Title: METHODS FOR ASSAYING MITOCHONDRIAL INTERMEMBRANE SPACE PROTEIN TRANSLOCATION

Staurosporine/200nm

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Cytosol: cytochrome c

HA

PKB

Abstract: Compositions and methods are provided for identifying agents that alter mitochondrial intermembrane space protein (MISP) translocation. The screening methods generally detect agents that alter the level of detectable extramitochondrial MISP following exposure of a cell to an agent known or suspected to induce mitochondrial intermembrane space protein translocation. Such agents may be used, for example, in the treatment of a variety of conditions associated with altered mitochondrial function.
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METHODS FOR ASSAYING MITOCHONDRIAL INTERMEMBRANE SPACE PROTEIN TRANSLOCATION

TECHNICAL FIELD

The invention relates generally to methods for detecting the translocation of mitochondrial intermembrane space proteins. More specifically, the invention relates to compositions and screening methods for use in identifying agents that alter the translocation of epitope tagged mitochondrial intermembrane space proteins, which occurs for example as a result of altered outer mitochondrial membrane permeability, in response to various agents including, for example, apoptogens.

BACKGROUND OF THE INVENTION

Mitochondria are the main energy source in cells of higher organisms, and provide direct and indirect biochemical regulation of a wide array of cellular respiratory, oxidative and metabolic processes. Such processes include electron transport chain (ETC) activity, which drives oxidative phosphorylation to produce metabolic energy in the form of adenosine triphosphate (ATP), and which controls mitochondrial regulation of intracellular and intramitochondrial calcium homeostasis.

Mitochondrial ultrastructural characterization reveals the presence of an outer mitochondrial membrane that serves as an interface between the organelle and the cytosol, a highly folded inner mitochondrial membrane that appears to form attachments to the outer membrane at multiple sites, and an intermembrane space between the two mitochondrial membranes. The subcompartment within the inner mitochondrial membrane is commonly referred to as the mitochondrial matrix. (For a review, see, e.g., Ernster et al., 1981 J. Cell Biol. 91:227s.) The cristae, originally postulated to occur as infoldings of the inner mitochondrial membrane, have recently been characterized using three-dimensional electron tomography as also including tube-like conduits that may form networks, and that can be connected to the inner membrane and/or the intermembrane space by open, circular (30 nm diameter) junctions (Perkins et al., 1997, Journal of Structural Biology 119:260-272). While the outer membrane is freely permeable to ionic and non-ionic solutes having molecular
weights less than about ten kilodaltons, the inner mitochondrial membrane exhibits selective and regulated permeability for many small molecules, including certain cations, and is impermeable to large (> \sim 10 \text{kDa}) molecules.

Altered or defective mitochondrial activity, including but not limited to failure at any step of the ETC, may result in catastrophic mitochondrial collapse that has been termed "permeability transition" (PT) or "mitochondrial permeability transition" (MPT). This collapse can also be less catastrophic, causing MPT within local domains of an individual mitochondrion. According to generally accepted theories of mitochondrial function, proper ETC respiratory activity requires maintenance of an electrochemical potential (\Delta \Psi_m) in the inner mitochondrial membrane by a coupled chemiosmotic mechanism. Altered or defective mitochondrial activity may dissipate this membrane potential, thereby preventing ATP biosynthesis and halting the production of a vital biochemical energy source. In addition, mitochondrial intermembrane space proteins such as cytochrome c may be transported or may leak out of the mitochondria after permeability transition initiating the genetically programmed cell suicide sequence known as apoptosis or programmed cell death (PCD).

Thus, in addition to their role in energy production in growing cells, mitochondria (or, at least, mitochondrial components) participate in apoptosis (Newmeyer et al., 1994, \textit{Cell} 79:353-364; Liu et al., 1996, \textit{Cell} 86:147-157). Apoptosis is apparently also required for, \textit{inter alia}, normal development of the nervous system and proper functioning of the immune system. Moreover, some disease states are thought to be associated with either insufficient (\textit{e.g.}, cancer, autoimmune diseases) or excessive (\textit{e.g.}, stroke damage, AD-associated neurodegeneration) levels of apoptosis. For general reviews of apoptosis, and the role of mitochondria therein, see Green and Reed (1998, \textit{Science} 281:1309-1312), Green (1998, \textit{Cell} 94:695-698) and Kroemer (1997, \textit{Nature Medicine} 3:614-620). Hence, agents that affect apoptotic events, including those associated with mitochondrial components, might have a variety of palliative, prophylactic and therapeutic uses.

At least one recognized apoptotic mechanism therefore involves the release of cytochrome c from the mitochondrial intermembrane space to the cytosol, a process referred to as translocation of the mitochondrial intermembrane space protein (\textit{e.g.}, Single et al., 1998 \textit{Cell Death Diff.} 5:1001). Improved understanding of the regulation of mitochondrial outer
membrane permeability is therefore desirable in order to diagnose and/or screen for agents useful for treating conditions associated with altered (e.g., increased or decreased) apoptosis.

Currently, the mechanism of cytochrome c translocation from the mitochondrial intermembrane space is not well understood, and may be specific to cytochrome c or may, alternatively, be accompanied by translocation of one or more additional intermembrane space proteins (Singe et al., 1998). Several distinct translocation models have been proposed, including (i) a model whereby specific channels for cytochrome c release are opened by pro-apoptotic Bcl-2 family members, (ii) a model wherein MPT underlies cytochrome c translocation and (iii) a model wherein mitochondrial outer membrane swelling and rupture permit nonspecific translocation of cytochrome c and other intermembrane space proteins such as adenylate kinase (see, e.g., Kohler et al., 1999 FEBS Lett. 447:10-12, and references cited therein). Technical problems related to the reliable detection of these and other intermembrane space proteins, however, have prevented further determination of mitochondrial events during translocation. For example, modifications to intermembrane space proteins that dramatically increase their molecular mass may disrupt their ability to translocate if mitochondrial outer membrane permeability is a function of solute size. As another example, the high degree of immunological crossreactivity between the mitochondrial and cytosolic isoforms of adenylate kinase (AK) may preclude reliable determination of translocated mitochondrial AK isoforms in the cytosol.

To provide improved diagnostics and therapeutics for disorders in which intermembrane space protein translocation may be a pathogenic event, agents that alter such translocation may be beneficial, and assays to specifically detect such agents are needed. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

The present invention is directed to compositions and methods for detecting mitochondrial intermembrane space protein translocation, including screening assays for agents that alter such translocation. Accordingly, it is an aspect of the invention to provide methods of detecting mitochondrial intermembrane space protein translocation, comprising contacting a sample comprising a cell (which may optionally be permeabilized) containing a
mitochondrion, or an isolated mitochondrion, or an apoptosis reconstitution system, with an agent known or suspected to induce the translocation of one or more mitochondrial intermembrane space proteins, under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, the mitochondrion comprising at least one mitochondrial intermembrane space protein fusion polypeptide, wherein the fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain; and contacting the sample with a detectable ligand that specifically binds to the affinity domain under conditions and for a time sufficient for said detectable ligand to bind to said fusion polypeptide in extramitochondrial spaces in order to form detectable ligand:fusion polypeptide complexes, and detecting the ligand:fusion polypeptide complexes, wherein an increasing signal from said detectable ligand:fusion polypeptide complexes corresponds to an increasing degree of intermembrane space protein translocation.

It is another aspect of the present invention to provide a method of identifying an agent that alters (e.g., enhances or inhibits) the translocation of one or more mitochondrial intermembrane space proteins, comprising contacting a biological sample comprising a mitochondrion with a candidate agent suspected of being able to alter mitochondrial intermembrane space protein translocation under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, the mitochondrion comprising at least one mitochondrial intermembrane space protein fusion polypeptide, wherein the fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain; contacting the sample with a detectable ligand that specifically binds to the affinity domain under conditions and for a time sufficient for the detectable ligand to bind to said fusion polypeptide in extramitochondrial spaces in order to form detectable ligand:fusion polypeptide complexes, and detecting the ligand:fusion polypeptide complexes; and comparing the level of extramitochondrial ligand:fusion polypeptide complexes detected in a control sample lacking the candidate agent to the level of extramitochondrial ligand:fusion polypeptide complexes detected in the sample having the candidate agent; wherein an altered level of signal of detectable ligand:fusion polypeptide complexes in the sample having the candidate agent, relative to the level of signal of detectable ligand:fusion polypeptide complexes in the control sample that lacks said
candidate agent, indicates that the candidate agent is an agent that alters mitochondrial intermembrane space protein translocation.

In another aspect of the present invention, an agent that alters mitochondrial intermembrane space protein translocation is provided, and the object of the invention is to provide a method of identifying a compound that alters (e.g., enhances or inhibits) the activity of the agent that alters mitochondrial intermembrane space protein translocation. Such a method comprises contacting a sample comprising a mitochondrion with, sequentially in either order or simultaneously, (i) an agent that alters mitochondrial intermembrane space protein translocation and (ii) a candidate compound suspected of being able to alter the activity of the agent, under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, the mitochondrion comprising at least one mitochondrial intermembrane space protein fusion polypeptide, wherein the fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain; contacting the sample with a detectable ligand that specifically binds to said affinity domain under conditions and for a time sufficient for the detectable ligand to bind to the fusion polypeptide in extramitochondrial spaces in order to form detectable ligand:fusion polypeptide complexes, and detecting the ligand:fusion polypeptide complexes; and comparing the level of extramitochondrial ligand:fusion polypeptide complexes detected in a control sample lacking said candidate compound to the level of extramitochondrial ligand:fusion polypeptide complexes detected in the sample having the candidate compound, wherein an altered level of signal of detectable ligand:fusion polypeptide complexes in the sample having the candidate compound, relative to the level of signal of detectable ligand:fusion polypeptide complexes in the control sample, indicates that the candidate compound is a compound that alters the activity of the agent.

As is appreciated by those skilled in the art, a trivial modification of the preceding methods is as follows. Rather than having two separate test and control samples, to which the candidate agent or compound is and is not added to, respectively, equivalent methods can be designed in which the control sample is assayed first in time and then used as the test sample. That is, the sample is assayed first in the absence of the candidate agent or
compound and thus serves as the control sample, after which the candidate agent or compound is added to the same sample, which is then assayed as the test sample.

In the preceding and other embodiments of the invention, the mitochrondrion is contained in a cell, which is a neural cell in certain embodiments, and in certain embodiments the cell is a neuroblastoma cell. In certain further embodiments the neuroblastoma cell is a SH-SY5Y cell. Regardless of cell type, the cell may be permeabilized by the addition of permeabilizing agents such as digitonin, streptolysin O, *Staphylococcus aureus* α-toxin (α-hemolysin), saponin (all available from Sigma Chemical Co., St. Louis, MO; see Sigma catalog entitled “Biochemicals and Reagents for Life Science Research,” Anon., 1999, and references cited therein for these permeabilizing agents), or by physical manipulations, for example, electroporation. In other embodiments of the invention, the permeabilized cell is depleted of cytosol. In other embodiments of the invention, the mitochondrion is provided substantially isolated from other cellular components. In embodiments wherein isolated or purified mitochondria are used, release of a mitochondrial intermembrane space protein from mitochondria into their media is taken as a measure of protein “translocation” even though, to be precise, only dissociation from mitochondria actually occurs, as the cellular compartment(s) to which the proteins would translocate in intact cells are absent.

In certain embodiments, the mitochondrial intermembrane space protein is adenylate kinase-2, cytochrome c or sulfide oxidase, and in particularly preferred embodiments the mitochondrial intermembrane space protein is adenylate kinase-2. According to certain embodiments of the invention, the affinity domain is a hemagglutinin epitope tag, a FLAG® epitope tag, an XPRESS™ epitope tag, a myc epitope tag or a polyhistidine epitope tag and in particularly preferred embodiments the affinity domain comprises a hemagglutinin epitope tag. In some embodiments the detectable ligand comprises an antibody, and in certain further embodiments the antibody comprises a monoclonal antibody while in certain other further embodiments the antibody comprises a polyclonal antibody. In still other further embodiments the antibody comprises a single chain antibody, and in other embodiments the antibody comprises a fusion protein.

In certain embodiments, translocation of a mitochondrial intermembrane space protein occurs with some degree of activation of the mitochondrial permeability transition
(MPT) “pore” and/or biochemical events in apoptotic pathways. In certain other embodiments, however, protein translocation is observed in the absence of MPT; in these embodiments, the translocation of mitochondrial intermembrane space proteins can be detected and assayed in isolation from confounding signals that might result from MPT and/or apoptosis. Moreover, in some disorders, although translocation of mitochondrial intermembrane space proteins may not result in MPT and/or apoptosis per se, other pathological consequences may ensue. In particular, the translocation of cytochrome c from mitochondria under conditions wherein MPT and/or apoptosis are not seen to occur may be assayed according to the methods of the invention.

In certain embodiments of the present invention the cell comprises a polypeptide that is a Bcl-2 family member, and in certain further embodiments the cell comprises a nucleic acid expression construct comprising a promoter operably linked to a polynucleotide encoding a Bcl-2 family member. Bcl-2 family members include, by way of example and not limitation, Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, NR-13, BHRF1, LMW5-HL, ORF16, Ks-Bcl-2, E1B-19K (U.S. Patent No. 5,858,678) and Ced-9 (Adams et al., Science 281:1322-1326, 1998). The Bcl-2 family member polypeptide may be modified, for example by way of mutation, to contain one or more altered or substituted amino acid residues (see, for example, U.S. Patent No. 5,856,171).

In the preceding and other embodiments of the invention, the agent of the methods is an apoptogen, such as a pro-oxidant or a calcium ionophore, and in certain further embodiments the pro-oxidant is hydrogen peroxide, tert-butylhydroperoxide or peroxynitrite. In certain embodiments the calcium ionophore is ionomycin. In certain other embodiments of this aspect of the invention the apoptogen is an appropriate amount of atractyloside, bongkrekic acid, thapsigargin, glutamate, N-methyl-D-aspartic acid, carbachol, A23187 or ionomycin. In the case of permeabilized cells or isolated mitochondria, such apoptogens may further include Ca\(^{++}\), pro-apoptotic factors such as Bax (Jürgensmeier et al., Proc. Natl. Acad. Sci. U.S.A. 95:4997-5002, 1998; U.S. Patent No. 5,837,838), mutants of Bax (U.S. Patent No. 5,856,445) and Bid, and the like. In such embodiments, treatment with the agent may result in apoptosis whereas, in other embodiments, subcritical amounts of the apoptogen may
promote some degree of intermembrane space protein translocation without inducing apoptosis.

In other embodiments, the agent of the methods is an agent that can, at sufficiently high concentrations or periods of incubation, cause necrosis. In such embodiments, treatment with the agent may result in necrosis whereas, in other embodiments, subcritical amounts of the agent may promote some degree of intermembrane space protein translocation without inducing necrosis.

In other embodiments, the agent of the methods is an agent that promotes some degree of intermembrane space protein translocation but neither induces apoptosis nor causes necrosis. For example, such agents may induce MPT without inducing apoptosis or causing necrosis. When permeabilized cells are used in the methods of the invention, such agents may include, by way of non-limiting example, appropriate concentrations of MPT inducers such as t-BOOH, atractyloside, phenylarsine oxide, or gangliosides.

In some embodiments, a candidate compound is present and is being tested for its ability to alter (e.g., enhance or inhibit in a statistically significant manner) the activity of the agent that alters mitochondrial intermembrane space protein translocation. For example, the agent that alters mitochondrial intermembrane space protein translocation may be a calcium ionophore (when unpermeabilized cells are used) or Ca^{++} (when permeabilized cells or isolated mitochondria are used), and the compound that is being tested for its ability to alter the activity of such agents may be a Ca^{++} chelator. The invention may thus be used, for example, to evaluate the activity of compounds such as cell-permeant Ca^{++} chelators (Tymianski et al., J. Cereb. Blood Flow Metab. 14:911-923, 1994).

Turning to other aspects, the invention provides a nucleic acid expression construct comprising a promoter operably linked to a polynucleotide encoding an intermembrane space protein fusion polypeptide, wherein the fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain. Such cells can be cultured to produce the intermembrane space protein fusion polypeptide according to methods known in the art. Thus, in yet another aspect the invention provides an isolated intermembrane space protein fusion polypeptide, wherein the fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one
affinity domain. The intermembrane space fusion proteins of the invention may be incorporated into a containing device, such as a 96-well or 386-well microtiter plate, that may be combined with methods and mechanisms for high throughput screening of agents and compounds, according to methods known in the art.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entireties as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows western immunoblot analysis of transiently transfected 293 T and COS-1 cells expressing HA-tagged AK2 mitochondrial intermembrane space protein fusion polypeptide. Lane 1, molecular weight markers; lane 2, 293 T cells transfected with vector; lanes 3-6, 293 T cells transfected with an expression construct encoding an HA-tagged AK2 mitochondrial intermembrane space protein fusion polypeptide; lane 7, COS-1 cells transfected with vector; lanes 8-9, COS-1 cells transfected with an expression construct encoding an HA-tagged AK2 mitochondrial intermembrane space protein fusion polypeptide; lane 10, COS-1 cells taken through the transfection protocol without the addition of exogenous DNA. The sizes of the molecular weight markers (expressed in kilodaltons) are indicated on the lefthand side of the figure.

Figure 2 shows western immunoblot analysis of stable doubly transfected hybrid cells expressing HA-tagged AK2 mitochondrial intermembrane space protein fusion polypeptides and Bcl-2 polypeptides. Panel A, probed with antibody to Bcl-2; panel B, probed with antibody to HA tag. Lane 1, molecular weight markers; lanes 2-11, individually isolated potential double transfectants. The sizes of the molecular weight markers (expressed in kilodaltons) are indicated on the lefthand side of each panel. The isolate in lane 9, MC AK/Bcl2 #2, expresses both HA-tagged fusion and Bcl-2 polypeptides.

Figure 3 shows western immunoblot analysis of stable SH-SY5Y neuroblastoma cells expressing HA-tagged AK2 mitochondrial intermembrane space protein
fusion polypeptides. Lanes A and I, SH-SY5Y cells taken through the transfection protocol without the addition of exogenous DNA; lanes B-E, SH-SY5Y cells transfected with vector (pCDNA3) DNA; lanes F-H, SH-SY5Y cells transfected with expression constructs encoding HA-tagged AK2 fusion polypeptides.

Figure 4 shows tracings derived from mitochondria in a multiparameter chamber. The three panels on the left show results under the “non-MPT” conditions described herein, whereas the three panels on the right show results where ATP and Mg\(^{2+}\) have not been added.

Figure 5 is a Western immunoblot analysis of cells expressing an HA-tagged derivative of adenylate kinase 2 that were treated under “non-MPT” conditions.

Figure 6 shows light scattering tracings derived from mitochondria in a multiparameter chamber (left), and the corresponding Western analysis (right), for cells that have been treated with carboxyatractyloside (CAtr), Bongkrekic Acid (BkA) and alamethacin (Alm).

Figure 7 shows Ca\(^{2+}\) electrode tracings derived from mitochondria in a multiparameter chamber (left), and the corresponding Western analysis (right), for cells that have been treated with carboxyatractyloside (CAtr), Bongkrekic Acid (BkA) and alamethacin (Alm). “Bcl-2,” cells overexpressing Bcl-2; “puro,” control cells comprising a puromycin resistance gene.

Figure 8 is a Western immunoblot analysis of cells expressing an HA-tagged derivative of adenylate kinase 2 that were exposed to etoposide.

Figure 9 is a Western immunoblot analysis of cells expressing an HA-tagged derivative of adenylate kinase 2 that were exposed to thapsigargin.

Figure 10 is a Western immunoblot analysis of cells expressing an HA-tagged derivative of adenylate kinase 2 that were exposed to staurosporine.

Figure 11 shows calcium and mitochondrial membrane potential measurements in digitonin-permeabilized and cytosol depleted cells.

Figure 12 shows absorbance measurements in digitonin-permeabilized and cytosol depleted cells.
Figure 13 shows Western immunoblot analysis of cytochrome c in digitonin-permeabilized and cytosol depleted cells.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides compositions and methods for use in detecting mitochondrial intermembrane space protein translocation, and for identifying agents that alter such translocation. The invention is directed in part to detecting mitochondrial intermembrane space protein translocation in a biological sample comprising a cell having a mitochondrion. In preferred embodiments, the cell comprises a host cell transfected with, and expressing, a recombinant nucleic acid expression construct encoding an intermembrane space protein fusion polypeptide comprising an affinity domain as provided herein, for example, a monoclonal antibody defined peptide epitope tag. In certain further embodiments, the cell comprises a host cell transfected with, and expressing or overexpressing, a recombinant nucleic acid expression construct encoding a protein or polypeptide that is a member of the Bcl-2 family, for example, Bcl-2, Bid, Bad or Bax (see, e.g., Reed, Cell 91:559, 1997; Adams et al., Science 281:1322-1326, 1998). The method of the invention comprises, in part, contacting the biological sample with an agent known or suspected to induce the translocation of one or more mitochondrial intermembrane space proteins, under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, and contacting the sample with a detectable ligand as provided herein in order to detect extramitochondrial intermembrane space protein fusion polypeptide, i.e., fusion protein that has translocated.

According to the present invention, any mitochondrial intermembrane space protein (MISP) may be selected for use in a MISP fusion polypeptide as provided herein, as long as the MISP is a protein or polypeptide that is naturally present in a mitochondrial intermembrane space of any subject or biological source as described herein. Examples of MISPs include cytochrome c (Green et al., 1998 Science 281:1309; Liu et al., 1996 Cell 86: 147-157; Kluck et al., 1997 Science 275: 1132-36), pro-caspases 2, 3, and 9 (Mancini et al., 1995 J. Cell Biol. 140, 1485-95; Susin et al., 1999 J. Exp. Med. 189: 381-94), apoptosis inducing factor (Susin et al., 1999 Nature 397: 441-45), adenylate kinase (AK; e.g., Single et
al, 1998 Cell Death Diff. 5:1001), nucleoside diphosphate kinase, nucleoside monophosphate kinase (Tzagoloff, Chapter 2 in Mitochondria, Plenum Press, New York, NY, 1982, p. 28), and sulfide oxidase (e.g., Newmeyer presentation, 1998 Society of Toxicology 37th Annual Meeting, March 1998, see 1998 Toxicol. Sci. 42 (1-S):232, abstr. no. 1145), but the invention need not be so limited. In particularly preferred embodiments the MISP is AK.

By way of background, adenylate kinase (AK) is a ubiquitous enzyme involved in maintaining the homeostasis of cellular adenine and guanine nucleotide pools (Schulz, 1987 Cold Spring Harbor Symp. Quant. Biol. 52: 429-39; Tomasselli et al., Eur. J. Biochem. 93: 263-67,1979). To date, four isozymes (AK1, AK2, AK3, and AK4), each having a similar molecular mass of about 21-26 kDa have been characterized in vertebrates (Nobutomo et al., 1998 J. Biochem. 123, 128-135; Yineda et al., 1998 Mol. Brain Res. 62: 187-95). AK1, AK2 and AK3 are nuclear encoded proteins, but localize to different subcellular compartments: AK1 is present mainly in the cytosol, whereas mature AK2 and AK3 are imported into mitochondria, with AK2 distributing to the mitochondrial intermembrane space while AK3 is found in the mitochondrial matrix. AK2 is therefore a MISP, although in some tissues a subpopulation of the total intracellular AK2 pool may also be found in the cytosol (Nobutomo et al., 1998). At least two isoforms of vertebrate AK2 have been identified, cloned and sequenced (e.g., Nakazawa et al., 1990 Prog. Clin. Biol. Res. 344:495; Lee et al., 1998 J. Biochem. 123:47; Lee et al., 1996 Biochem. Mol Biol. Int. 39:833; Noma et al., 1998 Biochim. Biophys. Acta 1395:34). Subcellular distribution of AK4 has not been characterized, but AK4 is believed to localize to the mitochondrial matrix based on its primary structure (Yineda et al., 1998).

MISPs include full length proteins and polypeptides, fragments, and variants thereof, and further include MISP fusion polypeptides as provided herein. The portion of a MISP fusion polypeptide that is a MISP domain as provided herein may be a full length MISP or any analog, fragment or portion thereof, including a truncated MISP or a MISP variant. The fusion polypeptides of the present invention comprise at least one MISP protein domain and at least one affinity domain as provided herein; in preferred embodiments the MISP protein domain is fused in-frame to the affinity domain.
A truncated molecule, for example a truncated MISP polypeptide or nucleic acid, may be any molecule that comprises less than a full length version of the molecule. In certain preferred embodiments, the present invention provides truncated MISP polypeptides, and in certain embodiments the invention provides nucleic acids encoding such truncated polypeptides. Truncated nucleic acid molecules have less than the full length nucleotide sequence of a known or described nucleic acid molecule, where such a known or described nucleic acid molecule may be a naturally occurring, a synthetic or a recombinant nucleic acid molecule, so long as one skilled in the art would regard it as a full length molecule.

Thus, for example, truncated nucleic acid molecules that correspond to a gene sequence contain less than the full length gene where the gene comprises coding and non-coding sequences, promoters, enhancers and other regulatory sequences, flanking sequences and the like, and other functional and non-functional sequences that are recognized as part of the gene. In another example, truncated nucleic acid molecules that correspond to a mRNA sequence contain less than the full length mRNA transcript, which may include various translated and non-translated regions as well as other functional and non-functional sequences. In other preferred embodiments, truncated molecules are polypeptides that comprise less than the full length amino acid sequence of a particular protein. As used herein "deletion" has its common meaning as understood by those familiar with the art, and may refer to molecules that lack one or more portions of a sequence from either terminus or from a non-terminal region, relative to a corresponding full length molecule, for example, as in the case of truncated molecules provided herein. Truncated molecules that are linear biological polymers such as nucleic acid molecules or polypeptides may have one or more of a deletion from either terminus of the molecule or a deletion from a non-terminal region of the molecule, where such deletions may be deletions of 1-1500 contiguous nucleotide or amino acid residues, preferably 1-500 contiguous nucleotide or amino acid residues and more preferably 1-300 contiguous nucleotide or amino acid residues.

Analysis of MISP deletion mutants as provided herein permits identification of MISP structural domains that are responsible for particular functional properties, including, for example, the polypeptide regions that may mediate translocation of a particular MISP. Thus, the use of MISP truncation deletion mutants may permit molecular fine regulation of
mitochondrial function \textit{in vivo}. Accordingly, in certain embodiments of the invention, detectably altered (\textit{e.g.}, increased or decreased) MISP translocation in response to one or more translocation-inducing agents in cells transfected with MISP fusion polypeptides, or deletion or other mutants thereof, permits correlation of the presence of a particular MISP structural domain with a particular mitochondrial or cellular function or event (\textit{e.g.}, altered mitochondrial permeability, induction of apoptosis, stimulation of necrosis, etc.).

The affinity domain of the MISP fusion polypeptide permits reliable localization of the fusion polypeptide, including, for example, verification that the expressed fusion polypeptide is delivered to the mitochondrial intermembrane space and determination of whether the fusion polypeptide has translocated and may be detected as an extramitochondrial fusion polypeptide. Such extramitochondrial fusion polypeptides may include any mitochondrial intermembrane space protein (MISP) fusion polypeptide as provided herein that is not present in the mitochondrial intermembrane space, for example, a MISP fusion polypeptide that is present in the cytosol or a MISP fusion polypeptide that is present in a subcellular fraction prepared according to any of a variety of cell fractionation methods well known in the art and which is substantially free of mitochondria.

The affinity domain may also be useful for isolation and/or detection of the fusion polypeptide according to methodologies with which those having ordinary skill in the art will be familiar. In preferred embodiments the affinity domain of the fusion polypeptide is a small peptide that comprises fewer than 20 amino acids, more preferably fewer than 15 amino acids and still more preferably 12 or fewer amino acids. Without wishing to be bound by theory, in preferred embodiments the present invention provides a MISP fusion polypeptide comprising an affinity domain that is a small (\textit{e.g.}, <20 amino acids) peptide, such that the influence of the affinity domain on MISP fusion polypeptide translocation may be minimized where the translocation mechanism may be sensitive to MISP molecular mass. In addition, as noted above, the affinity domain of the MISP fusion polypeptide provides a specific and reliable detectable translocation marker. Thus, for example, in certain embodiments the present invention provides a method of detecting MISP translocation that may be qualitative (\textit{e.g.}, determination of whether MISP fusion polypeptide localizes mitochondrial or extramitochondrially under particular conditions), and in certain
embodiments the invention provides a method of quantitatively detecting MISP translocation (e.g., quantifying mitochondrial and/or extramitochondrial MISP fusion polypeptide levels under particular conditions).

MISP fusion polypeptides described herein may be detected, for example, by fluorescence, phosphorescence, radiography, bioluminescence, chemiluminescence or any other method for detecting such polypeptides with which those having ordinary skill in the art will be familiar. MISP fusion polypeptides include fusion proteins that may in certain embodiments be detected, isolated and/or purified by protein-protein affinity (e.g., receptor-ligand), hydrophobicity, metal affinity or charge affinity methods. In preferred embodiments, detection of the affinity domain is employed, for example, through the use of a detectable ligand that specifically binds to the affinity domain under conditions that can be readily identified by a person having ordinary skill in the art without undue experimentation. Detectable ligands may include antibodies that are specific for the affinity domain, and may also include other ligands able to specifically interact with an affinity domain, as described in greater detail below. In certain other embodiments the subject invention fusion proteins may be detected by specific protease cleavage of a fusion protein having a sequence that comprises a protease recognition sequence, such that the MISP domain polypeptide may be separable from the affinity domain polypeptide sequence. In particularly preferred embodiments, for example, each MISP polypeptide sequence is fused in-frame to an affinity domain polypeptide sequence. As used herein, the term “isolated” means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living animal is not isolated, but the same nucleic acid or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such nucleic acids could be part of a vector and/or such nucleic acids or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

Polypeptide sequences present in MISP fusion polypeptides may thus facilitate affinity detection and isolation of MISP fusion polypeptides and may include, for example, affinity domains that are poly-His or the defined antigenic peptide epitopes described in U.S. Patent No. 5,011,912 and in Hopp et al., (1988 Bio/Technology 6:1204), or the XPRESS™
epitope tag (Invitrogen, Carlsbad, CA), or the myc epitope tag (e.g., Roche Molecular Biochemicals, Indianapolis, IN). The affinity domain sequence may also be a hexa-histidine tag as supplied, for example, by a pBAD/His (Invitrogen) or a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host. Alternatively and in particularly preferred embodiments, the affinity domain sequence may be a hemagglutinin (HA) tag. The HA tag corresponds to an antibody defined epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, *Cell* 37:767) and which is useful when mammalian host cells, for example COS-7 cells, are used.

MISP fusion polypeptides may further comprise immunoglobulin constant region polypeptides added to MISP domain sequences to facilitate detection, isolation and/or localization of MISPs. The immunoglobulin constant region polypeptide preferably is fused to the C-terminus of a MISP domain polypeptide. General preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (1991 *Proc. Nat. Acad. Sci. USA*, 88:10535) and Byrn et al. (1990 *Nature*, 344:677). A gene fusion encoding the MISP:Fc fusion protein is inserted into an appropriate expression vector. In certain embodiments of the invention, MISP:Fc fusion polypeptides may be allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding dimeric MISP fusion proteins.

MISP fusion polypeptides having specific binding affinities for pre-selected antigens by virtue of affinity domain polypeptides comprising immunoglobulin V-region domains encoded by DNA sequences linked in-frame to sequences encoding a MISP are also within the scope of the invention, including variants and fragments thereof, as provided herein. General strategies for the construction of fusion proteins having immunoglobulin V-region fusion polypeptides are disclosed, for example, in EP 0318554; U.S. 5,132,405; U.S. 5,091,513; and U.S. 5,476,786.

Fusion proteins may in certain embodiments comprise a MISP domain polypeptide fused to one or more other affinity domain polypeptides having desirable affinity properties (e.g., receptor-ligand). Some specific examples of such additional affinity domain polypeptides having affinity properties include, without limitation, enzymes such as
glutathione-S-transferase (GST) and *Staphylococcus aureus* protein A polypeptide. Protein A encoding nucleic acids and their use in constructing fusion proteins having affinity for immunoglobulin constant regions are disclosed generally, for example, in U.S. Patent 5,100,788. Other useful affinity domain polypeptides for construction of MISP fusion polypeptides may include streptavidin fusion proteins, as disclosed, for example, in WO 89/03422; U.S. 5,489,528; U.S. 5,672,691; WO 93/24631; U.S. 5,168,049; U.S. 5,272,254 and elsewhere, and avidin fusion proteins (see, e.g., EP 511,747). As provided herein and in the cited references, MISP domain polypeptide sequences may be fused to affinity domain polypeptide sequences that may be full length fusion polypeptides and that may alternatively be variants or fragments thereof.

The detectable ligand may be any molecule, receptor, counterreceptor, antibody or the like with which the affinity tag may interact through a specific binding interaction as provided herein. In preferred embodiments of the invention, the detectable ligand is an antibody. Antibodies that are specific for a MISP fusion polypeptide affinity domain are readily generated as monoclonal antibodies or as polyclonal antisera, or may be produced as genetically engineered immunoglobulins (Ig) that are designed to have desirable properties using methods well known in the art. For example, by way of illustration and not limitation, antibodies may include recombinant IgGs, chimeric fusion proteins having immunoglobulin derived sequences or "humanized” antibodies that may all be used for detection of a human mesothelin polypeptide according to the invention. Many such antibodies have been disclosed and are available from specific sources or may be prepared according to well known methodologies.

The term “antibodies” includes polyclonal antibodies, monoclonal antibodies, fragments thereof such as F(\(\text{ab}'\))\(_2\), and Fab fragments, as well as any naturally occurring or recombinantly produced detectable ligands, which as provided herein are molecules that specifically bind to the affinity domain of a MISP fusion polypeptide, for example, an anti-HA monoclonal antibody that binds to an AK2-HA fusion polypeptide. Antibodies are defined to be “immunospecific” or specifically binding if they bind a cognate antigen (e.g., an affinity domain of a MISP fusion polypeptide) with a \(K_a\) of greater than or equal to about \(10^4\) M\(^{-1}\), preferably of greater than or equal to about \(10^5\) M\(^{-1}\), more preferably of greater than or
equal to about $10^6 \text{ M}^{-1}$ and still more preferably of greater than or equal to about $10^7 \text{ M}^{-1}$. Affinities of detectable ligands such as antibodies can be readily determined using conventional techniques, for example those described by Scatchard et al., *Ann. N.Y. Acad. Sci.* 51:660 (1949). Determination of other proteins as detectable ligands that bind to an affinity domain of a MISP fusion polypeptide can be performed using any of a number of known methods for identifying and obtaining proteins that specifically interact with other proteins or polypeptides, for example, a yeast two-hybrid screening system such as that described in U.S. Patent No. 5,283,173 and U.S. Patent No. 5,468,614, or the equivalent. The present invention also includes the use of a MISP fusion polypeptide, and peptides based on the amino acid sequence of a MISP fusion polypeptide, to prepare binding partners and antibodies that specifically bind to such a fusion polypeptide.

As noted above, antibodies may generally be prepared by any of a variety of techniques known to those of ordinary skill in the art (*see*, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988). As also noted above, within certain embodiments, the use of antigen-binding fragments of antibodies may be preferred. Such fragments include Fab fragments, which may be prepared using standard techniques (*e.g.*, by digestion with papain to yield Fab and Fc fragments). The Fab and Fc fragments may be separated by affinity chromatography (*e.g.*, on immobilized protein A columns), using standard techniques. *See*, e.g., Weir, D.M., *Handbook of Experimental Immunology*, 1986, Blackwell Scientific, Boston. Single chain antibodies for use in the present invention may also be generated and selected by a method such as phage display (*see*, e.g., U.S. Patent No. 5,223,409; Schlebusch et al., 1997 *Hybridoma* 16:47; and references cited therein).

There are a variety of assay formats known to those of ordinary skill in the art for using an antibody to detect a polypeptide, such as an affinity domain polypeptide, in a sample. *See*, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. For example, the assay may be performed in a Western blot format, wherein a protein preparation from the biological sample is submitted to gel electrophoresis, transferred to a suitable membrane and allowed to react with the antibody. The presence of the antibody on the membrane may then be detected using a suitable detection reagent, as described below.
A “biological sample” for use according to the invention may comprise any tissue or cell preparation in which a cell comprising a mitochondrion capable of maintaining a membrane potential when supplied with one or more oxidizable substrates such as glucose, malate or galactose is present; or in the case of isolated mitochondria or permeabilized cells, glucose, galactose, pyruvate, succinate, glutamate, malate, acetoacetate or β-hydroxybutyrate; and wherein the mitochondrion comprises at least one MISP fusion polypeptide as provided herein. Mitochondrial membrane potential may be determined according to methods with which those skilled in the art will be readily familiar, including but not limited to detection and/or measurement of detectable compounds such as fluorescent indicators, optical probes and/or sensitive pH and ion-selective electrodes (See, e.g., Ernster et al., 1981 J. Cell Biol. 91:227s and references cited therein; see also Haugland, 1996 Handbook of Fluorescent Probes and Research Chemicals- Sixth Ed., Molecular Probes, Eugene, OR, pp. 266-274 and 589-594.). By “capable of maintaining a potential” it is meant that such mitochondria have a membrane potential that is sufficient to permit the accumulation of a detectable compound (e.g., DASPMI [2,4-dimethylaminostyryl-N-methylpyridinium], TMRM [tetramethylrhodamine methyl ester], etc.) used in the particular instance. A biological sample may, for example, be derived from a normal (i.e., healthy) individual or from an individual having a disease associated with altered mitochondrial function. Biological samples may be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture or any other tissue or cell preparation from a subject or a biological source. The subject or biological source may be a human or another biological organism, including a genetically engineered organism, such as a non-human animal, a plant, a unicellular organism or a multicellular organism or mitochondria prepared therefrom. The subject or biological source may also be a primary cell culture or culture adapted cell line including but not limited to genetically engineered cell lines that may contain chromosomally integrated or episomal recombinant nucleic acid sequences, immortalized or immortalizable cell lines, somatic cell hybrid or cytoplasmic hybrid “cybrid” cell lines, differentiated or differentiatable cell lines, transformed cell lines and the like. A biological sample may, for example, be derived from a recombinant cell line or from a transgenic animal.
In particularly preferred embodiments the biological sample comprises a cell that is a host cell or mitochondria prepared therefrom. Host cells are genetically engineered (transduced, transformed or transfected) with one or more vectors and/or expression constructs as described herein. Engineered host cells may be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying particular genes such as genes encoding MISP fusion proteins and/or genes encoding Bcl-2 family members. Suitable culture conditions for particular host cells will be readily apparent to the ordinarily skilled artisan.

A host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell. Representative examples of appropriate host cells according to the present invention include, but need not be limited to, fungal cells, such as yeast; insect cells, such as Drosophila S2 and Spodoptera Sf9; animal cells, such as MDCK, Hep-2, CHO or COS; human cells such as Jurkat or 293 cells; plant cells, or any suitable cell already adapted to in vitro propagation or so established de novo. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

In certain particularly preferred embodiments, various mammalian cell culture systems can be employed that express a recombinant MISP fusion polypeptide as provided herein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. In certain other particularly preferred embodiments, it may be desirable to select a cell line of a specific developmental lineage (e.g., neural, hematopoietic, musculoskeletal, epithelial, etc.) or capable of differentiation into cells of one or more specific developmental lineages for use in the present invention. In one such highly preferred embodiment the cell line is a neural cell line and in another such embodiment the cell line is a neuroblastoma cell line, for example, the human SH-SY5Y cell line (e.g., Biedler et al., 1978 Cancer Res. 38:3751).

In some preferred embodiments, host cells may be cybrids (e.g., cytoplasmic hybrid cells comprising a common nuclear component but having mitochondria derived from different individuals). Methods for preparing and using cybrids are described in U.S. Patent

Mammalian expression vectors suitable for use in preparation of the nucleic acid expression construct of the present invention typically will comprise an origin of replication, a suitable promoter and enhancer, and also any additional necessary sequences, for instance, ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences and/or 5′ flanking non-transcribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Introduction of the construct into the host cell can be effected by a variety of methods with which those skilled in the art will be familiar, including but not limited to, for example, calcium phosphate transfection, liposome-mediated transfection, transfection with naked DNA, biolistic particle-mediated transfection, DEAE-Dextran mediated transfection, vector-mediated gene delivery or electroporation (e.g., Davis et al., 1986 Basic Methods in Molecular Biology; Ausubel et al., 1993 Current Protocols in Molecular Biology, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., Boston, MA; Sambrook et al., 1989 Molecular Cloning, Second Ed., Cold Spring Harbor Laboratory, Plainview, NY).

As noted above, the invention is directed in pertinent part to a method comprising contacting a biological sample with an agent that is, in certain embodiments, an apoptogen. A variety of apoptogens are known to those familiar with the art and may include by way of illustration and not limitation apoptogens that, when added to cells under appropriate conditions with which those skilled in the art will be familiar, require specific receptors such as the tumor necrosis factor, FasL, glutamate, NMDA (N-methyl-D-aspartic acid), IL-3, corticosterone, mineralcorticoid or glucocorticoid receptor(s). Apoptogens may further include other agents, and thus include atractyloside; bongkrekic acid; thapsigargin; carbachol (carbamoylcholine chloride); herbimycin A (Mancini et al., 1997 J. Cell. Biol.
138:449-469); paraquat (Costantini et al., 1995 Toxicology 99:1-2); ethylene glycols; protein kinase inhibitors, for example, staurosporine, calphostin C, caffeic acid phenethyl ester, chelerythrine chloride, and genistein; 1-(5-isouquinolinesulfonyl)-2-methylpiperazine; N-[2-((p-bromocinnamyl)amino)ethyl]-5-5-isouquinolinesulfonamide; KN-93; quercitin; sphingosine or its derivatives, for example ceramide (N-palmitoylsphingosine) or d-erythro-sphingosine derivatives; UV radiation; ionophores, for example, ionomycin, valinomycin and other ionophores known in the art; MAP kinase inducers, for example, anisomycin and anandamide; cell cycle blockers, for example, aphidicolin, colcemid, 5-fluorouracil and homoharringtonine; acetylcholineesterase inhibitors, for example, berberine; anti-estrogens, for example, tamoxifen; pro-oxidants, for example, tert-butyl hydroperoxide, peroxynitrite, hydrogen peroxide and nitric oxide donors including but not limited to L-arginine, 5,5'-dinitrosodithiol, N-hydroxy-L-arginine, S-nitroso-N-acetylpenicillamine, S-nitrosoglutathione, NOR-1, NOR-3, NOR4, 4-phenyl-3-furoxancarbonitrile, 3-morpholinosydnonimine, sodium nitroprusside and streptozotocin; glutathione depleting agents such as ethacrynic acid (Meister, 1995 Biochim. Biophys. Acta. 1271:35); free radicals such as nitric oxide; inorganic metal ions, for example, cadmium; DNA synthesis inhibitors, for example actinomycin D, or topoisomerase inhibitors, such as etoposide; DNA intercalators, for example, doxorubicin, bleomycin sulfate, hydroxyurea, methotrexate, mitomycin C, camptothecin, and daunorubicin; protein synthesis inhibitors such as cycloheximide, puromycin, and rapamycin; agents that effect microtubule formation or stability, for example, vinblastine, vincristine, colchicine, 4-hydroxyphenylretinamide, and paclitaxel; and other MPT inducers, for example, Bax protein (Jurgenmeier et al., 1998 PNAS 95:4997-5002), calcium and inorganic phosphate (Kroemer et al., 1998 Ann. Rev. Physiol. 60:619).

Any of a variety of well established assays for determining the induction of apoptosis may be employed to verify readily and without undue experimentation that a biological sample and an apoptogen have been contacted under conditions (e.g., concentration, media formulation, temperature, pH, cell density, etc.) and for a time sufficient to induce apoptosis, based on the disclosure herein. For example, cells may be examined for morphological, permeability or other changes that are indicative of an apoptotic state. Such
changes include, but are not limited to, altered morphological appearance (such as plasma membrane blebbing, cell shape change, loss of substrate adhesion properties or other morphological changes that can be readily detected by those skilled in the art using light microscopy); fragmentation and disintegration of chromosomes (which may be apparent by microscopy and/or through the use of DNA specific or chromatin specific dyes that are known in the art, including fluorescent dyes); and/or altered plasma membrane permeability properties, as may be readily detected through the use of vital dyes (e.g., propidium iodide, trypan blue) or by the detection of lactate dehydrogenase leakage into the extracellular milieu. Within another apoptosis assay, translocation of cell membrane phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane may be evaluated by measuring outer leaflet binding by the PS-specific protein annexin (Martin et al., J. Exp. Med. 182:1545, 1995; Fadok et al., J. Immunol. 148:2207, 1992).

In yet another apoptosis assay, induction of specific protease activity in a family of apoptosis-activated proteases known as the caspases may be measured, for example by determination of caspase-mediated cleavage of specifically recognized protein substrates. Such substrates may include, for example, poly-(ADP-ribose) polymerase (PARP) or other naturally occurring or synthetic peptides and proteins cleaved by caspases that are known in the art (see, e.g., Ellerby et al., 1997 J. Neurosci. 17:6165). The synthetic peptide Z-Tyr-Val-Ala-Asp-AFC (SEQ ID NO:1), wherein “Z” indicates a benzoyl carbonyl moiety and AFC indicates 7-amino-4-trifluoromethylcoumarin (Kluck et al., 1997 Science 275:1132; Nicholson et al., 1995 Nature 376:37), is one such substrate. Other substrates include nuclear proteins such as U1-70 kDa and DNA-PKcs (Rosen and Casciola-Rosen, 1997 J. Cell. Biochem. 64:50; Cohen, 1997 Biochem. J. 326:1).

The present invention thus provides certain advantages with regard to regulation of mitochondrial function, and in particular regulation of the mitochondrial permeability “pore” and MPT, as described herein. The invention also pertains to compositions and methods that relate to the mitochondrial adenine nucleotide translocator (ANT). By way of background, four of the five multisubunit protein complexes (Complexes I, III, IV and V) that mediate ETC activity are localized to the inner mitochondrial membrane, which is the most protein rich of biological membranes in cells (75% by weight); the
remaining ETC complex (Complex II) is situated in the matrix. ANT represents the most abundant of the inner mitochondrial membrane proteins. In at least three distinct chemical reactions known to take place within the ETC, positively-charged protons are moved from the mitochondrial matrix, across the inner membrane, to the intermembrane space. This disequilibrium of charged species creates an electrochemical potential of approximately 220 mV referred to as the "protonmotive force" (PMF), which is often represented by the notation $\Delta \psi$ or $\Delta \psi_m$ and represents the sum of the electric potential and the pH differential across the inner mitochondrial membrane (see, e.g., Ernster et al., 1981 *J. Cell Biol.* 91:227s and references cited therein).

This membrane potential drives ANT-mediated stoichiometric exchange of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) across the inner mitochondrial membrane, and provides the energy contributed to the phosphate bond created when ADP is phosphorylated to yield ATP by ETC Complex V, a process that is "coupled" stoichiometrically with transport of a proton into the matrix. Mitochondrial membrane potential, $\Delta \psi_m$, is also the driving force for the influx of cytosolic Ca$^{2+}$ into the mitochondrion. Under normal metabolic conditions, the inner membrane is impermeable to proton movement from the intermembrane space into the matrix, leaving ETC Complex V as the sole means whereby protons can return to the matrix. When, however, the integrity of the inner mitochondrial membrane is compromised, as occurs during MPT that may accompany a disease associated with altered mitochondrial function, protons are able to bypass the conduit of Complex V without generating ATP, thereby "uncoupling" respiration because electron transfer and associated proton pumping yields no ATP. Thus, mitochondrial permeability transition involves the opening of a mitochondrial membrane "pore", a process by which, *inter alia*, the ETC and ATP synthesis are uncoupled, $\Delta \psi_m$ collapses and mitochondrial membranes lose the ability to selectively regulate permeability to solutes both small (e.g., ionic Ca$^{2+}$, Na$^+$, K$^+$, H$^+$) and large (e.g., proteins).

As noted above, the binding of the adenine nucleotide translocator (ANT) is responsible for mediating transport of ADP and ATP across the mitochondrial inner membrane. ANT has also been implicated as the critical component of the mitochondrial permeability transition pore, a Ca$^{2+}$ regulated inner membrane channel that plays an important
modulating role in apoptotic processes. Additionally, ANT activity appears to be related to changes in ANT polypeptide conformation within the mitochondrial membrane, as evidenced by studies using agents that are capable of binding to ANT (Block et al., 1986 *Meths. Enzymol.* 125:658). Binding interactions between ANT and a variety of small molecule agents are known to those familiar with the art. For example, these interactions include binding to ANT by atractyloside, carboxyatractyloside, palmitoyl-CoA, bongkrekic acid, thyroxin, eosin Y and erythrosin B. (See, e.g., Stubbs, 1979 *Pharm. Ther.* 7:329; Klingenberg et al., 1978 *Biochim. Biophys. Acta* 503:193; Sterling, 1986 *Endocrinol.* 119:292; Majima et al., 1998 *Biochem.* 37:424; Block et al. 1986 *Meths. Enzymol.* 125:658; for erythrosin B and additional ANT inhibitors, see Beavis et al. 1993 *J. Biol. Chem.* 268:997; Powers et al. 1994 *J. Biol. Chem.* 269:10614.) According to certain embodiments of the invention, one or more of such agents may be contacted with a biological sample as provided herein.

Without wishing to be bound by theory, it is unresolved whether this pore is a physically discrete conduit that is formed in mitochondrial membranes, for example by assembly or aggregation of particular mitochondrial and/or cytosolic proteins and possibly other molecular species, or whether the opening of the “pore” may simply represent a general increase in the porosity of the mitochondrial membrane.

MPT may also be induced by compounds that bind one or more mitochondrial molecular components. Such compounds include, but are not limited to, atractyloside and bongkrekic acid, which are known to bind to ANT. Methods of determining appropriate amounts of such compounds to induce MPT are known in the art (see, e.g., Beutner et al., 1998 *Biochim. Biophys. Acta* 1368:7; Obatomi and Bach, 1996 *Toxicol. Lett.* 89:155; Green and Reed, 1998 *Science* 281:1309; Kroemer et al., 1998 *Annu. Rev. Physiol.* 60:619; and references cited therein). Thus certain mitochondrial molecular components, such as ANT, may contribute to the MPT mechanism. As noted above, it is believed that adenine nucleotide translocator (ANT) mediates ATP/proton exchange across the inner mitochondrial membrane, and that ANT inhibitors such as atractyloside or bongkrekic acid induce MPT under certain conditions. Hence, it is desirable to obtain ANT in sufficient quantities for structural and functional assays that provide, for example, ANT ligands and other agents that
interact with ANT, which will be useful for therapeutic management of mitochondrial pore activity. See also U.S. 09/161,172, entitled “Compositions and Methods for Identifying Agents that Alter Mitochondrial Permeability Transition Pores”, which is hereby incorporated by reference.

As described herein, the mitochondrial permeability transition “pore” may not be a discrete assembly or multisubunit complex, and the term thus refers instead to any mitochondrial molecular component (including, e.g., a mitochondrial membrane per se) that regulates the inner membrane selective permeability where such regulated function is impaired during MPT. As used herein, mitochondria are comprised of “mitochondrial molecular components”, which may be any protein, polypeptide, peptide, amino acid, or derivative thereof; any lipid, fatty acid or the like, or derivative thereof; any carbohydrate, saccharide or the like or derivative thereof; any nucleic acid, nucleotide, nucleoside, purine, pyrimidine or related molecule, or derivative thereof, or the like; or any other biological molecule that is a constituent of a mitochondrion. “Mitochondrial molecular components” includes but is not limited to “mitochondrial pore components”. A “mitochondrial pore component” is any mitochondrial molecular component that regulates the selective permeability characteristic of mitochondrial membranes as described above, including those responsible for establishing ΔΨm and those that are functionally altered during MPT.

As noted above, any of a variety of well known criteria may be employed for determining whether a fusion polypeptide is an extramitochondrial fusion polypeptide as provided herein. For example, cell fractionation techniques for the enrichment and detection of mitochondria and/or biochemical markers characteristic of these organelles may be used to determine that a particular subcellular fraction containing a detectable MISP fusion polypeptide as provided herein is substantially free of mitochondria (see, e.g., Ernster et al., 1981 J. Cell Biol. 91:227s). Such methods may be particularly preferred in embodiments of the invention that may be usefully performed on a large scale, for example high throughput screening assays for identifying an agent that alters MISP translocation as provided herein. As another example, intracellular localization of a MISP fusion polypeptide may be conducted to detect an extramitochondrial localization of a MISP fusion polypeptide, such as
by immunoelectron microscopy, immunofluorescence microscopy (e.g., including laser-scanning confocal microscopy) or the like.

In certain embodiments the present invention provides a method of identifying an agent that alters (e.g., increases or decreases in a statistically significant manner) mitochondrial intermembrane space protein translocation, by detecting such translocation, as described above, and comparing the level of extramitochondrial fusion polypeptide detected in the absence of a candidate agent to the level of extramitochondrial fusion polypeptide detected in the presence of the candidate agent. In certain preferred embodiments, such a method of identifying an agent that alters translocation comprises a high throughput screening assay, for example, where conditions are determined that permit contacting the detectable ligand with extramitochondrial but not with mitochondrial MISP fusion polypeptide. For instance, selective solubilization conditions may permeabilize the plasma membranes of cells without altering mitochondrial permeability, such that absolute (e.g., absence versus presence) or relative (e.g., quantitative) comparison may be made of the level of extramitochondrial (e.g., cytosolic) MISP fusion polypeptide. Those having familiarity with the art will appreciate that other conditions may be employed to detect extramitochondrial fusion polypeptide, such that the scope and spirit of the invention are not intended to be limited except by the appended claims.

A candidate agent may alter MISP translocation directly (e.g., by physical contact with one or more MISPs) or indirectly (e.g., by interaction with one or more additional molecular components, such as mitochondrial molecular components present in a host cell, where such additional components alter MISP translocation in response to contact with the agent). In some embodiments, the candidate agent may be a peptide, polypeptide, protein or small molecules. Typically, and in more preferred embodiments such as for high throughput screening, candidate agents are provided as “libraries” or collections of compounds, compositions or molecules. Such molecules typically include compounds known in the art as “small molecules” and having molecular weights less than $10^5$ daltons, preferably less than $10^4$ daltons and still more preferably less than $10^3$ daltons. For example, members of a library of test compounds can be administered to a plurality of biological
samples as provided herein, and then assayed for their ability to alter MISP translocation in a cell-based assay.

Candidate agents further may be provided as members of a combinatorial library, which preferably includes synthetic agents prepared according to a plurality of predetermined chemical reactions performed in a plurality of reaction vessels. For example, various starting compounds may be prepared employing one or more of solid-phase synthesis, recorded random mix methodologies and recorded reaction split techniques that permit a given constituent to traceably undergo a plurality of permutations and/or combinations of reaction conditions. The resulting products comprise a library that can be screened followed by iterative selection and synthesis procedures, such as a synthetic combinatorial library of peptides (see e.g., PCT/US91/08694 and PCT/US91/04666) or other compositions that may include small molecules as provided herein (see e.g., PCT/US94/08542, EP 0774464, U.S. 5,798,035, U.S. 5,789,172, U.S. 5,751,629). Those having ordinary skill in the art will appreciate that a diverse assortment of such libraries may be prepared according to established procedures, and tested using a MISP translocation assay according to the present disclosure.

Agents that alter MISP translocation, and that preferably also inhibit or delay the onset of apoptosis, may be used for a variety of purposes. For example, such agents may be used to alter (e.g., enhance or inhibit) initiation of an apoptotic cascade by a mitochondrion. The mitochondrion may be isolated or may be present within a cell. Briefly, a mitochondrion is contacted with an agent as described above under conditions and for a time sufficient to translocate one or more MISPs. As described herein, any of a variety of standard techniques may be used to detect MISP translocation by the mitochondrion (including determination of extramitochondrial MISPs) and/or to detect apoptosis.

Thus, as provided herein, any experimentally measurable consequence for cells containing mitochondria undergoing MISP translocation may be used, including, for example, detection of the loss of MISPs as provided herein to the cytoplasm, activation of one or more caspases as a downstream event in the apoptotic signaling cascade (see above), cell death and any other phenotypic, biochemical, biophysical, metabolic, respiratory or other useful parameter the alteration of which may depend on MISP translocation. Agents
identified according to the methods of the present invention that are suitable for treatment of a disease associated with altered mitochondrial function may potentiate, impair or alter the frequency and/or occurrence of MISP translocation and/or translocation-related regulatory mechanisms. Particularly preferred are agents that inhibit the appearance of one or more of the above indicators of MISP translocation. Such agents may also be used to alter survival of a cell. Briefly, a cell is contacted with an agent under conditions and for a time sufficient to modulate cell survival. Cell survival may then be assayed using standard techniques.

Within other aspects, an agent may be administered to a patient for treatment or prevention of diseases associated with altered mitochondrial function. Preferred agents for such uses inhibit MISP translocation. Diseases associated with altered mitochondrial function include, but are not limited to, AD, diabetes mellitus; Parkinson’s Disease; Huntington’s disease; dystonia; Leber’s hereditary optic neuropathy; schizophrenia; mitochondrial encephalopathy, lactic acidosis, and stroke (MELAS); cancer; psoriasis; hyperproliferative disorders; mitochondrial diabetes and deafness (MIDD) and myoclonic epilepsy ragged red fiber syndrome. Such diseases may be diagnosed using standard clinical criteria, which are well known in the art.

The agents that alter MISP translocation are preferably part of a pharmaceutical composition when used in the methods of the present invention. The pharmaceutical composition will include at least one of a pharmaceutically acceptable carrier, diluent or excipient, in addition to one or more MISP translocation-altering agents and, optionally, other components.

“Pharmaceutically acceptable carriers” for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985). For example, sterile saline and phosphate-buffered saline at physiological pH may be used. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used.

“Pharmaceutically acceptable salt” refers to salts of the compounds of the present invention derived from the combination of such compounds and an organic or
inorganic acid (acid addition salts) or an organic or inorganic base (base addition salts). The compounds of the present invention may be used in either the free base or salt forms, with both forms being considered as being within the scope of the present invention.

The pharmaceutical compositions that contain one or more MISP translocation-altering agents may be in any form which allows for the composition to be administered to a patient. For example, the composition may be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral (e.g., sublingually or buccally), sublingual, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal, intrathecal, intracavernous, intrameatal, intraurethral injection or infusion techniques. The pharmaceutical composition is formulated so as to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of one or more compounds of the invention in aerosol form may hold a plurality of dosage units.

For oral administration, an excipient and/or binder may be present. Examples are sucrose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose and ethyl cellulose. Coloring and/or flavoring agents may be present. A coating shell may be employed.

The composition may be in the form of a liquid, e.g., an elixir, syrup, solution, emulsion or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred compositions contain, in addition to one or more MISP translocation-altering agents, one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent may be included.

A liquid pharmaceutical composition as used herein, whether in the form of a solution, suspension or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides
which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

A liquid composition intended for either parenteral or oral administration should contain an amount of MISP translocation-altering agent such that a suitable dosage will be obtained. Typically, this amount is at least 0.01 wt% of an MISP translocation-altering agent in the composition. When intended for oral administration, this amount may be varied to be between 0.1 and about 70% of the weight of the composition. Preferred oral compositions contain between about 4% and about 50% of MISP translocation-altering agent(s). Preferred compositions and preparations are prepared so that a parenteral dosage unit contains between 0.01 to 1% by weight of active compound.

The pharmaceutical composition may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, beeswax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device. Topical formulations may contain a concentration of the MISP translocation-altering agent of from about 0.1 to about 10% w/v (weight per unit volume).

The composition may be intended for rectal administration, in the form, e.g., of a suppository which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter and polyethylene glycol.
In the methods of the invention, the MISP translocation-altering agent(s) may be administered through use of insert(s), bead(s), timed-release formulation(s), patch(es) or fast-release formulation(s). It will be evident to those of ordinary skill in the art that the optimal dosage of the MISP translocation-altering agent(s) may depend on the weight and physical condition of the patient; on the severity and longevity of the physical condition being treated; on the particular form of the active ingredient, the manner of administration and the composition employed. It is to be understood that use of a MISP translocation-altering agent in chemotherapy can involve such an agent being bound to another compound, for example, a monoclonal or polyclonal antibody, a protein or a liposome, which assist the delivery of said compound.

EXAMPLES

The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

RECOMBINANT EXPRESSION CONSTRUCT ENCODING AK2-HA FUSION POLYPEPTIDE

A. PCR Amplification of AK2 cDNAs

Total cellular RNA was prepared from human brain and heart tissue samples. The RNA was purified using TriZOL (Gibco/Life Technologies, Rockville, MD) essentially according to the manufacturer’s instructions, followed by treatment with RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, IN) using 1 ul of DNase I (10 U/ul) in a buffer containing 40 mM Tris-HCl, pH 7.0, 6 mM magnesium chloride and 2 mM calcium chloride for 30 minutes at 37°C. This treatment was followed by two phenol/chloroform extractions, one chloroform extraction and an ethanol precipitation in the presence of sodium acetate. The RNA pellet was collected by centrifugation, washed with 70% ethanol, air dried, and resuspended in RNase-free sterile water. The RNA was reverse transcribed to generate
cDNA using RNase H-deficient Reverse Transcriptase (SUPERSCRIPT™; Life Technologies).

AK2 cDNAs were amplified by polymerase chain reactions (PCR) in a thermal cycler using the following primers, AMPLITAQ™ DNA Polymerase (Perkin-Elmer, Norwalk, CT), and reagents and buffers supplied in a GENEAMP™ PCR Reagent Kit (Perkin-Elmer), according to the manufacturer’s instructions.

For human AK2A, the following primers were used:

ADK2 Primer (sense):

5’-GGGCCCAGGTACCAGACTTCGGCGGACAT (SEQ ID NO:2);

and ADK2Arev Primer (antisense):

5’-GGGCCCAGCGCAGCGGATAAACATAACAAGTC (SEQ ID NO:3).

For human AK2B, the following primers were used:

ADK2 Primer (sense) as shown above; and

ADK2Brev Primer (antisense):

5’-GGGCCCAGCGCAGCGGTTGTTGCTTTGGA (SEQ ID NO:4).
PCR products were cloned into the PCR 2.1 TA cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and the inserts were sequenced using ABI 373 Sequencer (P-E Applied Biosystems Division, Foster City, CA) to confirm their identities.

B. Generation of AK2 Expression Constructs

AK2 inserts were recovered from the PCR 2.1 TA vector by digesting the vector with the restriction endonucleases \textit{XhoI} and \textit{NotI} (both enzymes from Roche Molecular Biochemicals). The digestion was carried out according to the manufacturer’s recommendations using manufacturer-supplied reaction buffers. Restricted DNAs were purified by horizontal agarose gel electrophoresis and band extraction using the GENE CLEAN\textsuperscript{®} kit (Bio 101, Vista, CA).

AK2 inserts were ligated into the mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA). The vector was first digested with the restriction endonucleases \textit{KpnI} and \textit{XhoI} (Roche Molecular Biochemicals) and ligated with an AK2 insert (prepared as described above) and a DNA fragment encoding an HA tag. The DNA fragment encoding the HA tag was obtained by annealing a pair of overlapping oligos, which formed the restriction sites for the restriction endonucleases \textit{NotI} and \textit{XhoI} at its 5’ and 3’ termini, respectively. The overlapping oligos were:

HA1 Oligo (sense):

5’-GGCCGCCCATGTATGATGGTCTGTGATTATGCTAGCCCTCTAGC (SEQ ID:5);

and HA1rev Oligo (antisense):

5’-TCGAGCTAGGGCTAGCATAATCGGAACATCATACATGGC (SEQ ID:6).

The resulting pCDNA3 vector containing AK2-HA construct is termed AK2A-HA/pCDNA3.
EXAMPLE 2:

**EXPRESSION OF AK2A-HA FUSION PROTEINS**

293T cells (American Type Culture Collection, Manassas, VA) transformed with SV40 T antigen, and COS-1 cells (ATCC; accession numbers 45504 and CRL-1650, respectively) were transfected with the AK2-HA/pCDNA3 expression construct described in Example 1 using lipofectamine (BRL/Life Technologies, Rockville, MD) essentially according to the manufacturer’s instructions. The transfected cells were selected for G418 (Sigma, St. Louis, MO) resistance and expanded in culture.

The AK2-HA transfected 293T and COS-1 cells were lysed and the total protein concentration of each lysate was determined using the BCA Protein Assay kit (Pierce Chemical Co., Rockford, IL). An equivalent amount of total protein from each lysate preparation was mixed with 2x Laemmli electrophoresis buffer, separated by SDS-PAGE, and the resolved proteins were electrophoretically transferred to a nitrocellulose membrane. The blot was probed with a mouse monoclonal anti-HA antibody (Boehringer Mannheim) and developed with a goat anti-mouse Ig antibody conjugated to HRP using an ECL kit (Amersham, Arlington Heights, IL).

The results (Figure 1) show that the AK2-HA fusion protein is expressed in 293T and COS-1 cells. The fusion protein was also detected using standard immunofluorescence microscopy (Harlow and Lane, *Antibodies: A Laboratory Manual*, 1988, Cold Spring Harbor Laboratory, NY) with a murine anti-HA as the primary antibody and a goat anti-mouse FITC-conjugate (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as the secondary antibody (FITC, fluorescein isothiocyanate, is a fluorescent tag).

EXAMPLE 3

**DOUBLE TRANSFECTION OF MAMMALIAN CELLS WITH AK2-HA AND BCL-2**

MC3, a pool of 3 mixed control cybrid cell lines produced from SH-SY5Y cells that have been repopulated with mitochondria from platelets of 3 healthy donors (Sheehan et al., 1997, *J. Neurosci*. 17: 4612) was co-transfected with the AK2-HA/pCDNA3
expression construct described in Example 1 and with the pBP-bcl-2 expression construct which directs the expression of the Bcl-2 gene and which contains a puromycin resistance genetic selection marker (Kane et al., 1993, *Science* 262: 1274). G418 and puromycin (Sigma) were used to select doubly transfected cells expressing both AK2-HA/pCDNA3 and pBP-bcl-2 constructs. Three stable co-transfected cell lines were obtained.

Expression of AK2A-HA in these cell lines was detected by western blot analysis (see Fig. 2A) and immunofluorescence analyses, as described in Example 2. Similarly, the expression of the Bcl-2 gene was detected using rabbit polyclonal anti-human Bcl-2 antibody (Pharmlingen, San Diego, CA) and goat anti-rabbit Ig conjugated with HRP (Amersham) as the primary and secondary antibodies, respectively (see Fig. 2B). All three cell lines overexpressed Bcl-2 protein. One cell line (Fig. 2, lane 9) also overexpressed the AK2A-HA fusion protein.

**EXAMPLE 4**

**TRANSECTION OF SH-SY5Y NEUROBLASTOMA CELLS WITH AK2-HA**

SH-SY5Y neuroblastoma cells were transfected with the AK2-HA/ pCDNA3 expression construct as described in Example 1 using lipofectamine (BRL-Life Technologies). The transfected cells were selected for G418 resistance and further screened for overexpression of the AK2A-HA fusion protein using western blot analysis and immunofluorescence analysis, as described in Example 2.

The results (see Figure 3) show that several stable SH-SY5Y cell lines were established that overexpress the AK2A-HA fusion protein. Mitochondrial and cytosolic fractions from these established SH-SY5Y transfectant cell lines were prepared essentially as described in copending U.S. patent application Serial No. 09/185,904. Western blot analysis of these fractions demonstrated that less than about 1% of the AK2A-HA fusion protein was present in the cytosolic fraction.
EXAMPLE 5

RELEASE OF CYTOCHROME C FROM MITOCHONDRIA WITHOUT MEMBRANE PERMEABILITY TRANSITION IS INFLUENCED BY AGENTS THAT BIND TO ANT

Mechanistic studies regarding the conditions under which cytochrome c can be released in response to $\text{Ca}^{2+}$ led to the discovery that release of the protein from mitochondria can, under certain conditions, occur independently of mitochondrial permeability transition (Andreyev et al., *FEBS Letters* 439:373-376, 1998). Conditions for such release include 3 mM ATP and 4 mM Mg$^{2+}$ (there is a total of 4 mM Mg$^{2+}$ in the medium, which makes 3 mM MgATP complex and 1 mM free Mg$^{2+}$) and relatively high levels of Ca$^{2+}$. ATP and Mg$^{2+}$ are believed to be generally protective to mitochondria for multiple reasons, only some of which are understood. For instance, ATP can co-precipitate with Ca$^{2+}$, effectively taking Ca$^{2+}$ out of solution. Although preferably greater than about 120 µM, and more preferably greater than about 300 µM, Ca$^{2+}$ is required to stimulate cytochrome c release under these conditions, brain mitochondria are known to not undergo MPT at concentrations of Ca$^{2+}$ that induce MPT in liver mitochondria (Andreyev and Fiskum, *Cell Death and Differentiation* 6:825-832, 1999), and these levels of Ca$^{2+}$ are within levels that have been proposed to be physiologically meaningful (Eimerl and Schramm, *J. Neurochem.* 62:1223-1226, 1994).

In order to characterize MPT-independent mitochondrial inner membrane protein translocation, the following experiments were carried out. SH-SY5Y neuroblastoma cells overexpressing HA-tagged adenylate kinase were permeabilized with digitonin in a multiparameter chamber in KCl-medium (125 mM KCl, 2 mM K$_2$HPO$_4$, 20 mM Hepes, pH adjusted to 7.0 with KOH at ambient temperature). Ca$^{2+}$ was then added, and the cells were incubated at ambient temperature in the multiparameter chamber from about 10 to about 20 minutes, depending upon the experiment.

As its name suggests, the multiparameter chamber is an instrument that simultaneously measures several aspects of mitochondrial activity in a sample. In particular, the multiparameter chamber comprises a first electrode that is responsive to changing levels of TPP$^+$, which reflects the mitochondrial membrane potential (the “TPP$^+$ electrode”); a second electrode that responds to changes in Ca$^{2+}$ concentrations (the “Ca$^{2+}$ electrode”); a
third electrode that is responsive to changes in levels of oxygen in the media, which reflects mitochondria-mediated cellular respiration (the “O₂ electrode”); and a light pipe that detects absorption at 660 nm (A₆₆₀), which reflects mitochondrial swelling. Certain aspects of the multiparameter chamber have been described by Andreyev et al. (FEBS Letters 439:373-376, 1998).

In the absence of ATP and Mg²⁺, relatively conventional mitochondrial permeability transition-like behavior was detected in multiparameter tracings, as indicated by a drop in membrane potential in response to 300 μM Ca²⁺ (Figure 4, upper right panel) that persisted unless a lower concentration (100 μM) of Ca²⁺ was used, or cyclosporin A was present. The multiparameter tracings of extramitochondrial Ca²⁺ (Figure 4, middle right panel) also suggested conventional mitochondrial permeability transition-like behavior, in that the only conditions tested under which the added Ca²⁺ could not be completely taken up by the mitochondria were those where 300 μM Ca²⁺ was present and cyclosporin A was absent. Similarly, there was a decrease in light scattering following Ca²⁺ accumulation, unless a lower concentration (100 μM) of Ca²⁺ was used, or unless cyclosporin A was present (Figure 4, bottom right panel).

In contrast, when ATP (3 mM) and Mg²⁺ (4 mM) were present, behaviors normally associated with MPT were not seen (accordingly, these conditions are referred to herein as “non-MPT conditions”). Specifically, in response to the highest load of Ca²⁺ (1.2 mM), the mitochondria were able to slowly reestablish some membrane potential (as indicated by readings from the TPP⁺ electrode with 2 μM TPP⁺ in the medium; Fig. 4, upper left panel). Moreover, the mitochondria were able to take up most of the added Ca²⁺ (Fig. 4, middle left panel), and demonstrated an increase only (among the parameters monitored) in light scattering (Fig. 4, bottom left panel), possibly reflecting some degree of Ca²⁺ precipitation.

Following the measurements in the multiparameter chamber, the suspensions were centrifuged at 12,000g, and the pellets and supernatants were assayed for cytochrome c and HA-tagged AK2 release by Western blot analysis. The HA-tagged AK2 was detected as described in Example 2, and cytochrome c was detected using an antibody specific therefor (Promega Corp., Madison, WI). AlamarBicin (Sigma Chemical Co., St. Louis, MO; 40
μg/mg protein) was used as a positive control. This agent permeabilizes phospholipid bilayers, thereby inducing the efflux of proteins by either direct release, or via osmotic swelling following formation of a non-specific pore. Measurement of the signaling kinase AKT was used to confirm similar loading of the gel.

The results of the Western analyses confirmed that, in the absence of ATP and Mg²⁺, low (100 μM) and high (300 μM) levels of Ca²⁺ stimulated the release of cytochrome c as well as HA-tagged adenylate kinase 2. In contrast, Western analyses of extracts from cells treated under “non-MPT” conditions (Figure 5) indicated that high (> 120 μM) levels of Ca²⁺ enhanced the release of cytochrome c from mitochondria, but did not enhance adenylate kinase release (Fig. 5, lanes 2 to 5). The released cytochrome c represented a fraction, albeit a potentially significant fraction, of the total cytochrome c pool, as can be seen by the amount of material released following treatment with alamethicin (Fig. 5, lane 6) and that which remains in the pellet, which contains mitochondria (Fig. 5, lane 7).

As discussed above, mitochondrial permeability transition may occur via the formation of a multimolecular complex, the “MPT pore.” It has been proposed that the MPT pore comprises one or more isoforms of the adenine nucleotide translocator (ANT) protein (for a review, see Green and Reed, Science 281:1309-1312, 1998). Two agents that bind to ANT and influence ANT conformation, bongkrekic acid (BkA) and carboxyatractyloside (CAtr), were assessed for their ability to alter cytochrome c release under the non-MPT conditions. BKA (Calbiochem, San Diego, CA) was used at a final concentration of 10 mM; CATR (Calbiochem) was used at a final concentration of 1 mM.

The results are shown in Figure 6. The tracings of light scattering (left panel, Fig. 6), indicative of mitochondrial volume changes, show that the addition of Ca²⁺ induced a large immediate increase in light scattering that was presumably associated with Ca²⁺ precipitation. This initial increase was followed by a slow decrease in light scattering that may be associated with some mitochondrial swelling; neither BkA nor CAtr markedly altered these changes in light scattering. Such a slow decrease did not reflect MPT, which instead would be expected to be associated with a rapid and/or more extensive decrease in light scattering. However, the Western blot analysis (Fig. 6, right panel) showed that, under these conditions, CAtr (lane 2) induced a profound increase in cytochrome c release whereas BkA
(lane 3) did not. Alamethacin (Alm; lane 4) was included as a positive control for cytochrome c release. These results provide evidence that agents that are capable of altering the conformation of ANT influenced cytochrome c release under conditions that did not promote MPT.


GT1-7 hypothalamic murine tumor cells infected with a recombinant retrovirus that overexpresses human Bcl-2 and carries a puromycin resistance gene, or with a control retrovirus having only the puromycin resistance gene, were used (Myers et al., J. Neurochem. 65:2432-2440, 1995). The addition of 2 mM Ca^{2+} to the medium resulted in an initial increase in free Ca^{2+} in the medium, followed by a decrease as the mitochondria of the permeabilized cells sequestered the Ca^{2+} (Figure 7, left panel). Subsequently, an increase in the signal from the Ca^{2+} electrode tracing was recorded, apparently at the point where mitochondrial release of this Ca^{2+} commenced. Notably, the tracings for Ca^{2+} in the control cells ("puro," control transfectants carrying only the puromycin resistance gene) and Bcl-2 overexpressers ("Bcl-2") were similar, yet the quantity of cytochrome c release, as judged by Western blot analysis (Fig. 7, right panel) was greater in the control cells (compare lanes 1 and 2).

The Bcl-2-overexpressing cells were also evaluated for their response to the ANT ligand carboxyactrycloxylsine (CAtr) under the "non-MPT" conditions described above. Treatment with 1 mM CAtr induced a change in the calcium tracing for the control cells ("puros"). Specifically, CAtr inhibited the ability of the control cells to take up much of the
Ca\(^{2+}\) (Fig. 7, left panel), but had little or no effect on the Bcl-2-overpressers in this regard. Nevertheless, CA\(_{tr}\) stimulated the release of cytochrome c in the Bcl-2-overpressing cells (compare lanes 2 and 4). These data suggest that the conformation of ANT, which is influenced by agents such as CA\(_{tr}\) and BkA, influences the release of cytochrome c under the “non-MPT” conditions described herein, and further suggest that CA\(_{tr}\) can, under these circumstances, override the protective effects of Bcl-2.

**EXAMPLE 6**

**DIFFERENTIAL RELEASE OF CYTOCHROME C AND ADENYLATE KINASE**

In this example, SY5Y cells stably expressing HA tagged adenylate kinase were treated with various apoptogens, harvested, and analyzed for cytochrome c and adenylate kinase release from the mitochondria. Cells were treated with apoptogens in growth media while still adherent to plastic culture dishes. Apoptogens included: staurosporine (200 nM, Sigma, St. Louis, MO), etoposide (10 \(\mu\)M, Sigma), thapsigargin (15 \(\mu\)M, Calbiochem, San Diego, CA), and actinomycin D (0.5 \(\mu\)g/ml, Calbiochem).

After treatment with the indicated apoptogens for various amounts of time (0-6 hours, with samples taken at hourly intervals), the cells were harvested following trypsinization, and an aliquot of harvested cells was pelleted by centrifugation and resuspended at a concentration of 2.7 x 10\(^7\) cells/ml in a KCl-sucrose media containing respiratory substrates and digitonin (150 mM sucrose, 50 mM KCl, 20 mM Hepes, 2 mM K\(_2\)HPO\(_4\), pH 7.0 containing 5 mM glutamate, 5 mM malate, 0.03% digitonin, 4 mM MgCl\(_2\), 1 mM EGTA, 3.0 mM ATP). The cell suspension was incubated for 20 minutes at room temperature while being stirred in a disposable spectrophotometer cuvette to keep the cells in suspension. After the incubation period, cytosol was separated from the pellet (which contained mitochondria, other organelles and plasma membrane) by centrifugation at 20,800 x g in a refrigerated microfuge for 10 minutes at 4\(^\circ\)C. The supernatant was transferred to a new tube and Complete Protease Inhibitors (Roche Molecular Biochemicals, Inc., Indianapolis, IN) were added according to the supplier’s recommendations; the pellets were solubilized in PLC lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 1% Triton X-
100, 1.5 mM MgCl₂, 1 mM EGTA, 100 mM NaF) with complete protease inhibitors in a volume equal to the original volume of the sample.

Proteins in the cytosol and pellet samples were separated by SDS-PAGE (Novex 4-20% Tris Glycine gels, Invitrogen, Inc., San Diego, CA), transferred to nitrocellulose membranes using a Novex transfer module, and analyzed by western blotting essentially as described above in Example 5. Membranes were initially probed with an anti-cytochrome c antibody (1:2000, Pharmingen, Inc., San Diego, CA) and then sequentially stripped and probed with an anti-HA antibody (1 μg/ml, Santa Cruz Biotech, Inc., Santa Cruz, CA) to detect the HA-adenylate kinase fusion protein, and an anti protein kinase B (PKB) antibody (Phospho-Akt antibody diluted 1:2000, New England Biolabs, Inc., Beverly, MA) to detect PKB/Akt, a soluble cytosolic marker used to confirm that individual gel lanes had been comparably loaded.

Figure 8 shows the western blot analysis of cytosolic and pellet fractions from cells that had been treated with 10 μM etoposide for the indicated amounts of time, and Figure 9 shows western blot analysis of cytosolic and pellet fractions from cells that had been treated with 15 μM thapsigargin for the indicated times. With increased time of exposure to the indicated apoptogen, increasing amounts of the two MISP markers, cytochrome c and HA-adenylate kinase, were detected in the cytosolic fraction. The results of western blot analysis of cytosolic and pellet fractions from cells that had been exposed to 200 nM staurosporine are shown in Figure 10, where an increasing amount of cytochrome c was detected with increased time of exposure to the apoptogen, but the level of HA-adenylate kinase in the cytosolic fraction did not increase over time. Comparable results were obtained when cytosolic and pellet fractions from cells that had been exposed to 0.5 μg/ml actinomycin D were analyzed. The observations are summarized in Table 1, where “+” indicates an increase in the amount of MISP that was detectable in the cytosolic fraction which correlated with increasing exposure of cells to the indicated apoptogen.

<table>
<thead>
<tr>
<th>Apoptogen</th>
<th>Cytochrome c Release</th>
<th>HA-AK2 Release</th>
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</thead>
<tbody>
<tr>
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Table 1. Differential release of cytochrome c and adenylate kinase
<p>| | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Staurosporine (200 nM)</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Etoposide (10 µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thapsigargin (15 µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Actinomycin D (0.5 µg/ml)</td>
<td>+</td>
<td>—</td>
</tr>
</tbody>
</table>

**Example 7**

**Mitochondrial Intermembrane Space Protein Translocation in Permeabilized Cells Depleted of Cytosol**

This example describes certain unexpected advantages associated with embodiments of the invention in which MISP translocation and mitochondrial activity were monitored in cells depleted of cytosol. SH-SY5Y cells overexpressing HA-tagged adenylate kinase were prepared and assayed essentially as described above in Examples 5 and 6, except that cytosol was depleted from cells in some samples according to a modification of procedures described by Fiskum et al. (1980 *Proc. Nat. Acad. Sci. USA* 77:3430-3434).

Briefly, for assay cells in which cytosol was retained, aliquots of cell suspensions containing approximately $30 \times 10^6$ cells were pelleted by centrifugation at 16,000 x g for two minutes at room temperature, resuspended in basal incubation medium (125 mM KCl, 20 mM HEPES-KOH, pH 7.0, 2 mM K$_2$HPO$_4$) and pelleted again. The intact cell pellet was transferred into the multiparameter chamber as described above and assayed at 20°C in basal incubation medium containing either (i) 5 mM glutamate and 5 mM malate (Figs. 11-13), or (ii) 5 mM succinate and 2 µM rotenone (not shown), as respiratory substrates.

For assay cells in which cytosol was depleted ("cytosol depleted", Fig. 11 as indicated; Fig. 12, upper set of tracings; Fig. 13, lanes 1 and 3-8), cells were pelleted, resuspended and pelleted again as described above, then resuspended in basal incubation medium containing respiratory substrates and digitonin (as described in Examples 5 and 6, unless otherwise indicated). Digitonin permeabilized cells were again pelleted by centrifugation, and the supernatant fluid was removed. The pellet containing all cellular organelles (Fiskum et al., 1980) was transferred to the multiparameter chamber and assayed...
in digitonin-free incubation medium with respiratory substrates (e.g., glutamate/malate) as described in the preceding examples.

In certain samples containing digitonin-permeabilized and cytosol depleted cells, cyclosporin A (CsA, Sigma, St. Louis, MO) was present at 1 μM as indicated (Fig. 12; Fig. 13, lane 4). Other additions were made at indicated times (Figs. 11-12) of Ca\(^{2+}\) (300 μM), of the electron transport chain inhibitor antimycin (Fig. 11, AntA), or of the permeabilizing agent alamethacin (40 μg/ml) to determine maximal cytochrome c release. Figure 11 shows calcium and mitochondrial membrane potential (ΔΨ) traces for digitonin-permeabilized (but not cytosol depleted) cells ("Dig"), or for digitonin-permeabilized and cytosol depleted cells ("CD"). Traces were from readings taken as described in Example 5, with additions of calcium or antimycin A at indicated times. Ca\(^{2+}\) uptake in the absence of cytosol appeared to be solely driven by mitochondrial respiration, whereas permeabilized cells that retained cytosol appeared to utilize ATPase and respiratory activities to provide energy for Ca\(^{2+}\) uptake (Fig. 11).

Figure 12 shows changes in light absorbance by cytosol depleted (Fig. 12, upper panel) and by digitonin-permeabilized (but not cytosol depleted) cells at 660 nm, apparently due to cell swelling that accompanies mitochondrial permeability transition. As shown in Fig. 12, the degree of cell swelling in MPT appeared greater when cytosol was depleted, and this observation correlated with a quantitatively greater degree of cytochrome c release in cells lacking cytosol (Fig. 13). Following incubations in the multiparameter chamber, cells were pelleted and the supernatants assayed for cytochrome c by western immunoblot analysis as described above (Fig. 13). Cytochrome c detected in the supernatants from cytosol depleted cells is shown in Figure 13, lane 1 (control cells following exposure to 1 mM EGTA with no added Ca\(^{2+}\)); lane 3 (300 μM Ca\(^{2+}\) per Fig. 12, upper panel); lane 4 (300 μM Ca\(^{2+}\) plus 1 μM CsA per Fig. 12, upper panel); lane 5 (0.06% digitonin permeabilization control post-multiparameter chamber); lane 6 (0.09% digitonin permeabilization control post-multiparameter chamber); lane 7 (0.03% digitonin permeabilization control post-multiparameter chamber); and lane 8 (alamethacin per Fig. 12, upper panel). Fig. 13, lane 2 shows cytochrome c detected in the supernatant from digitonin-permeabilized (but not cytosol depleted) cells following exposure to 300 μM Ca\(^{2+}\).
lane 9 shows a cytochrome c protein standard used as a positive control for reactivity of the anti-cytochrome c antibody.

Thus, according to certain embodiments of the present invention there are provided methods for detecting MISP translocation, for identifying an agent that alters MISP translocation, and for identifying a compound that alters the activity of an agent that alters MISP translocation, each of said methods comprising in pertinent part the use of a sample comprising a mitochondrion contained within a cell that is permeabilized and that is depleted of cytosol. Determination of when a cell is depleted of cytosol may be accomplished by any of a variety of methods well known in the art, for example, those described in Fiskum et al. (1980), including quantitative methods for monitoring the degree of cytosolic depletion by determining any of a number of known cytosolic markers, for example, the enzyme lactate dehydrogenase (LDH), or by monitoring the effects of the depletion method on cellular architecture. Preferably, a cell depleted of cytosol is essentially completely depleted of cytosol, which refers to depletion of cytosol that results in there being no remaining detectable cytosolic marker associated with the cell, according to criteria such as those described Fiskum et al. (1980). In other embodiments, a cell that is depleted of cytosol may be substantially depleted of cytosol, which may include a cell from which greater than 40 percent, preferably greater than 60 percent, and more preferably greater than 75 percent of at least one detectable cytosolic marker (e.g., LDH) is no longer associated with the cell using criteria known to the art, relative to control cells from which cytosol has not been depleted.

Accordingly, it will be appreciated that the invention also contemplates compositions and methods for detecting MISP translocation, and for detecting agents that alter (e.g., increase or decrease in a statistically significant manner) MISP translocation, and for detecting compounds that alter the activity of such agents, which methods may relate to reintroducing to a sample comprising a mitochondrion (e.g., a cytosol depleted cell as provided herein) one or more cytosolic molecular components. Without wishing to be bound by theory, differences in the results obtained when cytosol is present and when cytosol has been depleted as observed in MISP translocation and/or in mitochondrial activity as determined using the multiparameter chamber described herein, may be attributable to the presence or activity of one or more cytosolic molecular components. Such cytosolic
components may include, for example, ATP or other biochemistry molecules such as metabolites, catabolites, intermediates, cofactors, substrates, catalysts and the like. Such cytosolic components may also include, for example, one or more of a protein, peptide, glycopeptide or glycoprotein, nucleic acid or polynucleotide (including, for example, DNA or RNA), lipid including a glycolipid, proteolipid or phospholipid, or a carbohydrate, or any combination of such species, that may be present in cytosol. Isolation of cytosolic molecular components may be achieved according to any of a number of well known biochemical and chemical separation strategies known to the art, including but not limited to radiolabeling or otherwise detectably tagging cytosolic components in a biological sample, or to cell fractionation, density sedimentation, differential extraction, salt precipitation, ultrafiltration, gel filtration, ion-exchange chromatography, partition chromatography, hydrophobic chromatography, electrophoresis, affinity techniques or any other suitable separation method. Antibodies to partially purified components may be developed according to methods known in the art and may be used to detect and/or to isolate such components.

Affinity techniques may be particularly useful in the context of the present invention, and may include any method that exploits a specific binding interaction between a cytosolic component and an agent identified according to the invention that interacts with the cytosolic component. For example, because agents that influence MPT and/or MISP translocation can be immobilized on solid phase matrices, an affinity binding technique for isolation of the cytosolic component(s) may be particularly useful. Alternatively, affinity labeling methods for biological molecules, in which a PT-active (or a MISP-active) agent may be modified with a reactive moiety, are well known and can be readily adapted to the interaction between the agent and a cytosolic component, for purposes of introducing into the cytosolic component a detectable and/or recoverable labeling moiety. (See, e.g., Pierce Catalog and Handbook, 1994 Pierce Chemical Company, Rockford, IL; Scopes, R.K., Protein Purification: Principles and Practice, 1987, Springer-Verlag, New York; and Hermanson, G.T. et al., Immobilized Affinity Ligand Techniques, 1992, Academic Press, Inc., California; for details regarding techniques for isolating and characterizing biological molecules, including affinity techniques.
Characterization of cytosolic component molecular species, isolated by affinity techniques described above or by other biochemical methods, may be accomplished using physicochemical properties of the cytosolic component such as spectrometric absorbance, molecular size and/or charge, solubility, peptide mapping, sequence analysis and the like. (See, e.g., Scopes, supra.) Additional separation steps for biomolecules may be optionally employed to further separate and identify molecular species that co-purify with such cytosolic components that influence MPT and/or MISP translocation and/or other mitochondrial activities such as those detected using the multiparameter chamber described above. These are well known in the art and may include any separation methodology for the isolation of proteins, lipids, nucleic acids, carbohydrates, or other biological molecules of interest, typically based on physicochemical properties of the newly identified components of the complex. Examples of such methods include RP-HPLC, ion exchange chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography, native and/or denaturing one- and two-dimensional electrophoresis, ultrafiltration, capillary electrophoresis, substrate affinity chromatography, immunoaffinity chromatography, partition chromatography or any other useful separation method.

For example, sufficient amounts of a cytosolic protein may be obtained for partial structural characterization by microsequencing. Using the sequence data so generated, any of a variety of well known suitable strategies for further characterizing the cytosolic components may be employed. For example, nucleic acid probes may be synthesized for screening one or more appropriately chosen cDNA libraries to detect, isolate and characterize a cDNA encoding such component(s). Other examples may include use of the partial sequence data in additional screening contexts that are well known in the art for obtaining additional amino acid and/or nucleotide sequence information. See, e.g., Molecular Cloning: A Laboratory Manual, Third Edition, edited by Sambrook, Fritsch & Maniatis, Cold Spring Harbor Laboratory, 1989. Such approaches may further include nucleic acid library screening based on expression of library sequences as polypeptides, such as binding of such polypeptides to PT-active or MISP-active agents identified according to the present invention; or phage display screening approaches or dihybrid screening systems based on protein-protein interactions with known mitochondrial proteins, and the like, any of which
may be adapted to screening for mitochondrially active cytosolic components provided by the present invention, using routine methodologies with which those having ordinary skill in the art will be familiar. (See, e.g., Bartel et al., In *Cellular Interactions in Development: A Practical Approach*, Ed. D.A. Harley, 1993 Oxford University Press, Oxford, United Kingdom, pp. 153-179, and references cited therein.) Preferably extracts of cultured cells, and in particularly preferred embodiments extracts of biological tissues or organs may be sources of novel mitochondrially active cytosolic proteins or other cytosolic factors. Preferred sources may include blood, brain, fibroblasts, myoblasts, liver cells or other cell types.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.
CLAIMS

We claim:

1. A method of detecting mitochondrial intermembrane space protein translocation, comprising:

   (a) contacting a sample comprising a mitochondrion with an agent known or suspected to induce mitochondrial intermembrane space protein translocation under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, said mitochondrion comprising at least one mitochondrial intermembrane space protein fusion polypeptide, wherein said fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain; and

   (b) contacting said sample with a detectable ligand that specifically binds to said affinity domain under conditions and for a time sufficient for said detectable ligand to bind to said fusion polypeptide in extramitochondrial spaces in order to form detectable ligand:fusion polypeptide complexes, and detecting said ligand:fusion polypeptide complexes,

   wherein an increasing signal from said detectable ligand:fusion polypeptide complexes corresponds to an increasing degree of intermembrane space protein translocation.

2. A method of identifying an agent that alters mitochondrial intermembrane space protein translocation, comprising:

   (a) contacting a sample comprising a mitochondrion with a candidate agent suspected of being able to alter mitochondrial intermembrane space protein translocation under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, said mitochondrion comprising at least one mitochondrial intermembrane space protein fusion polypeptide, wherein said fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain;

   (b) contacting said sample with a detectable ligand that specifically binds to said affinity domain under conditions and for a time sufficient for said detectable
ligand to bind to said fusion polypeptide in extramitochondrial spaces in order to form detectable ligand:fusion polypeptide complexes, and detecting said ligand:fusion polypeptide complexes; and

(c) comparing the level of extramitochondrial ligand:fusion polypeptide complexes detected in a control sample lacking said candidate agent to the level of extramitochondrial ligand:fusion polypeptide complexes detected in said sample having said candidate agent,

wherein an altered level of signal of detectable ligand:fusion polypeptide complexes in said sample having the candidate agent, relative to the level of signal of detectable ligand:fusion polypeptide complexes in said control sample, indicates that said candidate agent is an agent that alters mitochondrial intermembrane space protein translocation.

3. A method of identifying a compound that alters the activity of an agent that alters mitochondrial intermembrane space protein translocation, comprising:

(a) contacting a sample comprising a mitochondrion with, sequentially in either order or simultaneously, (i) an agent that alters mitochondrial intermembrane space protein translocation and (ii) a candidate compound suspected of being able to alter the activity of said agent, under conditions and for a time sufficient to induce mitochondrial intermembrane space protein translocation, said mitochondrion comprising at least one mitochondrial intermembrane space protein fusion polypeptide, wherein said fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain;

(b) contacting said sample with a detectable ligand that specifically binds to said affinity domain under conditions and for a time sufficient for said detectable ligand to bind to said fusion polypeptide in extramitochondrial spaces in order to form detectable ligand:fusion polypeptide complexes, and detecting said ligand:fusion polypeptide complexes; and

(c) comparing the level of extramitochondrial ligand:fusion polypeptide complexes detected in a control sample lacking said candidate compound to the
level of extramitochondrial ligand:fusion polypeptide complexes detected in said sample having said candidate compound,

wherein an altered level of signal of detectable ligand:fusion polypeptide complexes in said sample having said candidate compound, relative to the level of signal of detectable ligand:fusion polypeptide complexes in said control sample, indicates that said candidate compound is a compound that alters the activity of said agent.

4. The method of any one of claims 1, 2 or 3 wherein said mitochondrion is contained within a cell.

5. The method of claim 4 wherein said cell is permeabilized.

6. The method of any one of claims 1, 2 or 3 wherein said agent is selected from the group consisting of an apoptogen and an agent that causes necrosis.

7. The method of claim 6 wherein said apoptogen is selected from the group consisting of a pro-oxidant and a calcium ionophore.

8. The method of claim 7 wherein said pro-oxidant is selected from the group consisting of hydrogen peroxide, tert-butylhydroperoxide and peroxynitrite.

9. The method of claim 7 wherein said calcium ionophore is ionomycin.

10. The method of claim 6 wherein said apoptogen is selected from the group consisting of atractyloside, bongkrekic acid, thapsigargin, glutamate, N-methyl-D-aspartic acid, carbachol, and ionomycin.

11. The method of claim 4 wherein said cell is a neural cell.

12. The method of claim 4 wherein said cell is a neuroblastoma cell.
13. The method of claim 12 wherein said neuroblastoma cell is an SH-SY5Y cell.

14. The method of claim 4 wherein said cell is a cybrid cell.

15. The method of any one of claims 1, 2 or 3 wherein said mitochondrial intermembrane space protein is selected from the group consisting of adenylate kinase-2, cytochrome c and sulfide oxidase.

16. The method of any one of claims 1, 2 or 3 wherein said mitochondrial intermembrane space protein is adenylate kinase-2.

17. The method of any one of claims 1, 2 or 3 wherein said affinity domain is selected from the group consisting of a hemagglutinin epitope tag, a FLAG® epitope tag, an XPRESS™ epitope tag, a myc epitope tag and a polyhistidine epitope tag.

18. The method of any one of claims 1, 2 or 3 wherein said affinity domain comprises a hemagglutinin epitope tag.

19. The method of any one of claims 1, 2 or 3 wherein said detectable ligand comprises an antibody.

20. The method of claim 19 wherein said antibody is selected from the group consisting of a monoclonal antibody, a polyclonal antibody, and a single chain antibody.

21. The method of claim 19 wherein said antibody comprises a fusion protein.
22. The method of any one of claims 1, 2 or 3 wherein said sample comprises a polypeptide that is a Bcl-2 family member.

23. The method of claim 22 wherein said Bcl-2 family member is selected from the group consisting of Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, NR-13, BHRF1, LMW5-HL, ORF16, Ks-Bcl-2, E1B-19K, and Ced-9.

24. The method of claim 4 wherein said cell comprises a nucleic acid expression construct comprising a promoter operably linked to a polynucleotide encoding a polypeptide that is a Bcl-2 family member.

25. The method of claim 24 wherein said Bcl-2 family member is selected from the group consisting of Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, NR-13, BHRF1, LMW5-HL, ORF16, Ks-Bcl-2, E1B-19K and Ced-9.

26. A nucleic acid expression construct comprising a promoter operably linked to a polynucleotide encoding an intermembrane space protein fusion polypeptide, wherein said fusion polypeptide comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain.

27. The nucleic acid expression construct of claim 26, wherein said affinity domain comprises less than about 20 amino acids.

28. A host cell comprising the nucleic acid expression construct of claim 26.

29. The host cell of claim 28 further comprising a nucleic acid expression construct encoding a polypeptide that is a Bcl-2 family member.
30. The host cell of claim 29 wherein said Bcl-2 family member is selected from the group consisting of Bcl-2, Bcl-XL, Bcl-w, Mcl-1, A1, NR-13, BHRF1, LMW5-HL, ORF16, Ks-Bcl-2, E1B-19K and Ced-9.

31. An isolated intermembrane space protein fusion polypeptide that comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain.

32. The isolated intermembrane space protein fusion polypeptide of claim 31 wherein said affinity domain comprises less than about 20 amino acids.

33. A method of producing an isolated intermembrane space protein fusion polypeptide comprising culturing the host cell of claim 28.

34. A composition of matter comprising a containing device appropriate for high throughput screening of agents and compounds and an intermembrane space protein fusion polypeptide that comprises at least one mitochondrial intermembrane space protein domain and at least one affinity domain.

35. The composition of matter of claim 34 wherein said containing device is selected from the group consisting of a 96-well microtiter plate and a 386-well microtiter plate.

36. A method of screening compounds or agents using the composition of matter of claim 34.

37. A mechanism for screening compounds or agents comprising the composition of matter of claim 34.
38. The method of claim 5 wherein the permeabilized cell is depleted of cytosol.
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**Fig. 5**

Cytochrome c

AK-HA

AKY
Fig. 7
Fig. 11
**Fig. 12**

**CYTOSOL DEPLETED**