chain reaction) or restriction endonuclease treatment of human or other genomic DNA). It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

Stringent conditions for both DNA/DNA and DNA/RNA hybridization assays are as described by Sambrook et al. *Molecular Cloning, A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, herein incorporated by reference. For example, see page 7.52 of Sambrook et al.

Also within the invention is an isolated DNA at least 15 nucleotides in length (preferably at least 30, more preferably at least 100, and most preferably at least 450), including (a) a strand which hybridizes under stringent conditions to a DNA having the sequence of SEQ ID NO:6, (b) the complement thereof, or (c) a double-stranded DNA including both (a) and (b). Multiple copies of this isolated DNA (useful, for example, as a hybridization probe or PCR primer) can be produced by recombinant means, by transfecting a cell with a vector containing this DNA.

The invention includes modifications to bbp or bbpbδ DNA sequences such as deletions, substitutions and additions particularly in the non-coding regions of genomic DNA. Such changes are useful to facilitate cloning and modify gene expression.
Various substitutions can be made within the coding region that either do not alter the amino acid residues encoded or result in conservatively substituted amino acid residues. Nucleotide substitutions that do not alter the amino acid residues encoded are useful for optimizing gene expression in different systems. Suitable substitutions are known to those of skill in the art and are made, for instance, to reflect preferred codon usage in the particular expression systems.

The invention encompasses functionally equivalent variants and derivatives of bbp or bbpdb, which may enhance, decrease or not significantly affect the properties of BBP or BBP Binding Domain, respectively. "Derivatives" are defined herein as changes in the DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect its properties.

The above-mentioned polynucleotides can include control sequences that facilitate transcription (expression sequences) and translation of the coding sequences, such that the encoded polypeptide product is produced. Such control sequences include, but are not limited to, promoters, transcription termination sites, polyadenylation sites, ribosome binding sites, enhancers or sequences necessary for replication of a vector.

As described herein, hybridization analysis of Chinese Hamster somatic cell hybrids, each containing one human chromosome, indicated that bbp or genes closely related thereto, are located on chromosomes 2, 5 and X.

The complete Bak nucleotide and protein sequences are described in PCT Application No. PCT/US94/13930; Kiefer et al. (1995) Nature 374:736-739. As used herein, “bak” refers to the polynucleotide molecule, and derivatives thereof, described in the above-referenced patent application; “Bak” refers to the proteins encoded thereby and derivatives thereof.

The bbp or bbpbd cDNA or genomic DNA can be incorporated into vectors for further manipulation. Furthermore, the invention concerns a recombinant DNA which is a recombinant vector comprising at least one of the above mentioned genes.

The recombinant vectors of the invention comprise an origin of replication or an autonomously replicating sequence, one or more dominant marker sequences and, optionally, expression control sequences, signal sequences and additional restriction sites.

Preferably, the recombinant vector of the invention comprises an above described nucleic acid insert operably linked to an expression control sequence, in particular those described hereinafter.

Vectors typically perform two functions in collaboration with compatible host cells. One function is to facilitate the cloning of the bbp gene or bbpbd, i.e., to produce useable quantities of the nucleic acid (cloning vectors). The other function is to provide for replication and expression of the gene constructs in a suitable host, either by maintenance as an extrachromosomal element or by integration into the host chromosome (expression vectors). A cloning vector comprises the DNAs as described above, an origin of replication or an autonomously replicating sequence, selectable marker sequences, and optionally, signal sequences and additional restriction sites. An expression vector additionally comprises expression control sequences essential for the transcription and translation of the bbp gene or bbpbd. Thus, an expression vector refers to a recombinant DNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into a suitable host cell, results in expression
of the cloned DNA. Suitable expression vectors are well known in the art and include those that are replicable in eukaryotic and/or prokaryotic cells.

Most expression vectors are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *Escherichia coli* and then the same vector is transfected into yeast or mammalian cells even though it is not capable of replicating independently of the host cell chromosome. DNA can also be amplified by insertion into the host genome. However, the recovery, for example, of the genomic bbp gene is more complex than that of exogenously replicated vector because restriction enzyme digestion is required to excise the gene. DNA can be amplified by PCR and be directly transfected into the host cells without any replication component.

Advantageously, expression and cloning vectors contain a selection gene also referred to as selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, e.g., ampicillin, neomycin, methotrexate or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available from complex media.

Since the amplification of the vectors is conveniently done in *E. coli*, an *E. coli* genetic marker and an *E. coli* origin of replication are advantageously included. These can be obtained from *E. coli* plasmids, such as pBR322, Bluescript vectors or a pUC plasmid or any other similar plasmid vector.

Suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the bbp gene or bbpb, such as dihydrofolate reductase (DHFR, methotrexate resistance), thymidine kinase, or genes conferring resistance to G418 or hygromycin. The mammalian cell transfectants are placed under selection pressure which only those transfectants are uniquely adapted to survive which have taken up and are expressing the marker.
Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the bbp gene or bbpbd. Suitable promoters can be inducible or constitutive. The promoters are operably linked to the gene of interest by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native promoter sequence and many heterologous promoters can be used to direct amplification and/or expression of a gene or fragment of interest. However, heterologous promoters are preferred, because they generally allow for greater transcription and higher yields of BBP or BBP Binding Domain as compared to native promoter.

Promoters suitable for use with prokaryotic hosts include, for example, the β-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system and hybrid promoters such as the tac promoter. Their nucleotide sequences have been published thereby enabling the skilled worker to ligate them to the gene of interest using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems will also generally contain a Shine-Dalgarno sequence operably linked to the gene.

Gene transcription from vectors in mammalian host cells can be controlled by promoters compatible with the host cell systems, e.g., promoters derived from the genomes of viruses. Suitable plasmids for expression of bbp or fragments of bak, such as bbpbd, in eukaryotic host cells, particularly mammalian cells, include, but are not limited to, cytomegalovirus (CMV) promoter-containing vectors, Rous Sarcoma Virus (RSV) promoter-containing vectors and SV40 promoter-containing vectors and MMTV LTR promoter-containing vectors. Depending on the nature of their regulation, promoters can be constitutive or regulatable by experimental conditions. Transcription of bbp or bak fragments according to the invention by higher eukaryotes can be increased by inserting an enhancer sequence into the vector. These promoters can also be cell type-specific, that is, inducible only in a particular cell type and often only during a specific
period of time. The promoter can further be cell cycle specific, that is, induced or inducible only during a particular stage in the cell cycle.

The various DNA segments of the vector DNA are operably linked, i.e., they are contiguous and placed into a functional relationship to each other.

Construction of vectors according to the invention employs conventional ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored and religated in the form desired to generate the plasmids required. If desired, analysis to confirm correct sequences in the constructed plasmids is performed in a manner known in the art. Suitable methods for constructing expression vectors, preparing in vitro transcripts, introducing DNA into host cells, and performing analyses for assessing bbp expression and function are known to those skilled in the art. Gene presence, amplification and/or expression can be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA, dot blotting (DNA or RNA analysis), in situ hybridization, using an appropriately labeled probe based on a sequence provided herein, binding assays, immunodetection and functional assays.

Nucleotides can also be expressed in non-human transgenic animals, particularly transgenic warm-blooded animals. Methods for producing transgenic animals, including mice, rats, rabbits, sheep and pigs, are known in the art and are disclosed, for example by Hammer et al. (1985) Nature 315:680-683. An expression unit including a gene of interest together with appropriately positioned expression control sequences, is introduced into pronuclei of fertilized eggs. Introduction can be achieved, e.g., by microinjection. Integration of the injected DNA is detected, e.g., by blot analysis of DNA from suitable tissue samples. It is preferred that the introduced DNA be incorporated into the germ line of the animal so that it is passed to the animal's progeny.

Furthermore, a knock-out animal can be developed by introducing a mutation in the bbp or bbpbd sequence, thereby generating an animal which no longer expresses the functional bbp gene or bbpbd fragment. Such knock-out
animals are useful, e.g., for studying the role of the BBP or BBP Binding Domain in apoptosis or other physiological functions.

More specifically, a knock-out animal can be developed (i.e., an animal that does not express the endogenous gene of interest), in which one introduces a mutated or wild-type gene. Methods for producing knock-out mice are known in the art. The knock-out animals are useful not only for studying the role of a given protein, as exemplified by published studies (see, e.g., Conquet et al. (1994) Nature 372:237-243; Aiba et al. (1994) Cell 79:365-375; and Masu et al. (1995) Cell 80:757-765), but also and, in particular, for providing a mammalian animal model with a suitable genetic background for introducing and expressing transgenes encoding the protein.

The nucleic acid sequences provided herein can be employed to identify DNAs encoding related polypeptides. A method for identifying such DNA comprises contacting DNA with a nucleic acid probe described above and identifying DNA(s) which hybridize to that probe. bbp polynucleotides can also be used as probes or primers to detect a bbp gene or bbp RNA species to diagnose a phenotype characteristic of cells having elevated or reduced expression of BBP.

These methods are well known to those skilled in the art, and protocols can be found in a variety of sources, such as Ausubel (1993) Current Protocols in Molecular Biology, Green and Wiley, U.S.A., and periodic updates.

For an individual polypeptide related to BBP, the expression pattern in different tissues may vary. Thus, in order to isolate cDNA encoding a particular BBP-related polypeptide, it is advantageous to screen libraries prepared from different suitable tissues or cells. As a screening probe, there may be employed a DNA or RNA comprising substantially the entire coding region of bbp or a suitable oligonucleotide probe based on said DNA. A suitable oligonucleotide probe (for screening involving hybridization) is a single stranded DNA or RNA that has a sequence of nucleotides that includes at least 14 contiguous bases that are the same as (or complementary to) any 14 or more contiguous bases set forth in SEQ ID NO:6. The probe can be labeled with a suitable chemical moiety for
ready detection. The nucleic acid sequences selected as probes should be of sufficient length and be sufficiently unambiguous so that false positive results are minimized.

Preferred regions from which to construct probes include 5’ and/or 3’ coding sequences, sequences predicted to encode ligand binding sites, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labeled with suitable label means for ready detection upon hybridization. For example, a suitable label means is a radiolabel. The preferred method of labeling a DNA fragment is by incorporating $^{32}\text{P}$-labeled $\alpha$-dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labeled with $^{32}\text{P}$-labeled $\gamma$-ATP and polynucleotide kinase. However, other methods (e.g., non-radioactive) can also be used to label the fragment or oligonucleotide, including, e.g., enzyme labeling and biotinylation.

After screening the library, e.g., with a portion of DNA including substantially the entire bbp gene or a suitable oligonucleotide based on a portion of said DNA, positive clones are identified by detecting a hybridization signal; the identified clones are characterized by restriction enzyme mapping and/or DNA sequence analysis, and then examined, e.g., by comparison with the sequences set forth herein, to ascertain whether they include a full length bbp gene (i.e., if they include translation initiation and termination codons). If the selected clones are incomplete, they can be used to rescreen the same or a different library to obtain overlapping clones. If the library is genomic, then the overlapping clones can include exons and introns. If the library is a cDNA library, then the overlapping clones will include an open reading frame. In both instances, complete clones can be identified by comparison with the DNAs and deduced amino acid sequences provided herein.
Furthermore, in order to detect any abnormality of an endogenous bbp or bak, genetic screening can be carried out using a nucleotide sequence of the invention as hybridization probes. Genetic defects due to a mutation in a bbp gene or bbpb polynucleotides disclosed herein can be used to identify cells having a mutation in a bbp or bak gene using well known hybridization methods. In order to provide the specificity necessary to identify, for example, a point mutation in a bbp gene, one skilled in the art would know that an oligonucleotide probe should be at least about fourteen to sixteen nucleotides in length. In addition, the probe should incorporate a detectable moiety such as a radiolabel, a fluorochrome or a detectable binding agent such as biotin. Various detectable moieties and methods of incorporating the moiety into an oligonucleotide probe are well known in the art and are commercially available.

Also, based on the nucleic acid sequences provided herein antisense-type or ribozyme therapeutic agents can be designed and are included in the scope of the invention. For example, oligoribonucleotides, including antisense RNA and DNA molecules and ribozymes can function to inhibit translation of one or more components of a protein complex. S. T. Crooke and B. Lebleu, eds. Antisense Research and Applications (1993) CRC Press. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between -10 and +10 regions of the relevant nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA,
followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead or other motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences encoding protein complex components.

Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets can also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays. See, Draper PCT WO 93/23569.

Both anti-sense RNA and DNA molecules and ribozymes of the invention can be prepared by any method known in the art for the synthesis of RNA molecules. See, Draper, id. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art including, but not limited to, solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules can be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Various modifications to the DNA molecules can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences or ribo- or deoxyribo-
nucleotides to the 5’ and/or 3’ O-methyl rather than phosphodiesterase linkages within the oligodeoxyribonucleotide backbone.
It is envisaged that the nucleic acid of the invention can be readily modified by nucleotide substitution, nucleotide deletion, nucleotide insertion or inversion of a nucleotide stretch, and any combination thereof. Such modified sequences can be used to produce a mutant BBP which differs from those found in nature. Mutagenesis can be predetermined (site-specific) or random. A mutation which is not a silent mutation should not place sequences out of reading frames and preferably will not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins.

Given the guidance of the present invention, the nucleic acids of the invention are obtainable according to the methods well known in the art. The present invention further relates to a process for the preparation of such nucleic acids.

For example, a DNA of the invention is obtainable by chemical synthesis, by recombinant DNA technology or by PCR. Preparation by recombinant DNA technology can involve screening a suitable cDNA or genomic library. A suitable method for preparing a DNA or of the invention can, e.g., comprise the synthesis of a number of oligonucleotides, their amplification by PCR methods, and their splicing to give the desired DNA sequence. Suitable libraries are commercially available or can be prepared from individual tissues or cell lines.

Suitable antibodies for use herein are generated by using BBP or fragments of BBP as an antigen. Antibodies to a BBP Binding Domain or Bak are also suitable for use herein. Methods of detecting proteins using antibodies and of generating antibodies using proteins or synthetic peptides are known in the art and are not described in detail herein.

The present invention further encompasses antibodies or antigen binding fragments that recognize BBP or BBP Binding Domain, or fragments thereof. Such antibodies can be useful e.g. for immunoassays including immunohistochemistry, in the detection of a substances that bind to BBP or BBP Binding Domain, or, alternatively, for use in purification or identification of BBP or BBP Binding Domain or antigenically related polypeptides, or for diagnostic or
therapeutic purposes. For example, an antibody can be used to detect the presence of a BBP in a biological sample. The cells can either be lysed or permeabilized to the antibody, both are acceptable methods of exposing the contents of the cells to antibodies, using methods known in the art.

Antibodies which can be used as therapeutic or diagnostic agents include polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as a protein-protein complex, or individual components. For the production of polyclonal antibodies, various host animals can be immunized by injection with the antigen such as rabbits, mice, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, such as Freund's (complete and incomplete), mineral gels (e.g., lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol), and potentially useful human adjuvants such as BCG (bacille Calmette-Guérin) and Corynebacterium parvum.

A monoclonal antibody, which is a substantially homogeneous population of antibodies to a particular antigen, can be obtained by techniques providing for the production of antibody molecules by continuous cell lines in culture. Examples of such techniques include the hybridoma technique described by Kohler and Milstein (1975) *Nature* 256:495-497, the human B-cell hybridoma technique (Olsson et al. *J. Immunol. Methods* 61:17-32; and Cole et al. (1984) *Mol. Cell. Biochem.* 62:109-120), and the EBV-hybridoma technique (Roder et al. (1986) *Methods Enzymol.* 121:140-167). The hybridoma producing the mAb can be cultivated *in vitro* or *in vivo*. 
In addition, techniques developed for the production of “chimeric antibodies” (Morrison et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Neuberger et al. (1984) *Nature* 312:604-608; and Takeda et al. (1985) *Nature* 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.


Antibody fragments containing specific binding sites of a complex can be generated by known techniques. For example, such fragments include the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries can be constructed (Huse et al. (1989) *Science* 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity to BBP or BBP Binding Domain.

The invention further provides methods for detecting agents that can modulate the interaction between a BBP and a Bak protein or BBP Binding Domain of the Bak protein. “Modulate” in this sense means to alter the ability of a BBP to associate with a Bak protein. Thus, modulation includes enhancement of the association between a BBP and a Bak protein, as well as dissociation of a bound complex formed by the association of a BBP and a Bak protein. Methods for detecting an agent that can modulate the interaction between a Bak and a BBP
are known in the art and include, but are not limited to, yeast two-hybrid screening, phage display and assays such as the one described in Example 6.

As used herein, the term "agent" means a chemical or biological molecule such as a simple or complex organic molecule, a peptide, a protein or an oligonucleotide. An "effective agent" is an agent that, in fact, alters an interaction of proteins involved in apoptosis. An effective agent is therefore also a potential therapeutic agent.

The screening assay described herein is particularly useful in that it can be automated, which allows for high through-put screening of randomly designed agents to identify useful drugs, which can alter the ability of a BBP and a Bak protein to associate. For example, a drug can alter the ability of a BBP and a Bak protein to associate by decreasing or inhibiting the binding affinity of a BBP and a Bak protein. Such a drug can be useful where it is desirable to increase the concentration of unbound Bak in a cell, for example, so that free Bak is available to induce apoptosis. Alternatively, a drug can be useful for increasing the affinity of binding of a BBP and a Bak protein.

The drug screening assay can utilize BBP or a BBP fusion protein such as a BBP-glutathione-S-transferase (gst). The BBP or BBP fusion protein is characterized, in part, by having an affinity for a solid substrate as well as having an affinity for a Bak protein. For example, when BBP is used in the assay, the solid substrate can contain a covalently attached anti-BBP antibody. Alternatively, a BBP-gst fusion protein can be used in the assay. Where such a fusion protein is used in the assay, the solid substrate can contain covalently attached glutathione, which is bound by the gst component of the BBP-gst fusion protein.

The drug screening assay can be performed by allowing the BBP or BBP-fusion protein to bind to the solid support, then adding a Bak protein and a drug to be tested. Control reactions will not contain the drug. Following incubation of the reaction mixture under conditions known to be favorable for the association, for example, of BBP and Bak in the absence of a drug, the amount of Bak
specifically bound to BBP in the presence of a drug can be determined. For ease of detecting binding, the Bak protein can be labeled with a detectable moiety, such as a radionuclide or a fluorescent label, using methods well known in the art. By comparing the amount of specific binding of BBP and Bak in the presence of a drug as compared to the control level of binding, a drug that increases or decreases the binding of a BBP and a Bak protein can be identified. Thus, the drug screening assay provides a rapid and simple method for selecting drugs having a desirable effect on the association of a BBP and a Bak protein.

A transcription activation assay such as the yeast two-hybrid system allows for the identification and manipulation of protein-protein interactions. Fields and Song (1989) Nature 340:245-246. The conceptual basis for a transcription activation assay is predicated on the modular nature of transcription factors, which consist of functionally separable DNA-binding and trans-activation domains. When expressed as separate proteins, these two domains fail to mediate gene transcription. However, the ability to activate transcription can be restored if the DNA-binding domain and the trans-activation domain are bridged together through a protein-protein interaction. These domains can be bridged, for example, by expressing the DNA-binding domain and trans-activation domain as fusion proteins (hybrids), where the proteins that are appended to these domains can interact with each other. The protein-protein interaction of the hybrids can bring the DNA-binding and trans-activation domains together to create a transcriptionally competent complex.

A transcription activation assay such as the yeast two hybrid system can also be useful as a screening assay to identify effective agents that alter interactions between a Bak and a BBP. A transcription activation assay can be used to screen a panel of agents to identify an effective agent, which can be useful for increasing or decreasing apoptosis in a cell.

An effective agent can be identified by detecting an altered level of transcription of a reporter gene. For example, the level of transcription of a reporter gene due to the bridging of a DNA-binding domain and trans-activation
domain by a BBP and a Bak protein can be determined in the absence and in the presence of an agent. An effective agent that increases the interaction between a Bak protein and a BBP can be identified by an increased level of transcription of the reporter gene as compared to the control level of transcription in the absence of the agent.

An agent that effectively increases the interaction of Bak and BBP, as detected by increased transcription of the reporter gene in a two-hybrid assay, can be used to alter the level of apoptosis in a cell. An effective agent that decreases the interaction of Bak and BBP also can be identified, in this case by detecting a decreased level of transcription of a reporter gene as compared to the level of transcription in the absence of the agent. Effective agents that result in decreased levels of apoptosis can be particularly useful as a medicament for treating a patient suffering from a disease characterized by a high level of apoptosis such as a neurodegenerative disease. Effective agents that result in increased levels of apoptosis can be useful, for example, to increase the level of apoptosis of a cancer cell, which is characterized by having a decreased level of apoptosis as compared to its normal cell counterpart. Thus, effective agents identified using the methods described herein are particularly useful as medicaments to increase or decrease the level of apoptosis in a cell in a subject.

In some cases, an agent may not be able to cross the yeast cell wall and, therefore, cannot enter the yeast cell to alter an interaction among members of the Bak protein family. The use of yeast spheroplasts, which are yeast cells that lack a cell wall, can circumvent this problem (Smith and Corcoran, In Current Protocols in Molecular Biology (ed.Ausubel et al.; Green Publ., NY 1989)). In addition, a potentially effective agent, upon entering a cell, may require "activation" by a cellular mechanism, which may not be present in yeast. Activation of an agent can include, for example, metabolic processing of the agent or a modification such as phosphorylation of the agent, which can be necessary to convert the agent into an effective agent. In this case, a mammalian cell line can be used to screen a panel of agents. A transcription assay such as the yeast two-hybrid system

It may be advantageous to employ a peptide analog of BBP or Bak, or a portion thereof, as a pharmaceutical agent or as a commercial research reagent. For example, a peptide analog of BBP having high affinity for binding Bak can be used as a competitive inhibitor of BBP:Bak complex formation by competing with native BBP for binding to Bak.

In addition to polypeptides consisting only of naturally-occurring amino acids, peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" and are usually developed with the aid of computerized molecular modeling. Eichler et al. (1995) Med. Res. Rev. 15:481-496; Moore et al. (1995) Adv. Pharmacol. 33:1-41; Moore (1994) Trends Pharmacol. Sci. 15:124-129; Saragovi et al. (1992) Biotechnol. 10:773-778.

Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), such as Bak, but have one or more peptide linkages optionally replaced by a linkage such as: —CH₂NH—, —CH₂S—, —CH₂—CH₂—, —CH=CH— (cis and trans), —COCH₂—, —CH(OH)CH₂—, and —CH₂SO—, by methods known in the art. See, for example, Spatola (1983) Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins B. Weinstein eds. Marcel Dekker, New York.

Such peptide mimetics can have significant advantages over polypeptide embodiments, including, for example, more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption,
potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecule(s) (e.g., are not contact points in Bak:BBP complexes) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (e.g., labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

The invention also provides methods for identifying polypeptide sequences which bind to a BBP polypeptide. For example, a yeast two-hybrid screening system can be used for identifying polypeptide sequences that bind to BBP. Yeast two-hybrid systems wherein one GAL4 fusion protein comprises a BBP polypeptide sequence, typically a full-length or near full-length BBP sequence, and the other GAL4 fusion protein comprises a cDNA library member can be used to identify cDNAs encoding proteins which interact with the BBP polypeptide, can be screened according to the general method of Chien et al. (1991) Proc. Natl. Acad. Sci. USA 88:9578-9582. Other methods for detecting BBP-binding proteins are known in the art and include, but are not limited to screening a phage display library with a BBP. U.S. Patent No. 5,223,409.

As used herein, the terms “suitable conditions” or “appropriate conditions” refer to temperature, pH, ionic strength, viscosity and biochemical parameters which are compatible with physiological protein-protein interactions. Suitable in vitro or in vivo reaction conditions for protein-protein interactions are generally physiological conditions. In general, in vitro physiological conditions comprise 50 - 200 mM NaCl or KCl, pH 6.5 - 8.5, 20°C - 45°C and 0.001 - 10 mM divalent cation (e.g., Mg++, Ca++); preferably about 150 mM NaCl or KCl, pH 7.2 - 7.6, 5 mM divalent cation, and often include 0.01 - 1.0 % nonspecific protein (e.g., bovine serum albumin). Particular aqueous conditions can be selected by the
practitioner according to conventional methods. Addition of other components is optional and the additional components and reaction conditions can vary according to a particular need.

In another embodiment, diagnostic methods are provided to detect the expression of BBP either at the protein level or the mRNA level. Any antibody that specifically recognizes BBP is suitable for use in BBP diagnostics. Abnormal levels of BBP may be found in the tissues of patients with diseases associated with inappropriate apoptosis; diagnostic methods are therefore useful for detecting and monitoring biological conditions associated with such apoptosis defects. Detection methods are also useful for monitoring the success of BBP-related therapies.

BBP protein expression can also be monitored by measuring the level of bbp mRNA. Any method for detecting specific mRNA species is suitable for use in this method. This is easily accomplished using the polymerase chain reaction (PCR). Alternatively, Northern blots can be utilized to detect bbp mRNA or bak mRNA by using probes specific to bbp or bppbd. Methods of utilizing PCR and Northern blots are known in the art and are not described in detail herein.

The invention further provides methods for modulating the activity of Bak or a Bak-related protein in a cell by introducing into the cell a nucleotide sequence encoding BBP or an antisense nucleotide sequence, which is complementary to a region of a gene encoding BBP and can hybridize to the gene or to an mRNA transcribed from the gene. As used herein, the term "modulate" means the level of a BBP expressed in a cell can be increased or decreased. Thus, the compounds described herein can be used as medicaments for the treatment of a pathology caused by an altered level of apoptosis.

The level of a gene product such as BBP or BBP Binding Domain can be increased in a cell using recombinant expression vectors and gene transfer technology to express a nucleic acid encoding BBP or BBP Binding Domain or an active fragment of BBP or BBP Binding Domain. Various expression vectors and methods for introducing such vectors into a cell are well known in the art and are
described, for example, in Sambrook et al. (1989). Viral vectors that are compatible with a targeted cell are particularly useful for introducing a nucleic acid encoding BBP or BBP Binding Domain into a cell. For example, recombinant adenoviruses having general or tissue-specific promoters can be used to deliver expression constructs into a variety of types of tissues and cells, including non-mitotic cells, and to drive the desired cDNA expression in the target cells. Recombinant adeno-associated viruses also are useful and have the added advantage that the recombinant virus can stably integrate into the chromatin of even quiescent non-proliferating cells such as neurons of the central and peripheral nervous systems. Lebkowski et al. (1988) *Mol. Cell. Biol.* 8:3988-3996.

Suitable indications for modulating endogenous levels of BBP or BBP Binding Domain are any in which BBP or BBP Binding Domain-mediated apoptosis is involved. These include, but are not limited to, various types of malignancies and other disorders resulting in uncontrolled cell growth such as eczema, or deficiencies in normal programmed cell death such as malignancies, including, but not limited to, B cell lymphomas.

Methods of treatment with BBP also include modulating cellular expression of BBP by increasing or decreasing levels of bbp mRNA or protein. Suitable methods of increasing cellular expression of BBP or BBP Binding Domain include, but are not limited to, increasing endogenous expression and transfecting the cells with vectors encoding bbp or bbpdbd, respectively.

For purposes of gene therapy, the level of BBP or a BBP Binding Domain can be increased in a cell using recombinant expression vectors and gene transfer technology to express bbp or bbpdbd or a portion thereof. Viral vectors that are compatible with a targeted cell are particularly useful for introducing a bbp or bbpdbd gene into a cell. For example, recombinant adenoviruses having general or tissue-specific promoters can be used to deliver bbp constructs into a variety of types of tissues and cells. Retroviral vectors are often used for *in vivo* targeting and therapy procedures. Retroviral vectors can be constructed either to function
as infectious particles or to undergo only a single initial round of infection. Methods for constructing and using viral vectors are known in the art and are reviewed, for example, in Miller and Rosman (1989) *Biotechniques* 7:980-982.

The invention encompasses *ex vivo* transfection with bbp or bbpdb, in which cells removed from animals including man are transfected with vectors encoding BBP or BBP Binding Domain and reintroduced into animals. Suitable transfected cells include individual cells or cells contained within whole tissues. In addition, *ex vivo* transfection can include the transfection of cells derived from an animal other than the animal or human subject into which the cells are ultimately introduced. Such grafts include, but are not limited to, allografts, xenografts, and fetal tissue transplantation. Essentially any cell or tissue type can be treated in this manner. Suitable cells include, but are not limited to, cardiomyocytes and lymphocytes. Transfection methods include, but are not limited to calcium phosphate precipitation and DEAE-dextran or lipofectin facilitated transfection; these methods are well known in the art and are described in, for example, Sambrook et al. (1989).

Further, the invention encompasses cells transfected *in vivo* by vectors containing bbp or bbpdb cDNA. Suitable methods of *in vivo* transfection are known in the art and include, but are not limited to, that described by Zhu et al. (1993) *Science* 261:209-211. Receptor-mediated DNA delivery approaches also can be used to deliver BBP or BBP Binding Domain expression plasmids into cells in a tissue-specific fashion using a tissue-specific ligand or antibody non-covalently complexed with DNA via bridging molecules. Curiel et al. (1992) *Hum. Gene Ther.* 3:147-154; and Wu and Wu (1987) *J. Biol. Chem.* 262:4429-4432. Direct injection of DNA or DNA encapsulated in cationic liposomes also can be used for stable gene transfer to non-dividing and dividing cells *in vivo*. Ulmer et al. (1993) *Science* 259:1745-1749. In addition, DNA can be transferred into a variety of cell types using the particle bombardment method. Heiser (1994) *Anal. Biochem.* 217:185-196; Yang et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:9568-9572. *In vivo* transfection by bbp may be particularly useful as a
prophylactic treatment for patients suffering from atherosclerosis. Modulation of the levels of BBP or BBP Binding Domain could serve as a prophylaxis for the apoptosis-associated reperfusion damage that results from cerebral and myocardial infarctions. In these patients with a high risk of stroke and heart attack, the apoptosis and reperfusion damage associated with arterial obstruction could be prevented or at least mitigated.

Infarctions are caused by a sudden insufficiency of arterial or venous blood supply due to emboli, thrombi, or pressure that produces a macroscopic area of necrosis; the heart, brain, spleen, kidney, intestine, lung and testes are likely to be affected. Apoptosis occurs to tissues surrounding the infarct upon reperfusion of blood to the area; thus, modulation of BBP or BBP Binding Domain levels, achieved by a biological modifier-induced change in endogenous production or by in vivo transfection, could be effective at reducing the severity of damage caused by heart attacks and stroke.

In addition, increasing endogenous expression of BBP can be accomplished by exposing the cells to biological modifiers that directly or indirectly increase levels of BBP either by increasing expression or by decreasing degradation of bbp mRNA. Suitable biological modifiers include, but are not limited to, molecules and other cells. Suitable molecules include, but are not limited to, drugs, cytokines, small molecules, hormones, combinations of interleukins, lectins and other stimulating agents e.g. PMA, LPS, bispecific antibodies and other agents which modify cellular functions or protein expression. Cells are exposed to such biological modifiers at physiologically effective concentrations, and the expression of BBP is measured relative to a control not exposed to the biological modifiers. Those biological modifiers which increase expression of BBP relative to the control are selected for further study.

The invention further encompasses a method of decreasing endogenous levels of BBP or BBP Binding Domain. The methods of decreasing endogenous levels of BBP or BBP Binding Domain include, but are not limited to, antisense nucleotide therapy and down-regulation of expression by biological modifiers.
Antisense therapy is known in the art and its application will be apparent to one of skill in the art. Homologous recombinant gene knock-out methods can also be used to decrease the level of BBP or BBP Binding Domain in a cell. Capecchi (1990) Nature 344:105.

The following examples are provided to illustrate but not limit the present invention. Unless otherwise specified, all cloning techniques were essentially as described by Sambrook et al. (1989) and all reagents were used according to the manufacturer's instructions.

**EXAMPLE 1**

**Identification and cloning of BBP cDNA**

BBP cDNA was discovered in human heart using the yeast two hybrid system. The requirement of the DNA binding and activation domains of the yeast transcriptional activator GAL4 for the induction of the GAL1 promoter and the ability of those functions to operate even when on separate fusion proteins provides the basis for the assay and allows detection of associating proteins. The strategy used to detect proteins with binding affinity for Bak is shown in Figure 1.

The GAL4-Bak fusion protein consisted of amino acids 1-147 of GAL4, a region that directs binding of GAL4 to the upstream activator sequence of the GAL1 promoter. The Bak protein, or BakΔ2-TM, whose amino acid sequences are shown in Figure 2 and Figure 3, respectively, served as the “bait”. The lacZ gene under the control of the GAL1 promoter constituted the first reporter system. The second reporter system consisted of the histidine reporter gene similarly under transcriptional control of the GAL1 promoter. Yeast possessing a dual reporter system responsive to transcriptional activation through the GAL1 promoter were transformed with a plasmid expressing a GAL4-Bak fusion protein. A cDNA library from human heart mRNA was purchased from Clontech and cloned into the vector pGAD10 such that members of the library would be constitutively expressed as a fusion protein with amino acids 768-881 of GAL4, a region.
containing the transcriptional activation domain. Yeast cells containing library proteins that interact with the bait protein, Bak or BakΔ2-TM, turn blue when grown on medium containing XGal, which yields a chromogenic product when acted on by β-galactosidase. Yeast cells were transformed by the lithium acetate method. Ito et al. (1983) *J. Bacteriol.* 153:163-168.

Full length bak or truncated bakΔ2-TM (aa71-187) constructs were amplified by PCR from cDNA as EcoRI fragments and cloned as in-frame fusions to the GAL4 DNA binding domain into the vectors pGBT9 and pAS2-1, respectively (Clontech, Palo Alto, CA). A human heart cDNA library of 3x10^6 independent clones in pGAD10 (Clontech) was amplified and cotransformed into yeast strain CG1945 (Clontech) with Bak bait plasmids. Histidine positive transformants were assayed for β-galactosidase activity by the filter method. Plasmid DNA was isolated from all His^+^/X-Gal^+^ clones by the rapid technique recommended by Clontech or by spheroplasting and subsequent alkaline lysis. This DNA was amplified by PCR to determine insert size. pGAD10 clones were isolated by transforming *E. coli* strain HB101 and selecting on M9 Leucine-Ampicillin plates. Plasmids were sorted by restriction enzyme digest and Southern blot and then sequenced.

Twenty-six clones activated both the β-galactosidase and histidine reporter genes. Sixteen clones encoded the same protein, which was termed BBP. DNA sequence analysis of BBP cDNA, shown in Figure 4, revealed an open reading frame encoding a 151 amino acid protein, with a predicted relative mass of 16 kD. TopPredII, a program for predicting membrane protein structure, indicated that BBP was a three transmembrane protein with hydrophilic N-terminal 42 amino acid region and a 58 amino acid loop region, both located on the cytoplasmic side of the membrane.
EXAMPLE 2
Northern Blot Analysis of cDNA Clones

Northern blot analysis was performed according to the method described by Lehrach et al. (1977) *Biochem.* 16:4743-4751 and Thomas (1980) *Proc. Natl. Acad. Sci. USA* 77:5201-5205. In addition, a human multiple tissue Northern blot was purchased from Clontech. The coding regions of BBP cDNA was labeled by the random priming method described by Feinberg and Vogelstein (1984) *Anal. Biochem.* 137:266-267. Hybridization and washing conditions were performed according to standard methods.

The results, presented in Figure 5, indicate that BBP shows a wide tissue distribution among the organs tested (heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas), with high levels of a 1.2 kb mRNA found in heart, skeletal muscle and pancreas.

EXAMPLE 3
BBP interacts with itself and with BAK

Using the yeast two hybrid system described above, the interactions between BBP and members of the bcl2 family of apoptosis modulators, as well as with BBP itself, were analyzed. BBP was expressed as a fusion protein with the activation domain (AD) of GAL4. Genes encoding BBP, Bak, Bcl2, Bclx, Bax and E1B19k proteins were inserted into expression vectors containing the coding sequence for the binding domain of GAL4 such that these proteins would be expressed as fusion proteins with the binding domain (BD) of GAL4. Results, summarized in Figure 6, indicated that BBP associates with itself and with Bak, but not with the other members of the bcl2 family that were tested.
EXAMPLE 4
Copy number and chromosomal location of bbp

Southern blot analysis was used to determine gene copy number and size of the bbp gene(s). Restriction analysis of human genomic DNA with several enzymes, including BamHI, EcoRI and HindIII, revealed multiple bands hybridizing to the bbp cDNA. EcoRI generated eight bands ranging in size from 1.5 to 15 kilobase pairs (kb). Similar patterns were seen with BamHI and HindIII. To investigate the number and distribution of bbp genes, a Somatic Cell Hybrid Panel of human chromosomes was obtained from Coriell Cell Repositories (Camden, NJ). Each mouse or Chinese Hamster somatic cell hybrid contains one human chromosome. Genomic DNA from these hybrids was digested with EcoRI along with human, mouse and Chinese Hamster genomic DNA controls. Under stringent hybridization conditions (0.1 X SSC, 0.1% SDS, 65°C) with the bbp cDNA, genes were found to reside on human chromosomes 2, 5 and X and align with eight bands seen with total human genomic DNA. Thus, there are at least three gene members in the BBP family.

EXAMPLE 5
Examination of Bak interactions with BBP

To delineate the regions of Bak and BBP that mediate the association of these two proteins, two series of experiments were conducted in which portions of BBP fused to glutathione-S-transferase (gst) were allowed to interact with Bak or derivatives thereof.

In the first series of experiments, gst fusion proteins were made that contained the two hydrophilic regions of BBP, full-length E1B19K or full-length BHRF-1. The hydrophilic regions of BBP are the N-terminal 42 amino acid
portion and an internal loop of BBP consisting of amino acids 86 through 133, and are referred to as BBP-Loop1 (BBP-L1) and BBP-Loop2 (BBP-L2), respectively. DNA sequences encoding the hydrophilic regions were amplified by polymerase chain reaction (PCR) and subcloned into pGEX-5X (Pharmacia) in-frame with gst.

Gst fusion proteins containing BBP-L1, BBP-L2, E1B19K or BHRF-1 were immobilized on a glutathione-Sepharose column. Yeast lysates expressing Bak or control vector were passed over the column, washed and Bak binding was assessed by Western blot analysis. As had been previously shown, Bak interacted with BHRF-1 and E1B19K. Bak was also shown to interact with the N-terminal 42 amino acid region of BBP and with the internal loop (Figure 7).

To define further the interaction between Bak and BBP, a peptide consisting of amino acids 1-50 of Bak was used in place of full-length Bak and binding to immobilized BBP-L1 or BBP-L2 was assessed. The synthetic peptide of the first 50 residues of Bak (Bak 1-50) was supplied by Pharmingen. This peptide is soluble in 1X phosphate-buffered saline (PBS).

For each of the three glutathione Sepharose/gst fusion protein complexes (representing BBP-L1, BBP-L2 and a control), 100 μg of Bak 1-50 (in 1 ml of PBS/0.1% triton X-100) was added to 50 μL of resin in an Eppendorf tube and left overnight at 4°C. Duplicate samples were prepared lacking the peptide in the PBS/0.1% triton X-100 buffer. The following day, the resins were washed 5 times with the PBS/0.1% triton X-100 buffer and then the resins boiled in 50 μL 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) sample loading buffer. A western was performed to assess the protein-protein interactions. The results, shown in Figure 8A, demonstrate that a region in the first 50 amino acids of Bak is capable of interacting with both BBP-L1 and BBP-L2.

In a second series of experiments, an N-terminal deletion of Bak was made and analyzed in a similar manner as above for binding to the hydrophilic regions of BBP. The N-terminal deletion mutant of Bak is termed BakΔ3, represents
amino acids 96-210 of full-length Bak and is shown in Figure 3. BakΔ3 was cloned into a baculovirus vector. The PCR amplified BakΔ3 was subcloned into the pBlueBac III vector (Invitrogen) and recombinant plasmids were isolated. Recombinants were sequenced by the dideoxy chain termination method (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467) using sequencing kits purchased from U.S. Biochemicals (Sequenase version 2.0). Recombinant viruses were generated by cotransfecting Spodoptera frugiperda clone 9 (Sf9) cells with the recombinant plasmid and wild-type AcMNPV viral DNA using cationic liposomes as described by supplier (Invitrogen). Recombinant viruses were identified by visual screening and PCR and propagation of recombinant baculovirus was performed as described by the supplier (Invitrogen).

For the production of recombinant BakΔ3, 1×10^7 Sf9 cells were cultured in each of twenty 75 cm² tissue culture flasks (Corning) containing 15 ml of Grace's complete insect cell media (Invitrogen) supplemented with 10% fetal bovine serum. After allowing the insect cells to attach to the plate for 1 hour, the cells were infected at a multiplicity of infection of 5 with baculovirus expressing bakΔ3. The plates were placed on a rocker for 1 hour and then the media was removed and replaced with 15 ml of fresh media. The plates were placed at 27°C and left for 72 hours. After allowing the Sf9 cells to express the BakΔ3, the cells were harvested, centrifuged, and washed with 1X PBS. The pellet was collected into a single 15 ml tube and washed again with 1X PBS. The pellet was then extracted with 0.5% NP-40 extraction buffer (10 mM HEPES, 142.5 mM KCl, 1 mM EGTA, 5 mM MgCl₂, 0.5% NP-40, pH 7.2). This was left overnight at 4°C with gentle shaking.

The solubilized cell extract was then sonicated, centrifuged, and one ml of each supernatant was mixed with 50 ml of a suspension of glutathione Sepharose to which either gst, gst-BBP-L1 or gst-BBP-L2 was bound. The extract containing BakΔ3 was kept at 4°C for 24 hours with the Sepharose-bound gst, gst-BBP-L1 or gst-BBP-L2 suspensions. The Sepharose-protein complexes were collected by centrifugation, washed three times with the buffer described above,
then suspended in 100 ml each of 1X SDS-PAGE buffer. Eight µL of each sample were run on a 15% SDS PAGE gel and, after the proteins were satisfactorily separated, transferred to nitrocellulose for immunoblotting. Complexes containing the Bak derivative were detected using rabbit IgG anti-Bak antibodies and the blot was developed using enhanced chemiluminescence (ECL) and anti-rabbit IgG/peroxidase conjugate. The results, shown in Figure 8B, indicate that N-terminal deletion of Bak represented by BakΔ3 is capable of interaction with BBP-L1, but is not capable of associating with the hydrophilic region represented by BBP-L2.

**EXAMPLE 6**

**Drug Screening Assay**

This example describes an assay useful for screening for agents such as drugs that alter the affinity of binding of Bak with BBP.

Specifically, this example presents a scheme for using a BBP such as the BBP-Loop1 or BBP-Loop2 in a drug screening assay that is suitable for automated high through-put random drug screening. A DNA sequence encoding BBP-Loop1 or BBP-Loop2 is amplified by PCR and subcloned into the pGEX-5X plasmid in frame with gst, allowing the production of Gst-BBP-L1 or Gst-BBP-L2 fusion proteins in *E. coli*. Gst-BBP-L1 or Gst-BBP-L2 are purified by standard methods using glutathione-Sepharose chromatography, then the fusion proteins are immobilized onto 96-well microtiter plates. Bak coupled to a second moiety that can serve as a detectable tag, e.g. green fluorescent protein (GFP; Prasher (1995) *Trends Genet.* 11:320-323), is added along with the agent whose ability to affect the binding between Bak and BBP is to be tested, including, but not limited to a small molecule or peptide. Control samples include no test agent. After a suitable incubation period, the test wells are washed, and fluorescence is monitored by measuring excitation at 488 nm and emission at 511 nm. Test wells showing a
significantly higher or significantly lower fluorescence compared with the control are then examined further to confirm an effect on Bak-BBP binding.

EXAMPLE 7

Analysis of Bak-BBP interaction

To further confirm Bak-BBP interactions and to determine regions of Bak that are important in binding to BBP, SPOTS peptide binding assays were performed.

Sixty-three Bak peptides, 15 amino acids in length, were synthesized on a membrane using a SPOTs kit according to suppliers instructions (Genosys Biotechnologies, The Woodlands, Texas). The peptides were overlapping, off register by 4 amino acids, and spanned amino acids 1-194 (see SEQ ID NO: 2). All regions of Bak were represented except for the transmembrane region. The order of synthesis was determined using the SPOTscan and SPOTslot programs provided by the suppliers.

SPOTs kit included proprietary membrane for peptide synthesis, derivatized amino acids, computer program for determining the order of synthesis, synthesis trough, 10X blocking buffer concentrate, and complete instructions.

Some reagents had to be obtained from outside sources. Dimethylformamide (DMF) was from EM Science (Gibbstown, New Jersey) while N-methylpyrrolidone (NMP) was from Applied Biosoftware (Foster City, California). Both were peptide synthesis grade. Molecular Sieves (4Å) were obtained from Aldrich (Milwaukee, Wisconsin).

Prior to the start of peptide synthesis, the solvents used (DMF and NMP) had to be further purified to remove any reactive amines or water. This was done using the molecular sieves which were activated by heating at 250°C for 24 hours, followed by drying in a vacuum dessicator containing Drierite (Aldrich) for another 48 hours. The activated sieves were mixed with the solvents (at 25% of the total solvent volume) and left for 72 hours.
Upon completion of peptide synthesis the membrane was washed in TBS (50 mM Tris, 150 mM NaCl, 5 mM KCl, pH 8) three times and then incubated in blocking buffer overnight (suppliers blocking buffer in TBS/0.05% Tween-20, 5% sucrose) at room temperature. The membrane was washed three times with TBS/0.05% Tween-20 and incubated overnight at room temperature in 25 mL of blocking buffer containing 2 mg purified GST-BBP loop 1, GST-BBP loop 2 or GST (negative control). The membrane was again washed three times with TBS/0.05% Tween-20, incubated with rabbit polyclonal anti-GST (1:1000; Pharmacia) for 1 hour at room temperature, washed again with TBS/0.05% Tween-20 and incubated with goat anti-rabbit IgG/peroxidase conjugate (Sigma, St. Louis, Missouri) for 1 hour at room temperature. The membrane was then developed using ECL method (Amersham, Chicago, Illinois).

The results were as follows. BBP loop 1 interacted most strongly with two contiguous clusters of Bak peptides (Figure 9). The first cluster overlapped the Bak BH3 domain (six peptides spanning amino acids 70 to 102) and the second cluster began upstream and extended into the N-terminal region of the BH1 domain (amino acids 103-126). Several weaker interactions occurred between BBP loop 1 and Bak peptides in two regions upstream of the Bak BH3 domain and a region between the Bak BH1 and BH2 domains. BBP loop 2 did not interact with any of the Bak peptides in the SPOTS assay (data not shown).

The BH3 domain of Bak has been shown to be important for the cell death promoting activity of Bak (Chittenden et al., 1995 EMBO J. 14, 5589-5596). Our SPOTS data suggest that BBP can cooperate with Bak in modulating apoptosis by binding to the BH3 domain of Bak. Interestingly, it was recently shown that in some cases Bak does not require its BH3 domain to promote apoptosis (Simonian et al., 1997, Oncogene 15, 1871-1875). BakΔ3, a construct which lacks the N-terminal 95 amino acids, including the BH3 domain, was shown to be extremely active in promoting cell death in insect cells (data not shown). BakΔ3 was also shown to bind to BBP loop 1 (Figure 8B).
Our SPOTS data support that the BBP interaction with BakΔ3 occurs in the region of Bak upstream and extending into the N-terminal region of the BH1 domain (amino acids 103-126 of SEQ ID No: 2, defined as “BBP Binding Domain 1”, nucleic acids 507-578 of SEQ ID NO: 1, defined as “bbpbd-1”) and also in the region immediately downstream from the BH1 domain (amino acids 138-156 of SEQ ID No: 2, defined as “BBP Binding Domain 2”, nucleic acids 611-668 of SEQ ID NO: 1, defined as “bbpbd-2”). Collectively, BBP Binding Domains 1 and 2 are defined as “BBP Binding Domains”. The results support that these regions are additional important death domains of Bak.

Although the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kiefer, Michael C.
     Gibson, Helen L.
     Fitzpatrick, Paul A.
     Barr, Philip J.

(ii) TITLE OF INVENTION: A NOVEL Bak BINDING PROTEIN, DNA
     ENCODING THE PROTEIN, AND METHODS OF USE THEREOF

(iii) NUMBER OF SEQUENCES: 7

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     (F) ZIP: 94804

(v) COMPUTER READABLE FORM:
     (A) MEDIUM TYPE: Floppy disk
     (B) COMPUTER: IBM PC compatible
     (C) OPERATING SYSTEM: PC-DOS/MS-DOS
     (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
     (A) APPLICATION NUMBER:
     (B) FILING DATE:
     (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
     (A) NAME: WILKE, KATHRYN P.
     (B) REGISTRATION NUMBER: 37,472
     (C) REFERENCE/DOCKET NUMBER: 23647-20021.40

(ix) TELECOMMUNICATION INFORMATION:
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     (B) TELEX: (510) 412-9109

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 2094 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: double
     (D) TOPOLOGY: linear

(ix) FEATURE:
     (A) NAME/KEY: CDS
     (B) LOCATION: 201..833

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
Pro Ile Leu Asn Val Leu Val Val Leu Gly Val Val Leu Leu Gly Gln  190 200

TTT GTG GTA CGA AGA TTC TTC AAA TCA TGACTCCCAA GGGTGCCCTT 853
Phe Val Val Arg Arg Val Phe Phe Lys Ser  
205

973

GCCATCTGCA TGGGCCCTGGAG AGACTGATAAA CTTGGGGGAG GAAGAGACTG GGAGCCACCT 1153

CTCCCCAGAA AGTGTTTACAG GTTTTGATCT TTTTATAATA CCCCCCCGAG AGCCCATCTCC 1213

CACATTCTA CCTGAGGCCA GGACGTCTGG GGTGTGGGGA TTGGTGGGTC TATGTCCCCC 1273

AGGATTCAAC TATTTCTGGAA GATCAGCACCC TAAAGAGATG GAACATGAGC CTGAGCCTGG 1333

GCCATCTGCA TGCTGCCAGA AGCAGGACCT AGTAGGAGAC GGGGCAAGC 1393

GACATCTGCA AGCTGTTGCTG TGGGTGGGTG GGGTGCTGAG ACCAGACGCTG 1513

TCTGAACTCA CAGTTGCAAGA GCCCTCAAGG CCACTCCCATG AAGGTCCTCCG CTGTCTCTCC 1573

CTCCCTCTCT CCTATAGAC ACTGTGCTCC AACCCATCTC CTACAGGTGA AGGCCTCTCA 1633

GCTGATTTGCT GAGGCTTGGG CTGTGGGTCG GAAGAGCAGC TATGTCCCCC 1693

GTTAGGACT TGTGTCTTCT TATCAAGGAA AAGGAGTGGG GAGTTGATCTG CACGCTTCTG 1753

AAATGGGAGA AGGACTATGA AACAGAATGC GAAATCCAGA GGGGTGGCTG CCCCCCATCC 1813

GCCCTGAGGA GAGGTGGAGAT GGGGGAGTGT TGAATCTACTG AACCTGCTTC 1873

CCTTCTCCTC TGCTGTCTTG GTGTTGGGCTG GACGAGTGGCTG AAGAATGGTGG 1933

TCTCTGTGGG CACAGCTGTTG GGGTGCTGAG AGGAGACTCC TTTATCTCTG 1993

AATGAGGAAG GGGAGAGGAC ATGGAGGCCA AGATCGCTCC TTAGCTAGA 2053

AAATATCTG TGGAAATCCCC CAAAAAAA AACGGAGATC C 2094

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 211 amino acids
  (B) TYPE: amino acid
  (D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Ser Gly Gln Gly Pro Gly Pro Pro Arg Gln Glu Cys Gly Glu  
1      5      10     15
Pro Ala Leu Pro Ser Ala Ser Glu Glu Gln Val Ala Gln Asp Thr Glu  
20     25     30
Glu Val Phe Arg Ser Tyr Val Phe Tyr Arg His Gln Gln Glu Gln Glu  
35     40     45
Ala Glu Gly Val Ala Ala Pro Ala Asp Pro Glu Met Val Thr Leu Pro  
50     55     60
Leu Gln Pro Ser Ser Thr Met Gly Gln Val Gly Arg Gln Leu Ala Ile  
65     70     75     80
Ile Gly Asp Asp Ile Asn Arg Arg Tyr Asp Ser Glu Phe Gln Thr Met  
85     90     95
Leu Gln His Leu Gln Pro Thr Ala Glu Asn Ala Tyr Glu Tyr Phe Thr  
100    105    110
Lys Ile Ala Thr Ser Leu Phe Glu Ser Gly Ile Asn Trp Gly Arg Val  
115    120    125
Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu Ala Leu His Val Tyr Gln  
130    135    140
His Gly Leu Thr Gly Phe Leu Gly Gln Val Thr Arg Phe Val Val Asp  
145    150    155    160
Phe Met Leu His His Cys Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly  
165    170    175
Trp Val Ala Ala Leu Asn Leu Gly Asn Gly Pro Ile Leu Asn Val Leu  
180    185    190
Val Val Leu Gly Val Leu Leu Gly Gln Phe Val Val Arg Arg Phe  
195    200    205
Phe Lys Ser  
210

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2094 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(iv) ANTI-SENSE: YES
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGATCCTCCGT TTTTTTTTGG AGGGGATGTC ACAGTTTATT TCCAACACT CAGAGGATAG 60
GGGGTGCCCT ATGGGGTCCCA TCTGCTTCCC CTCCCCCTCAT TTGGCTGATT CAAGAACTTC 120
TCCCCCTCTGA CCCCCAGCAC CATAGGGCGTC GCCAAATAGAG AAGGCACGCTC ACACCCGCCAC 180
CTTGGAGGGA CTTAGACGGG TGGAGGACTG GGAGGGTGTTG GGAACAGAGT TCAAGTATTCC 240
ACAGTTCCCA CATCTACACC CCTGATTAC ACCTGTGCCCAG AGCCATGAGG GAGGATCCAC 300
CTCTGGGATTT CCTAGAGGGG TTTGATAGGCT TTTCCCCTACT TAGAACCCTTC CAGATGAACT 360
CCCTACTTCTTTTCCCTGAT ATAAACAACCC AAGTCCTAAG CCGCTGATGTC TGGGCAAGGA 420
GGAGCCCTTAG CAGGCGCACTC ACCCAAGGCC CCCAGGGGTTGGTGAGAGCC TCCACCTGTA 480
GTGAATGGGTT TGGGAGAAGG TGTCTTAAAG GAGAGAGAAG GGGGAGAAG TGGAGGACCT 540
TGGGAGGAAG GGGTGGGGAG CTTCTGACAC GTGAGTTCTCAG ACAGCCTCTG TCTGGCACTCTC 600
CACCCCCACC CACACCCCAA GCCAGGAATAC CCGAGAGTCC CCACTGCAAA GGCATATGG 660
CCGGAGGGGAG GAACTGGGAG GGGGTAGAGG TTGAGCAGGA CTTGGGCCCCT CCTCTCTTAG 720
TAGGTCCTGC TCCTGGGACA CATGTCTTAGG GACGCCGAGG ACCAGGTCGA GTGCTTAGTCC 780
CCATCTCTTA GGGTGCTGAT CTTCAGCAAGT ATGTAAGATCC TGGGGAACAT AGACCCACCA 840
ATCCCAACAC CCCAGACGTCAT CGTGGGCTCAG GTGAAATGGTT GGAATGGGGC TCTCAACAGG 900
GTATTATAAA AAGCTTAAAC CGTTAAACACT TTTCTGGGGA GAATGGGCTC CCAGTCTCCTT 960
GCCCCCAGAAA GTATCTCGTC TCCCGGCGCC CTAGCAGACA GGGCTAAGGA GTTCCCGAGAG 1020
AGCTGAGGGA GGAGAGGCAC ACAGCCCTTG GGGAGGAGAG AGGSCACTAG CACCAGTCAA 1080
TGTTGAGGTTG CCTCTGAGCC CTGACTGGCC CCCCAGCCAG GCTCCGCAAG CTGGAGTGCA 1140
CAGTCTGCAA AGTCTTCTGTA CTCTTGAGGG GGGACCTGCG AAGGGAAACG AGAAGGGCAA 1200
GACTTGGCCTT AAGTCCTGGA AAGGGTCTGTA ACCGGAGGCC AAGGGGCACCA TTTGGGAGTCC 1260
ATGATTGGAA GAACTTTGCT ACCAACAAACT GGGCCACAGG AACCAAACAC AGAACCAACA 1320
GCAGCTGCCAAG CATGGGACCA TTGCGCAAGT TCAGGCGCTGC CACCAGGCC CCCCCTCTGTG 1380
CAATACACCG GCAAATCGCA TGAATGACGA TGAAGTGCAG CAGAAGGCGG GTCAAGGCTGC 1440
CTAGGAGGCC AAGTACGCCC TTGCTGTAGGA CGTGTAGGCG CAGACGGTAGC GCAAGGCCCA 1500
GAAGAGGCAC CACACGGCC CAAATTGATGC CACTCTCACA CAGGCTGAGTG GCCAATTTGG 1560
TGAAGTACTT ATAGGCAATT TCTGGGCTGG GCTGGCGGTT CTTGAACATG GTCTGGAACT 1620
CTGAGTCATA GCGTCGGTTG ATGTCGCTCC CAGTGTGCC GAGCTGCAGT TCCACCTGGC 1680
CCATGCGTGC CTGAGGTGTC AGAAGGAAGG TGACCATGC TCGGGGTGGCA GGGCGAGCCA 1740
CCCTTCACGC TCTCCGTCTC TGCTGATGGC GGTAAAAAC GTAGCTGCGG AAAACCTCCT 1800
CTGGTGCTGTG GGGTACCTGC TCTCTAGAAG CAGAGGGCA GGGAGGTCTT CCCACACTCT 1860
GCCTGGGAGG ACCTGGGCCCT TGCCCCGAAG CACATTTTCA GGTCTCAGTG GAGGACGGGA 1920
TCAGCCCTGC GGGATCCTGG CCCAACCAGG GTGCGTCAGC AGGGTGAGAA TGGAGTCCG 1980
AGGCGGCTGCG GGCTGCTGCT GCAAGGGGCT GAGTGCGAGC CCAGTTCCCA GGAATGGGCG 2040
TCAGTGCACTT CCCGCGATCT GGAATGAGCC TTTACTTGTG TCCTGTAGAT CCTC 2094

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..117
(D) OTHER INFORMATION: /note= "Bak (delta)2 (delta)TM"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Gln Val Gly Arg Gln Leu Ala Ile Ile Gly Asp Asp Ile Asn 1
   5
Arg Arg Tyr Asp Ser Glu Phe Gln Thr Met Leu Gln His Leu Gln Pro 20
   25
Thr Ala Glu Asn Ala Tyr Glu Tyr Phe Thr Lys Ile Ala Thr Ser Leu 35
   40
Phe Glu Ser Gly Ile Asn Trp Gly Arg Val Val Ala Leu Leu Gly Phe 50
   55
Gly Tyr Arg Leu Ala Leu His Val Tyr Gln His Gly Leu Thr Gly Phe 65
   70
Leu Gly Gln Val Thr Arg Phe Val Val Asp Phe Met Leu His His Cys 85
   90
Ile Ala Arg Trp Ile Ala Gln Arg Gly Gly Trp Val Ala Ala Leu Asn 100
105
Leu Gly Asn Gly Pro 115

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..116
(D) OTHER INFORMATION: /note = "Bak (delta)3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Leu Gln His Leu Gln Pro Thr Ala Glu Asn Ala Tyr Glu Tyr Phe
1      5      10
Thr Lys Ile Ala Thr Ser Leu Phe Glu Ser Gly Ile Asn Trp Gly Arg
20     25     30
Val Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu Ala Leu His Val Tyr
35     40     45
Gln His Gly Leu Thr Gly Phe Leu Gly Gln Val Thr Arg Phe Val Val
50     55     60
Asp Phe Met Leu His His Cys Ile Ala Arg Trp Ile Ala Gln Arg Gly
65     70     75     80
Gly Trp Val Ala Leu Asn Leu Gly Asn Gly Pro Ile Leu Asn Val
85     90
Leu Val Val Leu Gly Val Val Leu Gly Gln Phe Val Val Arg Arg
100    105    110
Phe Phe Lys Ser
115

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 975 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 26..478

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTCCTCGCGA GACAAGCTCA CCGAA ATG GCC GGC TCC AGT CAA GGA AAC TTT
Met Ala Ala Ser Ser Gln Gly Asn Phe

52

Met Ala Ala Ser Ser Gln Gly Asn Phe
215

220
GAG GGA AAT TTT GAG TCA CTG GAC CTT GCG GAA TTT GCT AAG AAG CAG Glu Gly Asn Phe Glu Ser Leu Asp Leu Ala Glu Phe Ala Lys Lys Gln 225 230
CCA TGG TGG CTG AAG CTG TTC GGG CAG GAA TCT GGA CCT TCA GCA GAA Pro Trp Trp Arg Lys Leu Phe Gly Gin Glu Ser Gly Pro Ser Ala Glu 240 245
AAG TAT AGC GTG GCA ACC CAG CTG TTC ATT GGA GGT GTC ACT GGA TGG Lys Tyr Ser Val Ala Thr Gin Leu Phe Ile Gly Gin Val Thr Gly Trp 255 260
TGC ACA GGT TTC ATA TTC CAG AAG GTT GGA AAG TTG GCT GCA ACA GCT Cys Thr Gly Phe Ile Phe Gin Lys Val Gly Lys Leu Ala Ala Thr Ala 270 275
280
GTG GGA GGT GGA TTT TTT CTC CTT CAG CTT GCA AAC CAT ACT GGG TAC Val Gly Gly Phe Phe Leu Leu Gin Leu Ala Asn His Thr Gly Tyr 285 290
295
300
ATC AAA GTT GAC TGG CAA CGA GTG GAG AAG GAC ATG AAG AAA GCC AAA Ile Lys Val Asp Trp Gin Arg Val Glu Asp Met Lys Lys Ala Lys 305 310
315
GAG CAG CTG AAG ATC GGT AAG AGC AAT CAG ATA CCT ACT GAG GTC AGG Glu Gin Leu Lys Ile Arg Lys Ser Asn Gin Ile Pro Thr Glu Val Arg 320 325
330
AGC AAA GCT GAG GAG GTG GTG TCA TTT GTG AAG AAG AAT GTT CTA GTA Ser Lys Ala Glu Val Ser Phe Val Lys Lys Val Asn Val Leu Val 335 340
345
ACT GGG GGA TTT TTC GGA GGC TTT CTG CTT GGC ATG GCA TCC Thr Gly Gly Phe Phe Gly Phe Leu Leu Gin Met Ala Ser 350 355
360
TAAGGAAGAT GACCTCATGT TCATTGTTCC TGGTPTTTTCC CAGCCACGAG CCTCTACACT 538
CCATCATAGG ACAAAGGCT CACTCCTCTC TTCTCCCATG CCCCCTCTTCC CTGCCATGGC 598
AAATCTGAGT GGCTCTCTTA AGCATCTGCT GTACAAAGTC AATGTGCCAC CATGAGCTTC 658
ATGCTGGCGA AAGAGACGAT AGTCCT TAGC TACTCCTCCA GTACACCCCC TACTTGGCCA 718
GCTGGTGGCC CAACAAGGAG GTGCTTTTGC CCCCCAGGCA GACACTTTAG AAGAGACAGT 778
ACAAGATGCA TGACGGTGGG GAGATGAGTG ATCCCTGAAAG GTGTCTCTCAAA ACTTGTTGATT 838
TGGAANAGAA AATAAGCACAT AGATAACCTT ATATGTGCTCT GTCTGAAAG GAAAGGAACT 898
GAAATCATTT TGGTTAAAGC ATGGAAAAAA AAAA AAAAAAAA AAAAAAAA AAAAAAAA AAAAAAAA 958
AAAAAAAG AAAA
(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 151 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Ala Ala Ser Ser Gln Gly Asn Phe Glu Gly Asn Phe Glu Ser Leu
  1     5      10     15
Asp Leu Ala Glu Phe Ala Lys Lys Gln Pro Trp Trp Arg Lys Leu Phe
 20    25     30
Gly Gln Glu Ser Gly Pro Ser Ala Glu Lys Tyr Ser Val Ala Thr Gln
 35    40     45
Leu Phe Ile Gly Gly Val Thr Gly Trp Cys Thr Gly Phe Ile Phe Gln
 50    55     60
Lys Val Gly Lys Leu Ala Ala Thr Ala Val Gly Gly Gly Phe Phe Leu
 65    70     75     80
Leu Gln Leu Ala Asn His Thr Gly Tyr Ile Lys Val Asp Trp Gln Arg
 85    90
Val Glu Lys Asp Met Lys Lys Ala Lys Glu Gln Leu Lys Ile Arg Lys
100   105    110
Ser Asn Gln Ile Pro Thr Glu Val Arg Ser Lys Ala Glu Glu Val Val
115   120    125
Ser Phe Val Lys Asn Val Leu Val Thr Gly Phe Phe Gly Gln
130   135    140
Phe Leu Leu Gly Met Ala Ser
145   150
CLAIMS

1. An isolated polypeptide comprising SEQ ID NO:7.

2. An isolated polypeptide, comprising a linear sequence of six or more amino acids of SEQ. ID NO:7.

3. An isolated polypeptide having at least one of the biological functions of the polypeptide of SEQ ID NO:7.

4. An isolated polypeptide comprising a fragment of SEQ ID NO:7, wherein said fragment binds to Bak protein under appropriate conditions.

5. A fusion polypeptide comprising the polypeptide of SEQ ID NO:7 or fragments thereof.

6. An isolated polynucleotide comprising SEQ. ID NO:6 and polynucleotide sequences complementary thereto.

7. An isolated polynucleotide comprising a fragment of at least 18 consecutive nucleotides of SEQ ID NO:6.

8. An isolated polynucleotide encoding the polypeptide of SEQ ID NO:7.

9. An isolated polynucleotide comprising a sequence that encodes a polypeptide having at least one of the biological functions of the polypeptide of SEQ ID NO:7 and a polynucleotide complementary thereto.

10. An isolated polynucleotide comprising a fragment of SEQ ID NO:6, wherein said fragment encodes a polypeptide that binds to Bak protein under appropriate conditions.
11. The isolated polynucleotide of claims 6, 7, 8, 9 or 10, which is operably linked to control sequences for expression.

12. A recombinant vector comprising the polynucleotide of claim 11.

13. Recombinant host cells modified to contain the polynucleotide of claim 12.

14. The recombinant host cells of claim 13, wherein the cells are bacterial.

15. The recombinant host cells of claim 13, wherein the cells are eukaryotic.

16. A method for screening potential therapeutic agents that modulate the interaction between Bak and BBP comprising the steps of:

(a) combining a Bak and a BBP under conditions in which they interact, to form a test sample;

(b) exposing the test sample to a potential therapeutic agent and;

(c) monitoring the interaction of the Bak and the BBP; wherein a potential therapeutic agent is selected for further study when it modifies the interaction compared to a control test sample to which no potential therapeutic agent has been added.

17. The method according to claim 16 wherein the potential therapeutic agent is selected from the group consisting of a pharmaceutical agent, a cytokine, a small molecule drug, a cell-permeable small molecule drug, a hormone, a
combination of interleukins, a lectin, a stimulating agent, a bispecific antibody, a
peptide mimetic, and an antisense oligonucleotide.

18. The method according to claim 16 wherein the Bak is selected from
the group consisting of Bak, a fragment of Bak sufficient to effect binding to a BBP,
and a fusion protein comprising a portion of Bak sufficient to effect binding to a BBP.

19. The method according to claim 18 wherein the fusion protein
comprises epitope-tagged Bak.

20. The method according to claim 16 wherein the BBP is selected from
the group consisting of epitope-tagged BBP and proteins homologous to SEQ ID
NO:7.

21. The method according to claim 16 wherein the monitoring step is
selected from the group consisting of co-precipitation, protein interactive trapping and
ELISA.

22. A composition comprising a monoclonal or polyclonal antibody or an
antigen-binding fragment thereof which forms a complex with a BBP but is
substantially unreactive with dissimilar proteins.

23. A method of detecting the presence of a BBP protein in a biological
sample comprising the steps of:
   a) obtaining a cell sample;
   b) exposing the contents of the cells to antibodies;
c) adding anti-BBP-specific antibodies to the cell sample;
d) maintaining the cell sample under conditions that allow the antibodies to complex with the BBP; and
e) detecting the antibody-BBP complexes formed.

24. A method for detecting the expression of a bbp gene in a biological sample comprising the steps of identifying the presence of RNA encoding the bbp.

25. The method according to claim 24 wherein the method for identifying the RNA encoding the bbp comprises Northern blotting.

26. A method identifying bbp mRNA comprising the steps of:

(a) obtaining a cell sample;

(b) obtaining RNA from the cell sample;

(c) performing a polymerase chain reaction on the RNA using primers corresponding to unique regions of bbp; and

(d) detecting the presence of products of the polymerase chain reaction.

27. A method of modulating apoptosis-induced cell death comprising modulating the endogenous levels of BBP.

28. The method according to claim 27 wherein the BBP levels are increased by modulating expression of an endogenous bbp gene.

29. The method according to claim 27 wherein the BBP levels are decreased by modulating expression of an endogenous bbp gene.
30. The method according to claim 27 wherein the BBP is encoded by an endogenous gene.

31. The method according to claim 27 wherein the BBP is encoded by a recombinant gene.

32. The method according to claim 31 wherein expression of the recombinant gene is under the control of an inducible promoter.

33. The method according to claim 32 wherein the recombinant gene is transfected into cells \textit{ex vivo} and further comprising the steps of reintroducing the transfected cells into an animal.

34. The method according to claim 32 wherein the recombinant gene is transfected into cells \textit{in vivo}.

35. A method of inducing apoptosis in a patient in need thereof comprising administering a therapeutically effective amount of the BBP.

36. An isolated polypeptide comprising amino acids 103-126 of SEQ ID No: 2, or derivatives thereof.

37. An isolated and purified peptide comprising a BBP Binding Domain.

38. An isolated polypeptide, comprising a linear sequence of six or more amino acids of a BBP Binding Domain.

39. An isolated polypeptide having at least one of the biological functions of a BBP Binding Domain.
40. An isolated polypeptide comprising a fragment of a BBP Binding Domain wherein said fragment binds to BBP protein under appropriate conditions.

41. A fusion polypeptide comprising a BBP Binding Domain or fragments thereof.

42. An isolated polynucleotide comprising nucleotides 507-578 of SEQ ID NO:1, and polynucleotide sequences complementary thereto.

43. An isolated polynucleotide comprising a fragment of at least 18 consecutive nucleotides of bbpbd-1.

44. An isolated polynucleotide comprising nucleotides 611-668 of SEQ ID NO:1, and polynucleotide sequences complementary thereto.

45. An isolated polynucleotide comprising a fragment of at least 18 consecutive nucleotides of bbpbd-2.

46. An isolated polynucleotide encoding a BBP Binding Domain.

47. An isolated polynucleotide comprising a sequence that encodes a polypeptide having at least one of the biological functions of a BBP Binding Domain and a polynucleotide complementary thereto.

48. The isolated polynucleotide of claims 42, 43, 44, 45, 46 or 47 which is operably linked to control sequences for expression.

49. A recombinant vector comprising the polynucleotide of claim 48.

50. Recombinant host cells modified to contain the polynucleotide of claim 48.
51. The recombinant host cells of claim 50, wherein the cells are bacterial.
52. The recombinant host cells of claim 50, wherein the cells are eukaryotic.
53. A method of modulating apoptosis-induced cell death comprising modulating the endogenous levels of a BBP Binding Domain.
54. The method according to claim 53 wherein the BBP Binding Domain levels are increased by modulating expression of an endogenous bak gene.
55. The method according to claim 53 wherein the BBP Binding Domain levels are decreased by modulating expression of an endogenous bak gene.
56. The method according to claim 53 wherein the BBP Binding Domain is encoded by an endogenous gene.
57. The method according to claim 53 wherein the BBP Binding Domain is encoded by a recombinant gene.
58. The method according to claim 57 wherein expression of the recombinant gene is under the control of an inducible promoter.
59. The method according to claim 57 wherein the recombinant gene is transfected into cells ex vivo and further comprising the steps of reintroducing the transfected cells into an animal.
60. The method according to claim 57 wherein the recombinant gene is transfected into cells in vivo.
61. A method of modulating apoptosis in a patient in need thereof comprising administering a therapeutically effective amount of a BBP Binding Domain.

62. An isolated polypeptide comprising amino acids 138-156 of SEQ ID No: 2, or derivatives thereof.
Figure 2B

830  840  850  860  870  880  890  900
AAA TCA TGA  CTTCC AAAGGGGTGCG  CTTCCCTGC TTCTCAACCG  CCGTGTCGGCT  AGCTTTAGCGA  AGCTTGCTTCG
TTT ACT ACT  CGAGTGTGAGG  GTTCCAGGAA  GCAAACAGGGG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG
K S

910  920  930  940  950  960  970  980
TTCTCTCTTC  CTTCCCTTAC  AGAGTGGAGGA  AGCTTTAGCGA  AGCTTGCTTCG  CGAGTGTGAGG  GTTCCAGGAA  GCAAACAGGGG
GGAACGGTTT  TGGAGTGGAGG  GTTCCAGGAA  GCAAACAGGGG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG

>Psil
990  1000  1010  1020  1030  1040  1050  1060
TGGGGGCGCG  TCAAGCTCTCA  GAAGGCACCTC  AACACTCTCAT  GGGCTCTGGG  GGTGACAGTC  ATCCAGCTTC  GGGCCAGAGG
ACCCTGGTTTC  AGTGGCAGGAC  GGGCAGAGGAC  GGGCCAGAGGAC  GGGCCAGAGGAC  GGGCCAGAGGAC  GGGCCAGAGGAC  GGGCCAGAGGAC

1070  1080  1090  1100  1110  1120  1130  1140
CTCTTCCTTC  AGCTTTAGCGA  AGCTTTAGCGA  GGGCTCTGGG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG
GGAACGGTTT  TGGAGTGGAGG  GTTCCAGGAA  GCAAACAGGGG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG

1150  1160  1170  1180  1190  1200  1210  1220
CTGGAACCA  TCTTCCTACA  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG

>Ala2
1230  1240  1250  1260  1270  1280  1290  1300
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1310  1320  1330  1340  1350  1360  1370  1380
ACCCTGACCA  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG

1390  1400  1410  1420  1430  1440  1450  1460
GAGGGGGGGG  TGGAGTGGAGG  GTTCCAGGAA  GCAAACAGGGG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG

1470  1480  1490  1500  1510  1520  1530  1540
CTGAGCTTGC  GGTGCAAAAG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG

1550  1560  1570  1580  1590  1600  1610  1620
AGCTTTAGCGA  TCTGACCATCC  CTTCCCTGC  TCTGACCATCC  CTTCCCTGC  TCTGACCATCC  CTTCCCTGC  TCTGACCATCC

1630  1640  1650  1660  1670  1680  1690  1700
TGGTGCTGCTG  GGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG

1710  1720  1730  1740  1750  1760  1770  1780
ACTGTTTGTG  TTTATGAGT  AAGGGAGGTG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG  AGTGAGATCG

>BaseH1
1790  1800  1810  1820  1830  1840  1850  1860
TAGGATCC  AGAGGAGGTG  GTTCCAGGAA  GCAAACAGGGG  GGGAGGTGCG  TGAGAAGGACCG  TCGAATCGCTC  TCAGAAACCG
Figure 2C

> BssI

1870 1880 1890 1900 1910 1920 1930 1940
TTGACTCTG TTCCGCCACC CTCATGCTC CACCTCTGC TCGTCCTCT CAGGGTGGGG GGAGCAGAG CCGTTCTCTAT
AAGGGGGTGG AGGTACGAG GAGTGAGAC ATCCAGAGGA GTCCACCCCC CCAGCTGTCG GGAAGAGATA

TGGCAAGCCC TAGGGTCTTG GGCTGGAGGG GGAGAAGTT CTTGATTCAG CAAATCGAC GGAGGGGAGG CAGATGGAGC
ACCGTGCTGG ATCCCAAGACC CCCAGTCCCC CCCCCCTCAA GAAGAAGTGC GTTTTACGTC CCTCCCCCTCC GTCTACGTG

2030 2040 2050 2060 2070 2080 2090
CCATAGCCCA CCCCTATGCC CTGGGAATTT TGCAAATAAA CTGGCAATCC CCCTCAAAAA AAAAAAGGAG ATCC
GGTACCGGGT GGGGGTAGGG AGACCTCACAA ACCTTATTTT GACACGTTAG GGGAGTTTTT TTPTTTGCTC TAGG
Figure 4

SEQ ID NO.: 6

gcctcgagacaaagctcaacgaa
ATG GCC GCG TCC AGT CAA GGA AAC TTT GAG GGA AAT TTA GAG TCA
1 M A A S S Q G N F E G N F S
CTG GAC CTT GGG GAA TTT GCT AAG AAG CAG CCA TGG TGG GTG AAG
115 L D LA F AK K Q P W R K
CTG TTC GGG CAG GAA TCT GGA CCT TCA GCA GAA AAG TAT ACG GTG
160 L F G Q E S G P S A E K Y S V
GCA ACC CAG CTG TTC ATT GGA GGT GTC ACT GGA TGG TGC ACA GGT
205 A T Q L F I G G V T G W C T G
TTC ATA TTC CAG AAG GTT GGA AAG TTT GCT GCA ACA GCT GTG GGA
250 F I F Q K V G K L A A T A V G
GGT GGA TTT TTT CTC CTT CAG CTT GCA AAG CAT ACT GGG TAC ATC
295 G G F F L L Q L A N H T G Y I
AAA GTT GAC TGG CAA CGA GTG GAG AAG GAC ATG AAG AAA GCC AAA
340 K V D W Q R V E K D M K K K
GAG CAG CTG AAG ATC CAG AAG GTC TCC ACT TCA GAT GTG
385 E Q L K I R K S N Q I P T E V
AGG AGC AAA GCT GAG GAG GTG GTG TCA TTT GTG AAG AAG AGT GTT
430 R S K A E E V V S F V K K N V
CTA GTA ACT GGG GGA TTT TTC GCA GGC TTT CTG CCA ATG GCA
475 L V T G G F F G F L L G M A
TCC TAA ggaagatgacccatgtctatgtgtctgctcttttccgcaccagcagccttc
532 * S

tacactcccatcaagcagctagctcacctctccctctctctctctctctctcctgc 592
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6/11
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<th>bcl2</th>
<th>bclx</th>
<th>bax</th>
<th>E1b19K</th>
<th>pLAM5'</th>
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<tr>
<td>BBP</td>
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<td>-</td>
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Figure 6
Figure 7

C B C B C B C B C B C B yeast lysate

GST GST-BHRF-1 GST-E1B GST-BBP-L1 GST-BBP-L2 GST-BBP-L1+L2 resin

kDa

35.5
29.1
20.9
Amino Acids 1-194 of SEQ ID NO.: 2

1 MASGQGP|GPRQECGEPALPSASEEQVA|DTEEVFRSYV|FYRHQQEQE 48

49 AEGVAA|PADP|EMVTLPLQPSSTMGQVR|QLAI|G|DINRR|YD|SEFQ|TML 97

BH3

98 QHLQP|TAENA|YE|FT|KIAT|S|LFESG|INWGRVVALLGF|YR|LALHV|Y|QHG 146

BH1

147 LT|GFLGQVTRFVVDFMLHHCIARWIA|QRGWVAALNLGNGPILNVLVV 194

BH2
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

<table>
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<th>IPC(6)</th>
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<td>Please See Extra Sheet.</td>
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</table>

According to International Patent Classification (IPC) or to both national classification and IPC.

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

| U.S. | 435/ 7.1, 69.1, 69.7, 91.2, 172.3, 320.1; 514/ 2, 44; 536/23.5, 24.31; 530/350, 387.9, 388.1 |

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPLUS search terms: bak, bbp, binding proteins, bhrf1, PCR, interaction, antibody, treatment

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
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<td>X</td>
<td>OUDEJANS, et al. BHRF1, the Epstein-Barr Virus (EBV) Homologue of the BCL-2 Proto-oncogene, Is Transcribed in EBV-Associated B-Cell Lymphomas and in Reactive Lymphocytes 01 September 1995. Vol 86. No. 5. pages 1893-1902, especially pages 1895-1896.</td>
<td>24, 26</td>
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<tr>
<td>X</td>
<td>WO 96/33416 A1 (BARR et al) 24 October 1996, claims 1-6.</td>
<td>16-21</td>
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</table>

[X] Further documents are listed in the continuation of Box C.  

See patent family annex.

**Date of the actual completion of the international search**

21 APRIL 1998

**Date of mailing of the international search report**

2 JUN 1998

**Name and mailing address of the ISA/US Commissioner of Patents and Trademarks**

Box PCT  
Washington, D.C. 20231

Facsimile No.  (703) 305-3230

**Authorized officer**

DARYL A. BASHAM

**Telephone No.**

(703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)
<table>
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<td>28, 33, 35, 54, 59, 61, 62</td>
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**Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-15, 36-52 and 62
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
   Claims 1-15, 36-52 and 62 could not be searched with respect to the enumerated sequence identifiers recited in the claims because there was no computer readable form containing the sequences of the disclosure available.

3. □ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.
A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):
C12N 15/12, 12/64; G01N 33/53; C12Q 1/48, 1/68; C07K 14/435; A61K 38/16

A. CLASSIFICATION OF SUBJECT MATTER:
US CL:
435/ 7.1, 69.1, 69.7, 91.2, 172.3, 320.1; 514/ 2, 44; 536/23.5, 24.31; 530/350, 387.9, 388.1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 16-21, drawn to a method of screening using polypeptide.
Group II, claim(s) 22, drawn to an antibody.
Group III, claim(s) 23, drawn to a method of detecting protein using antibody.
Group IV, claim(s)24-26, drawn to method of detecting gene expression.
Group V, claim(s) 27-34 and 53-60, drawn to method of modulating BBP and BBP binding domain levels.
Group VI, claim(s) 35 and 61, drawn to method of inducing apoptosis comprising administration of BBP and a method of modulating apoptosis comprising administration of BBP binding domain.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The method of Group I is materially different from the methods of Groups III-VI because each is practiced with materially different process steps; the process steps are the special technical features which distinguishes each method from the others. Because the process steps do not share the same or a corresponding special technical feature, the unity of invention is lacking. The method of Group I and the antibody of Group II do not share a special technical feature as the antibody is not used in nor produced by the process steps comprising Group I. The special technical feature by which the method of Group I is defined distinguish Group I from the special technical feature which defines the antibody of Group II and as such they do not share the same or corresponding special technical features. The claims are not so linked by a special technical feature within the meaning of the PCT Rule 13.2 so as to form a single inventive concept.