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(71) Applicant *(for all designated States except US)*: **ForHumanTech. Co., Ltd.** [KR/KR]; 707 Kowoon Institute Of Technology Innovation, P.O. Box 77, Suwon 445-743 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LEE, Sang-Kyou** [KR/KR]; 2-503 Han Shin Han Gang Apartment, 49-8 Jam Won Dong, Seocho-gu, Seoul (KR). **HWANG, Ki-Chul** [KR/KR]; 107-1403 Life Apartment, Jung-gye-dong, Nowon-gu, Seoul (KR). **LEE, Seung-Kyou** [KR/KR]; 113-1102 Dong-a Solecity Apartment, 1162 Bojung-dong, Giheung-gu, Yongin-si, Kyeuggi-do (KR). **JANG, Yang-Soo** [KR/KR]; 101-701 Bangbae Dae-lim Arcroriver, 752-36 Babgbac-dong, Seocho-chu, Seoul (KR).

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(54) **Title:** PHARMACEUTICAL COMPOSITION FOR ALLEVIATION AND TREATMENT OF ISCHEMIC CONDITIONS AND METHOD FOR DELIVERING THE SAME

(57) **Abstract:** The present invention relates to pharmaceutical compositions for treating diseases and conditions caused by ischemia. The pharmaceutical compositions contain a conjugate of a phospholipase (PL) polypeptide and a protein transduction domain (PTD). PLC-δ plays a major role in the regulation of cytosolic calcium levels. During myocardial ischemia, cytosolic calcium accumulation mediates pathogenic changes. According to the present invention, ischemic diseases or conditions leading to hypoxia in tissues, such as the heart and the brain, can be prevented or alleviated by administration of a PTD-PL conjugate.

PHARMACEUTICAL COMPOSITION FOR ALLEVIATION AND TREATMENT OF ISCHEMIC CONDITIONS AND METHOD FOR DELIVERING THE SAME

BACKGROUND OF THE INVENTION

Field of the Invention

[0001] The present invention relates to a novel pharmaceutical composition for the treatment of ischemia, which contains a conjugate of a molecule of interest, such as a phospholipase polypeptide, and a protein transduction domain (PTD), as well as a method for delivering the same.

Background Art

[0002] The body is critically dependent on the heart to pump blood. A healthy heart pumps blood throughout the body for the delivery of oxygen and nutrients and the removal of harmful products of metabolism. Ischemia leads to rapid changes in myocardial metabolism and cardiac and cellular injury. The extent of the injury is dependent on the severity of ischemia and the timeliness of appropriate treatment. Continued ischemia can lead to total tissue necrosis in a few hours.

[0003] Reperfusion, although generally considered beneficial, causes tissue injury by several mechanisms. Clinically, in open heart surgery, heart transplantation, and reversal of heart disease, protection of the myocardium against injury by ischemia-reperfusion is an issue of utmost clinical interest. Exacerbation of hypoxic injury after restoration of oxygenation (reoxygenation) by reperfusion is an important mechanism of cellular injury in other types of organ transplantation and in hepatic, intestinal, cerebral, renal, and other ischemic syndromes.

[0004] Ischemia and simulated ischemic conditions cause an increase in active oxygen species and an overload of calcium ions (Ca^{2+}) (Bolli, R., *et al.*, *Physiol. Rev. 79*:609-634 (1999)). Cytosolic calcium accumulation has been proposed as a mediator of pathologic changes that occur during myocardial ischemia (Moraru, I.I., *et al.*, *Biochim. Biophys. Acta 1268*:1-8 (1995)). The increase in intracellular calcium results in the opening of mitochondrial permeability transition pores (mPTPs). The increase in intracellular calcium further enhances the opening of additional mPTPs and also activates a number of cytosolic proteins, such as phospholipases, protein kinases, proteases, and endonucleases

(Bolli, R., *et al.*, *Physiol. Rev. 79*:609-634 (1999)). It has been reported that when treatment with mPTP inhibitors cyclosporin A and sanglifehrin A is performed in post-ischemic reperfusion, the recovery of systolic function and the viability of cells increases by about 20% and 62%, respectively (Javadov, S.A., *et al.*, *J. Physiol. 549*:513-524 (2003)).

[0005] Phospholipases, such as phospholipase C (PLC), play an important role in the regulation of calcium homeostasis. To date, eleven mammalian PLC isozymes have been identified. These can be divided into four types: PLC-β, PLC-γ, PLC-δ and PLC-ε. PLC δ1 and γ1 are the predominant forms in normal cardiac cells (Hansen, C.A., *et al.*, *J. Mol. Cell. Cardiol. 27*:471-484 (1995); and Schnabel, P., *et al.*, *J. Mol. Cell. Cardiol. 28*:2419-2427 (1996)).

[0006] All PLC isozymes contain a C2 domain that is sensitive to Ca^{2+} activation (Hwang, K-C, *et al.*, *J. Steroid Biochem. 91*:131-138 (2004)). Among the PLC isoforms, PLC-δ1 is most sensitive to activation by intracellular Ca^{2+}. *Id.*

[0007] PLC hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) (Hwang, K-C, *et al.*, *J. Steroid Biochem. 91*:131-138 (2004)). DAG and IP3 stimulate the activity of protein kinase C (PKC) and the release of calcium ions from intracellular reservoirs to the cytoplasm. *Id.* The activation mechanism of PLC is well known through PLC-β which is activated by a G-protein-coupled receptor, receptor tyrosine kinase, and ras pathway, respectively (Rhee, S.G., *et al.*, *Annu. Rev. Biochem. 70*:281-312 (2001)).

[0008] Based on recent studies, it was reported that PLC-δ1 present in the mitochondrial membrane of liver cells functions to inhibit the inflow of calcium, when an excess of calcium is present in the cytoplasm (Hwang, K-C, *et al.*, *J. Steroid Biochem. 91*:131-138 (2004)). It was further shown that PLC-δ1 is present in normal myocardial cells in amounts at least 7 times greater than that of the other isozymes, and that, in an ischemic state, the amount of PLC-δ1 decreases both *in vitro* and *in vivo* (Hwang, K-C, *et al.*, *Steroid Biochem. 91*:131-138 (2004)). When treated with the calpain inhibitor calpastatin and the caspase inhibitor zVAD-fmk, the degradation of PLC-δ1 was inhibited. *Id.* In addition, when PLC-δ1 was overexpressed in cardiomyocytes, intracellular Ca^{2+} overload induced by ischemic conditions was dramatically rescued. *Id.*

[0009] These results demonstrate the critical role PLC-δ1 plays in cytosolic calcium homeostasis in normal hearts and its effect on calcium balance after myocardial infarction. Clearly, an effective method of transducing PLC-δ1 into the cytosol and nucleus of living cells to treat or prevent ischemia or ischemic conditions, without deleterious side effects, is needed.

[0010] Protein transduction domains (PTDs) have been used for delivery of biologically active molecules (Viehl C.T., *et al.*, *Ann. Surg. Oncol. 12*:517-525 (2005); Noguchi H., *et al.*, *Nat. Med. 10*:305-309 (2004); and Fu A.L., *et al.*, *Neurosci. Lett. 368*:258-62 (2004)). PTDs are low molecular-weight peptides that have been used for the penetration of physiologically active molecules into cells. However, to date no attempts have been made to use PTDs as a way of delivering phospholipase C *in vivo*.

BRIEF SUMMARY OF THE INVENTION

[0011] One object of the invention is to effectively treat ischemic diseases and conditions by delivering a polypeptide *in vivo* using a protein transduction domain (PTD).

[0012] To achieve the above object, the present invention provides a conjugate of a PTD and a phospholipase (PL) polypeptide (PTD-PL). The conjugate can be prepared by fusing a PTD-encoding gene with a phospholipase gene and expressing and isolating the fusion protein *in vitro* or *in vivo* using standard cloning techniques and routine methods known to those skilled in the art. The PTD-PL conjugate according to the present invention easily passes through membranes due to the intracellular penetration and delivery effects of PTD, for delivery to cells.

[0013] One embodiment of the present invention is the use of a PTD-PL conjugate to treat, decrease, or prevent intracellular calcium overload caused by ischemia or reperfusion.

[0014] A further embodiment is the use of a PTD-PL conjugate to reduce the concentration of free calcium ions in a cell.

[0015] The invention also encompasses methods of using a PTD-PL conjugate to reduce, treat, prevent or eliminate cardiac injury (*e.g.*, heart failure, and myocardial infarction) caused by hypoxia or ischemia.

[0016] The invention further encompasses methods of using a PTD-PL conjugate to prevent and/or treat cardiovascular disease, myocardial hypoxia or ischemic damage.

[0017] The invention also encompasses methods of using a PTD-PL conjugate to prevent stroke during heart failure.

[0018] An additional embodiment is the use of a PTD-PL conjugate to prevent or reduce ischemia-reperfusion injury in a subject suffering from hypothermia.

[0019] Another embodiment is the use of a PTD-PL conjugate to prevent organ or tissue damage during organ or tissue transplantation. A preferred embodiment is the use of PTD-PLC-δ for heart transplantation.

[0020] An additional embodiment of the present invention is the use of a PTD-PL conjugate in combination with one or more therapeutic compounds or constructs.

[0021] For all of the above embodiments, fusions of PTD with one or more fragments, derivatives or analogues of PL are also contemplated.

[0022] This invention enables administration of the PTD-PL conjugate via local administration routes, thereby minimizing or avoiding systemic side effects.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0023] Figure 1 shows the purification of PTD-PLC-δ1 using nickel beads. Lane 1 represents a standard molecular weight protein, and lane 2 represents the fusion protein PTD-PLC-δ1. Human PLC-δ1 is 756 aa in length and about 85 kDa.

[0024] Figures 2A and 2B show the penetration of PTD-PLC-δ1 according to the present invention into hypoxia-induced myocardial cells. Figure 2A shows the amount of PTD-PLC-δ1 protein with a concentration gradient of 0-500 nM in myocardial cells cultured under low-oxygen conditions for 12 hours. Figure 2B shows cell viability under these conditions. CTL is the control.

[0025] Figure 3 shows the overload of calcium in myocardial cells cultured under low-oxygen conditions (hypoxia) for 12 hours (lane 2), as compared to a control group incubated under oxygen conditions (lane 1), and when treated with 100 nM of the PTD-PLC-δ1 protein (lane 3). The relative change in intracellular free Ca^{2+} was determined by measuring fluorescent intensity.

[0026] Figure 4 shows the delivery and penetration of PTD-PLC-δ1 to the internal organs (heart, kidney and liver). In the heart, the phosphorylation of protein kinase C (PKC) is shown. Lane 1: normal; Lane 2: Hypoxia (1 hr) and reperfusion (3 hrs); Lane 3: Hypoxia (1 hr) and reperfusion (3 hrs) plus PTD-PLC-δ1.

[0027] Figure 5 shows the effect of PTD-PLC-δ1 on the treatment of a cardiac ischemic area in an animal myocardial infarct model. The survival of myocardial cells is visualized in the absence and presence of PTD-PLC-δ1. I/R stands for ischemia followed by reperfusion.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Calcium and free radicals act in concert to induce cardiac and neural injury in acute trauma, *e.g.*, ischemia and spinal cord injury. The present invention encompasses methods for treating or preventing ischemia or reperfusion-induced ischemia in cells, such as myocardial cells, with a PTD-PL conjugate.

[0029] One of the embodiments of the present invention is the use of a PTD-PL conjugate to treat, decrease, or prevent calcium overload, thereby reducing or inhibiting cardiac, cardiovascular or neural cell death (*e.g.*, myocyte, neuron). Calcium overload may be caused by, *e.g.*, hypoxia, ischemia, reperfusion, cardiovascular (heart) disease or injury, such as a myocardial infarction, or a neurological (brain) disease or injury, such as an ischemic or hemorrhagic stroke. Calcium overload can occur in various body parts or organs, including but not limited to, brain, spinal cord, heart, transplanted organ or transplanted limb, and can be restricted to one of these body parts. A preferred embodiment is the use of a PTD-PLC-δ conjugate to treat, decrease, or prevent calcium overload.

[0030] A further embodiment is the use of a PTD-PL conjugate to reduce the concentration of free calcium ions in a cell by administering to a cell an effective amount of PTD-PL.

[0031] The invention also relates to methods of treating, preventing or minimizing myocardial oxidative stress, such as is caused by hypoxia or ischemia, in a subject. This is done by administering to a subject in need thereof a therapeutically effective amount of PTC-PL which modulates myocardial oxidative stress such that the myocardial cells which are the target of the oxidative stress are protected from cell death. The cell death may be due, *e.g.*, to necrosis or apoptosis.

[0032] A further embodiment of the present invention is the use of a PTD-PLC-δ conjugate to reduce or inhibit cardiovascular or neural cell death. The cardiovascular cell can be, *e.g.*, a cardiac myocyte, ventricular myocyte, atrial myocyte, cardiac stem cell,

endothelial cell, vascular smooth muscle cell, pacemaker cell, myofibroblast or fibroblast. The neural cell can be, *e.g.*, a neuron.

[0033] The invention also encompasses methods of treating, preventing, reducing or eliminating cardiac or neural injury caused by hypoxia or ischemia in a subject, wherein PTD-PL is administered to a subject in need thereof, such that the hypoxia or ischemic-related injury is treated, prevented, reduced, or eliminated.

[0034] The cardiac injury that can be treated, prevented, reduced, or eliminated by the methods and compositions of the present invention includes all cardiac injury caused or affected by hypoxia and/or ischemia. Such injury includes, but is not limited to, cardiac post-ischemic reperfusion, congestive heart failure, myocardial infarction, cardiotoxicity caused by compounds such as drugs (*e.g.*, doxorubicin), cardiac damage due to infection (*e.g.*, syphilis, chronic *Trypanosoma cruzi* infection), fulminant cardiac amyloidosis, heart surgery, heart transplantation, and traumatic cardiac injury (*e.g.*, penetrating or blunt injury, or aortic valve rupture). All or a portion of the heart may be injured, including associated blood vessels and/or tissue, such as the pericardium. Administration of the compounds of the invention may be done where clinically necessary or desirable, *e.g.*, prior to post-ischemic reperfusion, at the onset of post-ischemic reperfusion, or during post-ischemic reperfusion.

[0035] The neural injury that can be treated, prevented, reduced, or eliminated by the methods and compositions of the present invention includes all neural injury caused or affected by hypoxia and/or ischemia. Such injury includes, but is not limited to, ischemia-reperfusion injury, neurotoxicity caused by compounds such as drugs, and neural damage due to parasitic infection.

[0036] The invention also encompasses methods of using a PTD-PL conjugate to prevent and/or treat cardiovascular disease, myocardial hypoxia or ischemic damage.

[0037] The invention further encompasses methods of preventing organ or tissue damage during organ or tissue transplantation, by administering to a donor PTD-PL prior to or concurrent with removal of said organ or tissue, such that damage caused by reperfusion of said organ or tissue is decreased or prevented. Specifically, the invention encompasses a method of preventing ischemia-reperfusion injury in a subject suffering from hypothermia, whereby the subject is pre-treated with a therapeutically effective amount of

a fusion polypeptide containing a protein transduction domain (PTD) and a phospholipase polypeptide.

[0038] In preferred embodiments, the organ or tissue to be transplanted is the heart or cardiac tissue. The PTD-PL may also be contacted with the organ or tissue following surgical removal of the organ or tissue from the donor. In some embodiments, the PTD-PL is added in addition to known organ or tissue preservation solutions, such as, the University of Wisconsin solution or Celsior solution (*see, e.g.*, Thabut *et al.*, *Am. J. Respir. Crit. Care Med. 164*:1204-8 (2001); Faenza *et al.*, *Transplantation 72*:1274-7 (2001)).

[0039] The invention still further encompasses methods of preventing stroke or the onset of stroke in a subject (*e.g.*, a human) suffering from heart failure, by treating a subject with PTC-PL and a pharmaceutically acceptable carrier. The PTC-PL may be administered prior to, or concomitant with, a surgical procedure that may increase the likelihood of a stroke in the patient. In one embodiment, the procedure is balloon angioplasty. Other procedures include coronary artery bypass surgery and valve replacement surgery. The PTC-PL may be administered prior to, concomitant with, or after anti-thrombogenic agents (*e.g.*, coumadin).

[0040] The invention includes methods for treating heart failure in a subject by administering PTD-PL alone or in combination with one or more additional therapeutic compounds. In some embodiments, the additional therapeutic compound includes, but is not limited to, an anti-platelet drug, an anti-coagulant drug, and an anti-thrombotic drug, or combinations thereof.

[0041] The invention still further encompasses a method of preventing reperfusion injury in a subject (such as a human) suffering from hypothermia, by treating the subject with PTD-PL and a pharmaceutically acceptable carrier. The subject may be treated with PTD-PL prior to or concomitant with the standard rewarming procedures for treating a person suffering from hypothermia as are generally known in the art.

Protein Transduction Domain (PTD)

[0042] The PTD effectively allows delivery or uptake of proteins, peptides and chemical compounds of interest *in vivo* and *in vitro* into cells by systemic or local administration. Administration routes include routes that are, *inter alia*, intramuscular, intraperitoneal,

intravenous, oral, nasal, subcutaneous, intradermal, mucosal, and inhalation. Thus, if the PTD is provided as a conjugate with a protein, peptide and/or chemical compound, the PTD can deliver the protein, peptide and/or chemical compound to a topical area, *e.g.*, skin, eyeball or airway.

[0043] For use as the PTD in the present invention, the present inventors constructed several peptides using a solid synthesis method, but it is to be understood that other kinds of PTD can be used depending on the desired delivery area and the kind of linker used. The PTD consists of 3-30 amino acids, preferably 5-15 amino acids, at least 10-30% of which are preferably arginine residues. However, PTDs without any arginine residues are also contemplated.

[0044] One embodiment involves the use of Hph-1-PTD, the PTD from the human (and mouse) transcription factor HPH-1 (YARVRRRGPRR) (SEQ ID NO:1). Another embodiment involves the use of the PTD of Sim-2 (AKAARQAAR) (SEQ ID NO:2).

[0045] Other embodiments include, but are not limited to, the PTDs of HIV-1 viral protein Tat (YGRKKRRQRRR) (SEQ ID NO:3), Antennapedia protein (Antp) of *Drosophila* (RQIKIWFQNRRMKWKK) (SEQ ID NO:4), HSV-1 structural protein Vp22 (DAATATRGRSAASRPTERPRAPARSASRPRRPVE) (SEQ ID NO:5), regulator of G protein signaling R7 (RRRRRRR) (SEQ ID NO:6), MTS (membrane translocating sequence), (AAVALLPAVLLALLAPAAADQNQLMP) (SEQ ID NO:7), and short amphipathic peptide carriers Pep-1 (KETWWETWWTEWSQPKKKRKV) (SEQ ID NO:8) and Pep-2 (KETWFETWFTEWSQPKKKRKV) (SEQ ID NO:9).

Phospholipase (PL)

[0046] To achieve the above object, the present invention provides a conjugate of a PTD and a polypeptide, such as the enzyme phospholipase, more specifically phospholipase C. There are four main types of PLC enzymes: PLC-β (beta), PLC-γ (gamma), PLC-δ (delta), PLC-ε (eta) and PLC-ζ (zeta). Each PLC type further consists of several subtypes, *e.g.*, β1, β3, β4, δ1, δ3.

[0047] One embodiment is the conjugate of a PTD with phospholipase C delta 1 (PLC-δ1). PLC-δ1, when overexpressed in hypoxic cardiomyocytes, rescues intracellular Ca^{2+} overload induced by ischemic conditions.

[0048] The nucleotide sequence of PLC-δ1 (SEQ ID NO:10) is: atggactcgg gccgggactt cctgaccctg cacggcctac aggatgatga ggatctacag gcgctgctga agggcagcca gctcctgaag gtgaagtcca gctcatggag gagagagcgc ttctacaagt tgcaggagga ctgcaagacc atctggcagg agtcccgcaa ggtcatgcgg accccggagt cccagctgtt ctccatcgag gacattcagg aggtgcgaat ggggcaccgc acggagggtc tggagaagtt cgcccgtgat gtgcccgagg accgctgctt ctccattgtc ttcaaggacc agcgcaatac actagacctc atcgccccat cgccagctga tgcccagcac tgggtgctgg ggctgcacaa gatcatccac cactcaggct ccatggacca gcgtcagaag ctacagcact ggattcactc ctgcttgcga aaagctgaca aaaacaagga caacaagatg agcttcaagg agctgcagaa cttcctgaag gagctcaaca tccaggtgga cgacagctat gcccggaaga tcttcaggga gtgtgaccac tcccagacag actccctgga ggacgaggag attgaggcct tctacaagat gctgacccag cgggtggaga tcgaccgcac cttcgccgag gccgcgggct caggggagac tctgtcggtg gatcagttag tgacgttcct gcagcaccag cagcgggagg aggcggcagg gcctgcgctg gccctctccc tcattgagcg ctacgagccc agcgagactg ccaaggcgca gcggcagatg accaaggacg gcttcctcat gtacttactg tcggctgacg gcagcgcctt cagcctggca caccgccgtg tctaccagga catgggccag ccacttagcc actacctggt gtcctcttca cacaacacct acctgctgga ggaccagcta gccgggccca gcagcactga agcctacatc cgggcactgt gcaaaggctg ccgatgcctg gagcttgact gctgggacgg gcccaaccag gaaccaatca tctaccacgg ctatactttc acttccaaga tcctcttctg cgatgtgctc agggccatcc gggactatgc cttcaaggcg tccccctacc ctgtcatcct atccctggag aaccactgca cactggagca gcagcgcgtg atggcgcggc acctgcatgc catcctgggc cccatgctgt tgaaccgacc actggatggg gtcaccaaca gcctgccctc ccctgagcaa ctgaagggga agatcctgct gaaggggaag aagctcgggg ggctcctgcc ccctggaggg gagggtggcc ctgaggccac tgtggtgtca gacgaagacg aggctgctga gatggaggat gaggcagtga ggagccgtgt gcagcacaag cccaaggagg acaagctcag gctagcacag gagctctctg acatggtcat ttactgcaag agtgtccact ttgggggctt ctccagtcct ggcacccctg gacaggcctt ctacgagatg gcgtccttct ctgagaaccg tgcccttcga ctgctccaag aatcaggaaa cggctttgtc cgccacaacg tggggcacct gagcagaatc tacccggctg gatggagaac agactcctcc aactacagcc ccgtggagat gtggaatggg ggctgccaga tcgtggccct gaatttccag acacctgggc cagagatgga cgtgtaccag ggccgcttcc aggacaacgg ggcctgtggg tacgtgctga agcccgcctt cctgcgagac cccaacggca cctttaaccc ccgcgccctg gctcaggggc cctggtgggc acggaagcgg ctcaacatca gggtcatttc ggggcagcag ctgccaaaag tcaacaagaa taagaattca attgtggacc ccaaagtgac agtggagatc catggcgtga gccgggacgt ggccagccgc cagactgctg tcatcaccaa caatggtttc aacccatggt gggacacgga gtttgcgttt gaggtagttg tgcctgacct tgccctcatc cgcttcttgg tggaagatta tgatgcctcc tccaagaatg acttcattgg ccagagtacc atccccttga acagcctcaa gcaaggatac cgccatgtcc acctcatgtc taagaacggg gaccagcatc catcagccac cctctttgtg aagatctccc tccaggacta g.

[0049] The amino acid sequence of PLC-δ1 (SEQ ID NO:11) is:
MDSGRDFLTLHGLQDDEDLQALLKGSQLLKVKSSSWRRERFYKLQEDCKTIWQE
SRKVMRTPESQLFSIEDIQEVRMGHRTEGLEKFARDVPEDRCFSIVFKDQRNTLDL
IAPSPADAQHWVLGLHKIIHHSGSMDQRQKLQHWIHSCLRKADKNKDNKMSFK
ELQNFLKELNIQVDDSYARKIFRECDHSQTDSLEDEEIEAFYKMLTQRVEIDRTFA
EAAGSGETLSVDQLVTFLQHQQREEAAGPALALSLIERYEPSETAKAQRQMTKD
GFLMYLLSADGSAFSLAHRRVYQDMGQPLSHYLVSSSHNTYLLEDQLAGPSSTE
AYIRALCKGCRCLELDCWDGPNQEPIIYHGYTFTSKILFCDVLRAIRDYAFKASPY
PVILSLENHCTLEQQRVMARHLHAILGPMLLNRPLDGVTNSLPSPEQLKGKILLK
GKKLGGLLPPGGEGGPEATVVSDEDEAAEMEDEAVRSRVQHKPKEDKLRLAQE
LSDMVIYCKSVHFGGFSSPGTPGQAFYEMASFSENRALRLLQESGNGFVRHNVG
HLSRIYPAGWRTDSSNYSPVEMWNGGCQIVALNFQTPGPEMDVYQGRFQDNGA
CGYVLKPAFLRDPNGTFNPRALAQGPWWARKRLNIRVISGQQLPKVNKNKNSIV
DPKVTVEIHGVSRDVASRQTAVITNNGFNPWWDTEFAFEVVVPDLALIRFLVEDY
DASSKNDFIGQSTIPLNSLKQGYRHVHLMSKNGDQHPSATLFVKISLQD.

[0050] Another embodiment is the conjugate of a PTD with phospholipase C delta 3 (PLC-δ3). Human PLC-δ3 is 789 amino acids in length.

[0051] The nucleotide sequence of PLC-δ3 (SEQ ID NO:12) is: atgctgtgcg gccgctggag gcgttgccgc cgcccgcccg aggagccccc ggtggccgcc caggtcgcag cccaagtcgc ggcgccggtc gctctcccgt ccccgccgac tccctccgat ggcggcacca agaggcccgg gctgcgggcg ctgaagaaga tgggcctgac ggaggacgag gacgtgcgcg ccatgctgcg gggctcccgg ctccgcaaga tccgctcgcg cacgtggcac aaggagcggc tgtaccggct gcaggaggac ggcctgagcg tgtggttcca gcggcgcatc ccgcgtgcgc catcgcagca catcttcttc gtgcagcaca tcgaggcggt ccgcgagggc caccagtccg agggcctgcg gcgcttcggg ggtgccttcg cgccagcgcg ctgcctcacc atcgccttca agggccgccg caagaacctg gacctggcgg cgcccacggc tgaggaagcg cagcgctggg tgcgcggtct gaccaagctc cgcgcgcgcc tggacgccat gagccagcgc gagcggctag accactggat ccactcctat ctgcaccggg ctgactccaa ccaggacagc aagatgagct tcaaggagat caagagcctg ctgagaatgg tcaacgtgga catgaacgac atgtacgcct acctcctctt caaggagtgt gaccactcca acaacgaccg tctagagggg gctgagatcg aggagttcct gcggcggctg ctgaagcggc cggagctgga ggagatcttc catcagtact cgggcgagga ccgcgtgctg agtgcccctg agctgctgga gttcctggag gaccagggcg aggagggcgc cacactggcc cgcgcccagc agctcattca gacctatgag ctcaacgaga cagccaagca gcatgagctg atgacactgg atggcttcat gatgtacctg ttgtcgccgg agggggctgc cttggacaac acccacacgt gtgtgttcca ggacatgaac cagccccttg

cccactactt catctcttcc tcccacaaca cctatctgac tgactcccag atcggggggc ccagcagcac cgaggcctat gttagggcct ttgcccaggg atgccgctgc gtggagctgg actgctggga ggggccagga ggggagcccg tcatctatca tggccatacc ctcacctcca agattctctt ccgggacgtg gtccaagccg tgcgcgacca tgccttcacg ctgtcccctt accctgtcat cctatccctg gagaaccact gcgggctgga gcagcaggct gccatggccc gccacctctg caccatcctg ggggacatgc tggtgacaca ggcgctggac tccccaaatc ccgaggagct gccatcccca gagcagctga agggccgggt cctggtgaag ggaaagaagc tgcccgctgc tcggagcgag gatggccggg ctctgtcgga tcgggaggag gaggaggagg atgacgagga ggaagaagag gaggtggagg ctgcagcgca gaggcggctg gccaagcaga tctccccgga gctgtcggcc ctggctgtgt actgccacgc cacccgcctg cggaccctgc accctgcccc caacgcccca caaccctgcc aggtcagctc cctcagcgag cgcaaagcca agaaactcat tcgggaggca gggaacagct ttgtcaggca caatgcccgc cagctgaccc gcgtgtaccc gctggggctg cggatgaact cagccaacta cagtccccag gagatgtgga actcgggctg tcagctggtg gccttgaact tccagacgcc aggctacgag atggacctca atgccgggcg cttcctagtc aatgggcagt gtggctacgt cctaaaacct gcctgcctgc ggcaacctga ctcgaccttt gaccccgagt acccaggacc tcccagaacc actctcagca tccaggtgct gactgcacag cagctgccca agctgaatgc cgagaagcca cactccattg tggacccccc ggtgcgcatt gagatccatg gggtgcccgc agactgtgcc cggcaggaga ctgactacgt gctcaacaat ggcttcaacc cccgctgggg gcagaccctg cagttccagc tgcgggctcc ggagctggca ctggtccggt ttgtggtgga agattatgac gccacctccc ccaatgactt tgtgggccag tttacactgc ctcttagcag cctaaagcaa gggtaccgcc acatacacct gctttccaag gacggggcct cactgtcacc agccacgctc ttcatccaaa tccgcatcca gcgctcctga.

[0052] The amino acid sequence of PLC-δ3 (SEQ ID NO:13) is:

MLCGRWRRCRRPPEEPPVAAQVAAQVAAPVALPSPPTPSDGGTKRPGLRALKK
MGLTEDEDVRAMLRGSRLRKIRSRTWHKERLYRLQEDGLSVWFQRRIPRAPSQH
IFFVQHIEAVREGHQSEGLRRFGGAFAPARCLTIAFKGRRKNLDLAAPTAEEAQR
WVRGLTKLRARLDAMSQRERLDHWIHSYLHRADSNQDSKMSFKEIKSLLRMVN
VDMNDMYAYLLFKECDHSNNDRLEGAEIEEFLRRLLKRPELEEIFHQYSGEDRVL
SAPELLEFLEDQGEEGATLARAQQLIQTYELNETAKQHELMTLDGFMMYLLSPE
GAALDNTHTCVFQDMNQPLAHYFISSSHNTYLTDSQIGGPSSTEAYVRAFAQGCR
CVELDCWEGPGGEPVIYHGHTLTSKILFRDVVQAVRDHAFTLSPYPVILSLENHC
GLEQQAAMARHLCTILGDMLVTQALDSPNPEELPSPEQLKGRVLVKGKKLPAAR
SEDGRALSDREEEEEDDEEEEEEVEAAAQRRLAKQISPELSALAVYCHATRLRTL
HPAPNAPQPCQVSSLSERKAKKLIREAGNSFVRHNARQLTRVYPLGLRMNSANY
SPQEMWNSGCQLVALNFQTPGYEMDLNAGRFLVNGQCGYVLKPACLRQPDSTF
DPEYPGPPRTTLSIQVLTAQQLPKLNAEKPHSIVDPLVRIEIHGVPADCARQETDY

VLNNGFNPRWGQTLQFQLRAPELALVRFVVEDYDATSPNDFVGQFTLPLSSLKQ GYRHIHLLSKDGASLSPATLFIQIRIQRS.

[0053] An additional embodiment is the conjugate of a PTD with phospholipase C delta 4 (PLC-δ4). PLC-δ4 is also referred to as PLC-δ2. Human PLC-δ4 is 762 amino acids in length.

[0054] The nucleotide sequence of PLC-δ4 (SEQ ID NO:14) is: atggcgtccc tgctgcaaga ccagctgacc actgatcagg acttgctgct gatgcaggaa ggcatgccga tgcgcaaggt gaggtccaaa agctggaaga agctaagata cttcagactt cagaatgacg gcatgacagt ctggcatgca cggcaggcca ggggcagtgc caagcccagc ttctcaatct ctgatgtgga gacaatacgt aatggccatg attccgagtt gctgcgtagc ctggcagagg agctcccccc ggagcagggc ttcaccattg tcttccatgg ccgccgctcc aacctggacc tgatggccaa cagtgttgag gaggcccaga tatggatgcg agggctccag ctgttggtgg atcttgtcac cagcatggac catcaggagc gcctggacca atggctgagc gattggtttc aacgtggaga caaaaatcag gatggtaaga tgagtttcca agaagttcag cggttattgc acctaatgaa tgtggaaatg gaccaagaat atgccttcag tctttttcag gcagcagaca cgtcccagtc tggaaccctg gaaggagaag aattcgtaca gttctataag gcattgacta aacgtgctga ggtgcaggaa ctgtttgaaa gtttttcagc tgatgggcag aagctgactc tgctggaatt tttggatttc ctccaagagg agcagaagga gagagactgc acctctgagc ttgctctgga actcattgac cgctatgaac cttcagacag tggcaaactg cggcatgtgc tgagtatgga tggcttcctc agctacctct gctctaagga tggagacatc ttcaacccag cctgcctccc catctatcag gatatgactc aacccctgaa ccactacttc atctgctctt ctcataacac ctacctagtg ggggaccagc tttgcggcca gagcagcgtc gagggatata tacgggccct gaagcggggg tgccgctgcg tggaggtgga tgtatgggat ggacctagcg gggaacctgt cgtttaccac ggacacaccc tgacctcccg catcctgttc aaagatgtcg tggccacagt agcacagtat gccttccaga catcagacta cccagtcatc ttgtccctgg agacccactg cagctgggag cagcagcaga ccatggcccg tcatctgact gagatcctgg gggagcagct gctgagcacc accttggatg gggtgctgcc cactcagctg ccctcgcctg aggagcttcg gaggaagatc ctggtgaagg ggaagaagtt aacacttgag gaagacctgg aatatgagga agaggaagca gaacctgagt tggaagagtc agaattggcg ctggagtccc agtttgagac tgagcctgag ccccaggagc agaaccttca gaataaggac aaaaagaaga aatccaagcc catcttgtgt ccagccctct cttccctggt tatctacttg aagtctgtct cattccgcag cttcacacat tcaaaggagc actaccactt ctacgagata tcatctttct ctgaaaccaa ggccaagcgc ctcatcaagg aggctggcaa tgagtttgtg cagcacaata cttggcagtt aagccgtgtg tatcccagcg gcctgaggac agactcttcc aactacaacc cccaggaact ctggaatgca ggctgccaga tggtggccat gaatatgcag actgcagggc ttgaaatgga catctgtgat gggcatttcc gccagaatgg cggctgtggc tatgtgctga agccagactt cctgcgtgat atccagagtt ctttccaccc tgagaagccc atcagccctt tcaaagccca gactctctta atccaggtga tcagcggtca gcaactcccc aaagtggaca agaccaaaga ggggtccatt gtggatccac tggtgaaagt gcagatcttt ggcgttcgtc tagacacagc acggcaggag accaactatg tggagaacaa

tggttttaat ccatactggg ggcagacact atgtttccgg gtgctggtgc ctgaacttgc catgctgcgt tttgtggtaa tggattatga ctggaaatcc cgaaatgact ttattggtca gtacaccctg ccttggacct gcatgcaaca aggttaccgc cacattcacc tgctgtccaa agatggcatc agcctccgcc cagcttccat ctttgtgtat atctgcatcc aggaaggcct ggaggggat gagtcctga.

[0055] The amino acid sequence of PLC-δ4 (SEQ ID NO:15) is: MASLLQDQLTTDQDLLLMQEGMPMRKVRSKSWKKLRYFRLQNDGMTVWHAR QARGSAKPSFSISDVETIRNGHDSELLRSLAEELPLEQGFTIVFHGRRSNLDLMAN SVEEAQIWMRGLQLLVDLVTSMDHQERLDQWLSDWFQRGDKNQDGKMSFQEV QRLLHLMNVEMDQEYAFSLFQAADTSQSGTLEGEEFVQFYKALTKRAEVQELFE SFSADGQKLTLLEFLDFLQEEQKERDCTSELALELIDRYEPSDSGKLRHVLSMDGF LSYLCSKDGDIFNPACLPIYQDMTQPLNHYFICSSHNTYLVGDQLCGQSSVEGYIR ALKRGCRCVEVDVWDGPSGEPVVYHGHTLTSRILFKDVVATVAQYAFQTSDYP VILSLETHCSWEQQQTMARHLTEILGEQLLSTTLDGVLPTQLPSPEELRRKILVKG KKLTLEEDLEYEEEEAEPELEESELALESQFETEPEPQEQNLQNKDKKKKSKPILC PALSSLVIYLKSVSFRSFTHSKEHYHFYEISSFSETKAKRLIKEAGNEFVQHNTWQ LSRVYPSGLRTDSSNYNPQELWNAGCQMVAMNMQTAGLEMDICDGHFRQNGG CGYVLKPDFLRDIQSSFHPEKPISPFKAQTLLIQVISGQQLPKVDKTKEGSIVDPLV KVQIFGVRLDTARQETNYVENNGFNPYWGQTLCFRVLVPELAMLRFVVMDYD WKSRNDFIGQYTLPWTCMQQGYRHIHLLSKDGISLRPASIFVYICIQEGLEGDES.

[0056] Additional PLC-δ enzymes, including but not limited to, PLC-δ5, are also contemplated as part of the present invention. The nucleotide and amino acid sequence of PLC-δ5 can be found in U.S. Patent No. 6,958,152, incorporated herein by reference.

[0057] Furthermore, additional PLC enzymes, including but not limited to, PLC-β1 (beta1), PLC-β2 (beta2), PLC-β3 (beta3), PLC-β4 (beta4), PLC-γ1 (gamma1), PLC-γ2 (gamma2), PLC-ε1a (eta1a), PLC-ε1ba (eta1b), and PLC-ζ (zeta) are also contemplated as part of the present invention.

[0058] The amino acid sequence of PLC-β1 (SEQ ID NO:16) is: MAGAQPGVHALQLKPVCVSDSLKKGTKFVKWDDDSTIVTPIILRTDPQGFFFYW TDQNKETELLDLSLVKDARCGRHAKAPKDPKLRELLDVGNIGRLEQRMITVVYG PDLVNISHLNLVAFQEEVAKEWTNEVFSLATNLLAQNMSRDAFLEKAYTKLKLQ VTPEGRIPLKNIYRLFSADRKRVETALEACSLPSSRNDSIPQEDFTPEVYRVFLNNL CPRPEIDNIFSEFGAKSKPYLTVDQMMDFINLKQRDPRLNEILYPPLKQEQVQVLI

EKYEPNNSLARKGQISVDGFMRYLSGEENGVVSPEKLDLNEDMSQPLSHYFINSS
HNTYLTAGQLAGNSSVEMYRQVLLSGCRCVELDCWKGRTAEEEPVITHGFTMTT
EISFKEVIEAIAECAFKTSPFPILLSFENHVDSPKQQAKMAEYCRLIFGDALLMEPL
EKYPLESGVPLPSPMDLMYKILVKNKKKSHKSSEGSGKKKLSEQASNTYSDSSSM
FEPSSPGAGEADTESDDDDDDDDCKKSSMDEGTAGSEAMATEEMSNLVNYIQPV
KFESFEISKKRNKSFEMSSFVETKGLEQLTKSPVEFVEYNKMQLSRIYPKGTRVDS
SNYMPQLFWNAGCQMVALNFQTMDLAMQINMGMYEYNGKSGYRLKPEFMRR
PDKHFDPFTEGIVDGIVANTLSVKIISGQFLSDKKVGTYVEVDMFGLPVDTRRKAF
KTKTSQGNAVNPVWEEEPIVFKKVVLPTLACLRIAVYEEGGKFIGHRILPVQAIRP
GYHYICLRNERNQPLTLPAVFVYIEVKDYVPDTYADVIEALSNPIRYVNLMEQRA
KQLAALTLEDEEEVKKEADPGETPSEAPSEARTTPAENGVNHTTTLTPKPPSQAL
HSQPAPGSVKAPAKTEDLIQSVLTEVEAQTIEELKQQKSFVKLQKKHYKEMKDL
VKRHHKKTTDLIKEHTTKYNEIQNDYLRRRAALEKSAKKDSKKKSEPSSPDHGSS
TIEQDLAALDAEMTQKLIDLKDKQQQQLLNLRQEQYYSEKYQKREHIKLLIQKLT
DVAEECQNNQLKKLKEICEKEKKELKKKMDKKRQEKITEAKSKDKSQMEEEKT
EMIRSYIQEVVQYIKRLEEAQSKRQEKLVEKHKEIRQQILDEKPKLQVELEQEYQ
DKFKRLPLEILEFVQEAMKGKISEDSNHGSAPLSLSSDPGKVNHKTPSSEELGGDI
PGKEFDTPL.

[0059] The amino acid sequence of PLC-β2 (SEQ ID NO:17) is:
MSLLNPVLLPPKVKAYLSQGERFIKWDDETTVASPVILRVDPKGYYLYWTYQSK
EMEFLDITSIRDTRFGKFAKMPKSQKLRDVFNMDFPDNSFLLKTLTVVSGPDMVD
LTFHNFVSYKENVGKAWAEDVLALVKHPLTANASRSTFLDKILVKLKMQLNSEG
KIPVKNFFQMFPADRKRVEAALSACHLPKGKNDAINPEDFPEPVYKSFLMSLCPR
PEIDEIFTSYHAKAKPYMTKEHLTKFINQKQRDSRLNSLLFPPARPDQVQGLIDKY
EPSGINAQRGQLSPEGMVWFLCGPENSVLAQDKLLLHHDMTQPLNHYFINSSHN
TYLTAGQFSGLSSAEMYRQVLLSGCRCVELDCWKGKPPDEEPIITHGFTMTTDIFF
KEAIEAIAESAFKTSPYPIILSFENHVDSPRQQAKMAEYCRTIFGDMLLTEPLEKFP
LKPGVPLPSPEDLRGKILIKNKKNQFSGPTSSSKDTGGEAEGSSPPSAPAVWAGEE
GTELEEEEVEEEEEEESGNLDEEEIKKMQSDEGTAGLEVTAYEEMSSLVNYIQPT
KFVSFEFSAQKNRSYVISSFTELKAYDLLSKASVQFVDYNKRQMSRIYPKGTRMD
SSNYMPQMFWNAGCQMVALNFQTMDLPMQQNMAVFEFNGQSGYLLKHEFMR
RPDKQFNPFSVDRIDVVVATTLSITVISGQFLSERSVRTYVEVELFGLPGDPKRRY

RTKLSPSTNSINPVWKEEPFVFEKILMPELASLRVAVMEEGNKFLGHRIIPINALNS
GYHHLCLHSESNMPLTMPALFIFLEMKDYIPGAWADLTVALANPIKFFSAHDTKS
VKLKEAMGGLPEKPFPLASPVASQVNGALAPTSNGSPAARAGAREEAMKEAAEP
RTASLEELRELKGVVKLQRRHEKELRELERRGARRWEELLQRGAAQLAELGPPG
VGGVGACKLGPGKGSRKKRSLPREESAGAAPGEGPEGVDGRVRELKDRLELELL
RQGEEQYECVLKRKEQHVAEQISKMMELAREKQAAELKALKETSENDTKEMKK
KLETKRLERIQGMTKVTTDKMAQERLKREINNSHIQEVVQVIKQMTENLERHQE
KLEEKQAACLEQIREMEKQFQKEALAEYEARMKGLEAEVKESVRACLRTCFPSE
AKDKPERACECPPELCEQDPLIAKADAQESRL.

[0060] The amino acid sequence of PLC-β3 (SEQ ID NO:18) is:
MAGAQPGVHALQLEPPTVVETLRRGSKFIKWDEETSSRNLVTLRVDPNGFFLYW
TGPNMEVDTLDISSIRDTRTGRYARLPKDPKIREVLGFGGPDARLEEKLMTVVSG
PDPVNTVFLNFMAVQDDTAKVWSEELFKLAMNILAQNASRNTFLRKAYTKLKL
QVNQDGRIPVKNILKMFSADKKRVETALESCGLKFNRSESIRPDEFSLEIFERFLNK
LCLRPDIDKILLEIGAKGKPYLTLEQLMDFINQKQRDPRLNEVLYPPLRPSQARLLI
EKYEPNQQFLERDQMSMEGFSRYLGGEENGILPLEALDLSTDMTQPLSAYFINSS
HNTYLTAGQLAGTSSVEMYRQALLWGCRCVELDVWKGRPPEEEPFITHGFTMTT
EVPLRDVLEAIAETAFKTSPYPVILSFENHVDSAKQQAKMAEYCRSIFGDALLIEP
LDKYPLAPGVPLPSPQDLMGRILVKNKKRHRPSAGGPDSAGRKRPLEQSNSALSE
SSAATEPSSPQLGSPSSDSCPGLSNGEEVGLEKPSLEPQKSLGDEGLNRGPYVLGP
ADREDEEEDEEEEEQTDPKKPTTDEGTASSEVNATEEMSTLVNYIEPVKFKSFEA
ARKRNKCFEMSSFVETKAMEQLTKSPMEFVEYNKQQLSRIYPKGTRVDSSNYMP
QLFWNVGCQLVALNFQTLDVAMQLNAGVFEYNGRSGYLLKPEFMRRPDKSFDP
FTEVIVDGIVANALRVKVISGQFLSDRKVGIYVEVDMFGLPVDTRRKYRTRTSQG
NSFNPVWDEEPFDFPKVVLPTLASLRIAAFEEGGKFVGHRILPVSAIRSGYHYVCL
RNEANQPLCLPALLIYTEASDYIPDDHQDYAEALINPIKHVSLMDQRARQLAALIG
ESEAQAGQETCQDTQSQQLGSQPSSNPTPSPLDASPRRPPGPTTSPASTSLSSPGQR
DDLIASILSEVAPTPLDELRGHKALVKLRSRQERDLRELRKKHQRKAVTLTRRLL
DGLAQAQAEGRCRLRPGALGGAADVEDTKEGEDEAKRYQEFQNRQVQSLLELR
EAQVDAEAQRRLEHLRQALQRLREVVLDANTTQFKRLKEMNEREKKELQKILD
RKRHNSISEAKMRDKHKKEAELTEINRRHITESVNSIRRLEEAQKQRHDRLVAGQ

QQVLQQLAEEEPKLLAQLAQECQEQRARLPQEIRRSLLGEMPEGLGDGPLVACA
SNGHAPGSSGHLSGADSESQEENTQL.

[0061] The amino acid sequence of PLC-β4 (SEQ ID NO:19) is:
MAKPYEFNWQKEVPSFLQEGTVFDRYEEESFVFEPNCLFKVDEFGFFLTWRSEGK
EGQVLECSLINSIRSGAIPKDPKILAALEAVGKSENDLEGRIVCVCSGTDLVNISFT
YMVAENPEVTKQWVEGLRSIIHNFRANNVSPMTCLKKHWMKLAFMTNTNGKIP
VRSITRTFASGKTEKVIFQALKELGLPSGKNDEIEPTAFSYEKFYELTQKICPRTDIE
DLFKKINGDKTDYLTVDQLVSFLNEHQRDPRLNEILFPFYDAKRAMQIIEMYEPD
EDLKKKGLISSDGFCRYLMSDENAPVFLDRLELYQEMDHPLAHYFISSSHNTYLT
GRQFGGKSSVEMYRQVLLAGCRCVELDCWDGKGEDQEPIITHGKAMCTDILFKD
VIQAIKETAFVTSEYPVILSFENHCSKYQQYKMSKYCEDLFGDLLLKQALESHPLE
PGRALPSPNDLKRKILIKNKRLKPEVEKKQLEALRSMMEAGESASPANILEDDNE
EEIESADQEEEAHPEFKFGNELSADDLGHKEAVANSVKKGLVTVEDEQAWMAS
YKYVGATTNIHPYLSTMINYAQPVKFQGFHVAEERNIHYNMSSFNESVGLGYLK
THAIEFVNYNKRQMSRIYPKGGRVDSSNYMPQIFWNAGCQMVSLNYQTPDLAM
QLNQGKFEYNGSCGYLLKPDFMRRPDRTFDPFSETPVDGVIAATCSVQVISGQFL
SDKKIGTYVEVDMYGLPTDTIRKEFRTRMVMNNGLNPVYNEESFVFRKVILPDL
AVLRIAVYDDNNKLIGQRILPLDGLQAGYRHISLRNEGNKPLSLPTIFCNIVLKTY
VPDGFGDIVDALSDPKKFLSITEKRADQMRAMGIETSDIADVPSDTSKNDKKGKA
NTAKANVTPQSSSELRPTTTAALASGVEAKKGIELIPQVRIEDLKQMKAYLKHLK
KQQKELNSLKKKHAKEHSTMQKLHCTQVDKIVAQYDKEKSTHEKILEKAMKKK
GGSNCLEMKKETEIKIQTLTSDHKSKVKEIVAQHTKEWSEMINTHSAEEQEIRDL
HLSQQCELLKKLLINAHEQQTQQLKLSHDRESKEMRAHQAKISMENSKAISQDKS
IKNKAERERRVRELNSSNTKKFLEERKRLAMKQSKEMDQLKKVQLEHLEFLEKQ
NEQLLKSCHAVSQTQGEGDAADGEIGSRDGPQTSNSSMKLQNAN

[0062] The amino acid sequence of PLC-γ1 (SEQ ID NO:20) is:
MAGAASPCANGCGPGAPSDAEVLHLCRSLEVGTVMTLFYSKKSQRPERKTFQVK
LETRQITWSRGADKIEGAIDIREIKEIRPGKTSRDFDRYQEDPAFRPDQSHCFVILY
GMEFRLKTLSLQATSEDEVNMWIKGLTWLMEDTLQAPTPLQIERWLRKQFYSVD
RNREDRISAKDLKNMLSQVNYRVPNMRFLRERLTDLEQRSGDITYGQFAQLYRS
LMYSAQKTMDLPFLEASTLRAGERPELCRVSLPEFQQFLLDYQGELWAVDRLQV
QEFMLSFLRDPLREIEEPYFFLDEFVTFLFSKENSVWNSQLDAVCPDTMNNPLSHY

WISSSHNTYLTGDQFSSESSLEAYARCLRMGCRCIELDCWDGPDGMPVIYHGHTL
TTKIKFSDVLHTIKEHAFVASEYPVILSIEDHCSIAQQRNMAQYFKKVLGDTLLTK
PVEISADGLPSPNQLKRKILIKHKKLAEGSAYEEVPTSMMYSENDISNSIKNGILYL
EDPVNHEWYPHYFVLTSSKIYYSEETSSDQGNEDEEEPKEVSSSTELHSNEKWFH
GKLGAGRDGRHIAERLLTEYCIETGAPDGSFLVRESETFVGDYTLSFWRNGKVQH
CRIHSRQDAGTPKFFLTDNLVFDSLYDLITHYQQVPLRCNEFEMRLSEPVPQTNA
HESKEWYHASLTRAQAEHMLMRVPRDGAFLVRKRNEPNSYAISFRAEGKIKHCR
VQQEGQTVMLGNSEFDSLVDLISYYEKHPLYRKMKLRYPINEEALEKIGTAEPDY
GALYEGRNPGFYVEANPMPTFKCAVKALFDYKAQREDELTFIKSAIIQNVEKQEG
GWWRGDYGGKKQLWFPSNYVEEMVNPVALEPEREHLDENSPLGDLLRGVLDV
PACQIAIRPEGKNNRLFVFSISMASVAHWSLDVAADSQEELQDWVKKIREVAQT
ADARLTEGKIMERRKKIALELSELVVYCRPVPFDEEKIGTERACYRDMSSFPETK
AEKYVNKAKGKKFLQYNRLQLSRIYPKGQRLDSSNYDPLPMWICGSQLVALNFQ
TPDKPMQMNQALFMTGRHCGYVLQPSTMRDEAFDPFDKSSLRGLEPCAISIEVLG
ARHLPKNGRGIVCPFVEIEVAGAEYDSTKQKTEFVVDNGLNPVWPAKPFHFQISN
PEFAFLRFVVYEEDMFSDQNFLAQATFPVKGLKTGYRAVPLKNNYSEDLELASLL
IKIDIFPAKENGDLSPFSGTSLRERGSDASGQLFHGRAREGSFESRYQQPFEDFRIS
QEHLADHFDSRERRAPR RTRVNGDNRL

[0063] The amino acid sequence of PLC-γ2 (SEQ ID NO:21) is:
MSTTVNVDSLAEYEKSQIKRALELGTVMTVFSFRKSTPERRTVQVIMETRQVAW
SKTADKIEGFLDIMEIKEIRPGKNSKDFERAKAVRQKEDCCFTILYGTQFVLSTLSL
AADSKEDAVNWLSGLKILHQEAMNASTPTIIESWLRKQIYSVDQTRRNSISLRELK
TILPLINFKVSSAKFLKDKFVEIGAHKDELSFEQFHLFYKKLMFEQQKSILDEFKK
DSSVFILGNTDRPDASAVYLRDFQRFLIHEQQEHWAQDLNKVRERMTKFIDDTM
RETAEPFLFVDEFLTYLFSRENSIWDEKYDAVDMQDMNNPLSHYWISSSHNTYLT
GDQLRSESSPEAYIRCLRMGCRCIELDCWDGPDGKPVIYHGWTRTTKIKFDDVVQ
AIKDHAFVTSSFPVILSIEEHCSVEQQRHMAKAFKEVFGDLLLTKPTEASADQLPS
PSQLREKIIIKHKKLGPRGDVDVNMEDKKDEHKQQGELYMWDSIDQKWTRHYC
AIADAKLSFSDDIEQTMEEEVPQDIPPTELHFGEKWFHKKVEKRTSAEKLLQEYC
METGGKDGTFLVRESETFPNDYTLSFWRSGRVQHCRIRSTMEGGTLKYYLTDNL
TFSSIYALIQHYRETHLRCAEFELRLTDPVPNPNPHESKPWYYDSLSRGEAEDML
MRIPRDGAFLIRKREGSDSYAITFRARGKVKHCRINRDGRHFVLGTSAYFESLVEL

VSYYEKHSLYRKMRLRYPVTPELLERYNMERDINSLYDVSRMYVDPSEINPSMP
QRTVKALYDYKAKRSDELSFCRGALIHNVSKEPGGWWKGDYGTRIQQYFPSNY
VEDISTADFEELEKQIIEDNPLGSLCRGILDLNTYNVVKAPQGKNQKSFVFILEPKQ
QGYPPVEFATDRVEELFEWFQSIREITWKIDTKENNMKYWEKNQSIAIELSDLVV
YCKPTSKTKDNLENPDFREIRSFVETKADSIIRQKPVDLLKYNQKGLTRVYPKGQ
RVDSSNYDPFRLWLCGSQMVALNFQTADKYMQMNHALFSLNGRTGYVLQPES
MRTEKYDPMPPESQRKILMTLTVKVLGARHLPKLGRSIACPFVEVEICGAEYDNN
KFKTTVVNDNGLSPIWAPTQEKVTFEIYDPNLAFLRFVVYEEDMFSDPNFLAHAT
YPIKAVKSGFRSVPLKNGYSEDIELASLLVFCEMRPVLESEEELYSSCRQLRRRQE
ELNNQLFLYDTHQNLRNANRDALVKEFSVNENQLQLYQEKCNKRLREKRVSNS
KFYS

[0064] The amino acid sequence of PLC-ε1a (SEQ ID NO:22) is:
MADLEVYKNLSPEKVERCMSVMQSGTQMIKLKRGTKGLVRLFYLDEHRTRLRW
RPSRKSEKAKILIDSIYKVTEGRQSEIFHRQAEGNFDPSCCFTIYHGNHMESLDLIT
SNPEEARTWITGLKYLMAGISDEDSLAKRQRTHDQWVKQTFEEADKNGDGLLNI
EEIHQLMHKLNVNLPRRKVRQMFQEADTDENQGTLTFEEFCVFYKMMSLRRDL
YLLLLSYSDKKDHLTVEELAQFLKVEQKMNNVTTDYCLDIIKKFEVSEENKVKN
VLGIEGFTNFMRSPACDIFNPLHHEVYQDMDQPLCNYYIASSHNTYLTGDQLLSQ
SKVDMYARVLQEGCRCVEVDCWDGPDGEPVVHHGYTLTSKILFRDVVETINKH
AFVKNEFPVILSIENHCSIQQQRKIAQYLKGIFGDKLDLSSVDTGECKQLPSPQSLK
GKILVKGKKLPYHLGDDAEEGEVSDEDSADEIEDECKFKLHYSNGTTEHQVESFI
RKKLESLLKESQIRDKEDPDSFTVRALLKATHEGLNAHLKQSPDVKESGKKSHGR
SLMTNFGKHKKTTKSRSKSYSTDDEEDTQQSTGKEGGQLYRLGRRRKTMKLCRE
LSDLVVYTNSVAAQDIVDDGTTGNVLSFSETRAHQVVQQKSEQFMIYNQKQLTR
IYPSAYRIDSSNFNPLPYWNAGCQLVALNYQSEGRMMQLNRAKFKANGNCGYV
LKPQQMCKGTFNPFSGDPLPANPKKQLILKVISGQQLPKPPDSMFGDRGEIIDPFV
EVEIIGLPVDCCKDQTRVVDDNGFNPVWEETLTFTVHMPEIALVRFLVWDHDPIG
RDFVGQRTVTFSSLVPGYRHVYLEGLTEASIFVHITINEIYGKWSPLILNPSYTILHF
LGATKNRQLQGLKGLFNKNPRHSSSENNSHYVRKRSIGDRILRRTASAPAKGRKK
SKMGFQEMVEIKDSVSEATRDQDGVLRRTTRSLQARPVSMPVDRNLLGALSLPV
SETAKDIEGKENSLVQI

[0065] The amino acid sequence of PLC-ε1b (SEQ ID NO:23) is:

MADLEVYKNLSPEKVERCMSVMQSGTQMIKLKRGTKGLVRLFYLDEHRTRLRW
RPSRKSEKAKILIDSIYKVTEGRQSEIFHRQAEGNFDPSCCFTIYHGNHMESLDLIT
SNPEEARTWITGLKYLMAGISDEDSLAKRQRTHDQWVKQTFEEADKNGDGLLNI
EEIHQLMHKLNVNLPRRKVRQMFQEADTDENQGTLTFEEFCVFYKMMSLRRDL
YLLLLSYSDKKDHLTVEELAQFLKVEQKMNNVTTDYCLDIIKKFEVSEENKVKN
VLGIEGFTNFMRSPACDIFNPLHHEVYQDMDQPLCNYYIASSHNTYLTGDQLLSQ
SKVDMYARVLQEGCRCVEVDCWDGPDGEPVVHHGYTLTSKILFRDVVETINKH
AFVKNEFPVILSIENHCSIQQQRKIAQYLKGIFGDKLDLSSVDTGECKQLPSPQSLK
GKILVKGKKLPYHLGDDAEEGEVSDEDSADEIEDECKFKLHYSNGTTEHQVESFI
RKKLESLLKESQIRDKEDPDSFTVRALLKATHEGLNAHLKQSPDVKESGKKSHGR
SLMTNFGKHKKTTKSRSKSYSTDDEEDTQQSTGKEGGQLYRLGRRRKTMKLCRE
LSDLVVYTNSVAAQDIVDDGTTGNVLSFSETRAHQVVQQKSEQFMIYNQKQLTR
IYPSAYRIDSSNFNPLPYWNAGCQLVALNYQSEGRMMQLNRAKFKANGNCGYV
LKPQQMCKGTFNPFSGDPLPANPKKQLILKVISGQQLPKPPDSMFGDRGEIIDPFV
EVEIIGLPVDCCKDQTRVVDDNGFNPVWEETLTFTVHMPEIALVRFLVWDHDPIG
RDFVGQRTVTFSSLVPGYRHVYLEGLTEASIFVHITINEIYGKWSPLILNPSYTILHF
LGATKNRQLQGLKGLFNKNPRHSSSENNSHYVRKRSIGDRILRRTASAPAKGRKK
SKMGFQEMVEIKDSVSEATRDQDGVLRRTTRSLQARPVSMPVDRNLLGALSLPV
SETAKDIEGKENSLAEDKDGRRKGKASIKDPHFLNFNKKLSSSSSALLHKDTSQG
DTIVSTAHMSVTGEQLGMSSPRGGRTTSNATSNCQENPCPSKSLSPKQHLAPDPV
VNPTQDLHGVKIKEKGNPEDFVEGKSILSGSVLSHSNLEIKNLEGNRGKGRAATS
FSLSDVSMLCSDIPDLHSTAILQESVISHLIDNVTLTNENEPGSSISALIGQFDETNN
QALTVVSHLHNTSVMSGHCPLPSLGLKMPIKHGFCKGKSKSSFLCSSPELIALSSS
ETTKHATNTVYETTCTPISKTKPDDDLSSKAKTAALESNLPGSPNTSRGWLPKSPT
KGEDWETLKSCSPASSPDLTLEDVIADPTLCFNSGESSLVEIDGESENLSLTTCEYR
REGTSQLASPLKLKYNQGVVEHFQRGLRNGYCKETLRPSVPEIFNNIQDVKTQSIS
YLAYQGAGFVHNHFSDSDAKMFQTCVPQQSSAQDMHVPVPKQLAHLPLPALKL
PSPCKSKSLGDLTSEDIACNFESKYQCISKSFVTTGIRDKKGVTVKTKSLEPIDALT
EQLRKLVSFDQEDNCQVLYSKQDANQLPRALVRKLSSRSQSRVRNIASRAKEKQ
EANKQKVPNPSNGAGVVLRNKPSAPTPAVNRHSTGSYIAGYLKNTKGGGLEGRG
IPEGACTALHYGHVDQFCSDNSVLQTEPSSDDKPEIYFLLRL

[0066] The amino acid sequence of PLC-ζ (SEQ ID NO:24) is:
MEMRWFLSKIQDDFRGGKINLEKTQRLLEKLDIRCSYIHVKQIFKDNDRLKQGRI
TIEEFRAIYRIITHREEIIEIFNTYSENRKILLASNLAQFLTQEQYAAEMSKAIAFEIIQ
KYEPIEEVRKAHQMSLEGFTRYMDSRECLLFKNECRKVYQDMTHPLNDYFISSSH
NTYLVSDQLLGPSDLWGYVSALVKGCRCLEIDCWDGAQNEPVVYHGYTLTSKL
LFKTVIQAIHKYAFMTSDYPVVLSLENHCSTAQQEVMADNLQATFGESLLSDML
DDFPDTLPSPEALKFKILVKNKKIGTLKETHERKGSDKRGDNQDKETGVKKLPGV
MLFKKKKTRKLKIALALSDLVIYTKAEKFKSFQHSRLYQQFNENNSIGETQARKL
SKLRVHEFIFHTRKFITRIYPKATRADSSNFNPQEFWNIGCQMVALNFQTPGLPMD
LQNGKFLDNGGSGYILKPHFLRESKSYFNPSNIKEGMPITLTIRLISGIQLPLTHSSS
NKGDSLVIIEVFGVPNDQMKQQTRVIKKNAFSPRWNETFTFIIHVPELALIRFVVE
GQGLIAGNEFLGQYTLPLLCMNKGYRRIPLFSRMGESLEPASLFVYVWYVR

[0067] The present invention also provides a conjugate of a PTD and a fragment, derivative or analogue of PL. Specifically, a conjugate of a PTD and a calcium binding domain of PL is contemplated. The calcium binding domain of PL, C2, is sensitive to Ca^{2+} activation. The C2 domains of the PLC enzymes can be found at or near the following amino acids regions: amino acids 630 to 755 of SEQ ID NO:11 (PLC-δ1) (SEQ ID NO:25), amino acids 661 to 787 of SEQ ID NO:13 (PLC-δ3) (SEQ ID NO:26), amino acids 628 to 754 of SEQ ID NO:15 (PLC-δ4) (SEQ ID NO:27), amino acids 677 to 794 of SEQ ID NO:16 (PLC-β1) (SEQ ID NO:28), amino acids 679 to 797 of SEQ ID NO:17 (PLC-β2) (SEQ ID NO:29), amino acids 728 to 843 of SEQ ID NO:18 (PLC-β3) (SEQ ID NO: 30), amino acids 702 to 818 of SEQ ID NO:19 (PLC-β4) (SEQ ID NO:31), amino acids 1092 to 1212 of SEQ ID NO:20 (PLC-γ1) (SEQ ID NO:32), amino acids 1062 to 1187 of SEQ ID NO:21 (PLC-γ2) (SEQ ID NO:33), amino acids 734 to 855 of SEQ ID NO:22 (PLC-ε1a) (SEQ ID NO:34), amino acids 734 to 855 of SEQ ID NO:23 (PLC-ε1b) (SEQ ID NO:35), and amino acids 483 to 606 of SEQ ID NO:24 (PLC-ζ) (SEQ ID NO:36). Additional calcium-binding domains of PL, other than C2, are also contemplated.

[0068] The peptide conjugates of the invention can be prepared by fusing a PTD-encoding gene with a PL gene and expressing the fusion protein *in vitro* or *in vivo* using standard cloning techniques and routine methods known to those having ordinary skill in the art.

[0069] The PTD-PL conjugate can be linked to each other by a direct covalent bond, a peptide bond, or a linker. Particularly, the PTD-PL conjugate can be linked to each other by a linker containing a region that is cleaved specifically by a certain enzyme. In one embodiment, the linker DNA encodes a protease recognition sequence thereby allowing cleavage at the junction of the PTD and the PL. For example, the linker DNA may encode a caspase-3 recognition sequence (*e.g.*, an amino acid sequence comprising DEVD (SEQ ID NO:37)). Linkers without a cleavage site (non-cleavage linkers) may also be used. The length of the linker is typically between 1 and 10 amino acids, preferably between 1 and 5 amino acids. The linker may contain the amino acids Gly, Gly-Gly or Gly-Gly-Gly.

[0070] The PTD-PL conjugate according to the present invention easily passes through the cellular membrane into cells due to the intracellular penetration and delivery effects of the PTD.

[0071] The use of PTD-PL mRNA for all of the above indications is also contemplated.

[0072] A further embodiment involves the use of inhibitors that prevent the degradation of PTD-PL when administered *in vivo*. In ischemic heart and hypoxic neonatal cardiomyocytes, PLC-δ1 is selectively degraded. Degradation of PLC-δ1 is completely inhibited by the calpain inhibitor, calpastatin, and the caspase inhibitor zVAD-fmk. Thus, an additional embodiment of the present invention is the use of a PTD-calpain- or PTD-caspase-inhibitor to prevent the degradation of PL, thereby rescuing intracellular Ca^{2+} overload induced by ischemic conditions. Such a PTD-inhibitor can be administered alone or in combination with the PTD-PL fusion protein of the present invention and/or other compounds.

Definitions

[0073] For convenience, certain terms used in the specification, examples, and appended claims are collected here. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

[0074] As used herein, by the term "hypoxia" is meant insufficient levels of oxygen in blood or tissue (*e.g.*, myocardial infarction). Hypoxia may be the result of a shortage in blood supply caused by, for example, an obstruction of a blood vessel.

[0075] As used herein, by the term "ischemia" is meant an inadequate flow or shortage of blood to a part of the body, caused by constriction, obstruction or blockage of the blood vessels supplying it. Ischemia leads to tissue hypoxia. Hypoxia or ischemic-related injury includes cardiac injury.

[0076] As used herein, by the term "reperfusion" is meant the restoration of the flow of blood to a previously ischemic tissue or organ that has had its blood supply cut off, as after a heart attack or stroke.

[0077] As used herein, by the term "necrosis" is meant the death of cells or tissues through injury or disease, particularly in a localized area of the body such as the myocardium.

[0078] As used herein, by the term "apoptosis" is meant programmed cell death.

[0079] As used herein, the term "cardiac injury" is intended to encompass any chronic or acute pathological event involving the heart and/or associated tissues (*e.g.*, the pericardium, aorta and other associated blood vessels), including, but not limited to, ischemia-reperfusion injury, congestive heart failure, cardiac arrest, myocardial infarction, cardiotoxicity caused by compounds such as drugs (*e.g.*, doxorubicin, herceptin, thioridazine and cisapride), cardiac damage due to parasitic infection, bacteria, fungi, rickettsiae, or viruses (*e.g.*, syphilis, chronic *Trypanosoma cruzi* infection), fulminant cardiac amyloidosis, heart surgery, heart transplantation, and traumatic cardiac injury (*e.g.*, penetrating or blunt cardiac injury, or aortic valve rupture).

[0080] As used herein, the term "neural injury" is intended to encompass any chronic or acute pathological event involving the brain, spinal column, nerves, and/or associated tissues, including, but not limited to, ischemia-reperfusion injury, neurotoxicity caused by compounds such as drugs, and neural damage due to parasitic infection.

[0081] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and comprises any chain or chains of two or more amino acids joined together by peptide bonds. Thus, as used herein, terms including, but not limited to "peptide," "dipeptide," "tripeptide," "protein," "amino acid chain," "oligopeptide," "oligomer," or any other term used to refer to a chain or chains of two or more amino acids, are included in the definition of a "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with any of these terms. The term further includes polypeptides which have undergone post-translational

modifications, for example, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. The term "protein" is also intended to include fragments, analogues and derivatives of a protein wherein the fragment, analogue or derivative retains essentially the same biological activity or function as a reference protein.

[0082] The "fragment, derivative or analogue" of the protein may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably, a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code; or (ii) one in which one or more of the amino acid residues includes a substituent group; or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half life of the polypeptide (for example, polyethylene glycol); or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence which is employed for purification of the polypeptide. Such fragments, derivatives and analogues are deemed to be within the scope of those skilled in the art from the teachings herein.

[0083] Particularly preferred are variants, analogues, derivatives and fragments having the amino acid sequence of the protein in which several, *e.g.*, 5 to 10, 1 to 5, 1 to 3, 2, or 1 amino acid residues are substituted, deleted or added in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the protein of the present invention. Also especially preferred in this regard are conservative substitutions.

[0084] An example of a variant of the present invention is a fusion protein as defined above, apart from the substitution of one or more amino acids with one or more other amino acids. The skilled person is aware that various amino acids have similar properties. One or more such amino acids of a substance can often be substituted by one or more other such amino acids without eliminating a desired activity of that substance.

[0085] Thus the amino acids glycine, alanine, valine, leucine and isoleucine can often be substituted for one another (amino acids having aliphatic side chains). Of these possible substitutions it is preferred that glycine and alanine are used to substitute for one another (since they have relatively short side chains) and that valine, leucine and isoleucine are

used to substitute for one another (since they have larger aliphatic side chains which are hydrophobic). Other amino acids which can often be substituted for one another include: phenylalanine, tyrosine and tryptophan (amino acids having aromatic side chains); lysine, arginine and histidine (amino acids having basic side chains); aspartate and glutamate (amino acids having acidic side chains); asparagine and glutamine (amino acids having amide side chains); and cysteine and methionine (amino acids having sulphur containing side chains). Substitutions of this nature are often referred to as "conservative" or "semi-conservative" amino acid substitutions.

[0086] The terms "fusion protein," "fusion polypeptide," "chimeric protein, and "chimeric polypeptide" as used herein are interchangeable and refer to polypeptides and proteins which comprise a polypeptide or protein of interest and a protein transduction domain (PTD).

[0087] The term PTD-PL "conjugate" as used herein refers to both the fusion of a PTD protein with a PL protein, as well as, the fusion of a PTD-encoding gene with a PL gene construct.

[0088] The terms "protein of interest", "desired polypeptide", "desired protein" or "target protein" as used herein are interchangeable and refer to a whole protein molecule or a portion thereof. The other portion of the polypeptide or protein is capable of inducing a cellular response.

[0089] As used herein, the term "therapeutic agent" refers to a molecule, such as a protein, lipid, carbohydrate, nucleic acid or chemical compound, which when delivered to a subject, treats, *i.e.*, cures, ameliorates, or lessens the symptoms of, a given disease or condition (*e.g.*, ischemia or hypoxia) in that subject, or alternatively, prolongs the life of the subject by slowing the progress of a terminal disease or condition.

[0090] As used herein, the term "therapeutic fusion polypeptide" refers to a polypeptide which when delivered to a subject, treats, *i.e.*, cures, ameliorates, or lessens the symptoms of, a given disease or condition (*e.g.*, ischemia or hypoxia) in that subject, or alternatively, prolongs the life of the subject by slowing the progress of a terminal disease or condition.

Polypeptides

[0091] The therapeutic polypeptides of the present invention are the phospholipase proteins (PLs), including but not limited to, the phospholipase C (PLC) polypeptides. PLCs are broadly classified into four kinds: β, γ, δ and ε, and are present as a total of eleven isozymes. PLC-δ1 plays a major role in ischemia and apoptosis by regulating the opening of mitochondrial permeability transition pores (mPTP), the targeting of proteases activated by calcium ions, and intracellular calcium homeostasis.

[0092] Also included as polypeptides of the present invention are fragments, derivatives, analogs, or variants of the foregoing polypeptides, and any combination thereof, which are used to prevent or treat, *i.e.*, cure, ameliorate, lessen the severity of, or prevent or reduce ischemic or hypoxic conditions and/or neural injury.

[0093] Further embodiments of the invention include polypeptides, which comprise amino acid sequences at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical, to any of the amino acid sequences of the polypeptides described above.

[0094] As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:10 can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acids in the reference sequence are allowed.

Polynucleotides

[0095] Additionally, the present invention relates to polynucleotides which encode fusion proteins or chimeric proteins, recombinant expression vectors, plasmids and other polynucleotide constructs (collectively referred to as "expression vectors") containing the same, microorganisms transformed with these expression vectors, and processes for

obtaining these polynucleotides, and transformed cells using said vectors. Suitable host cells can be transformed with the expression vectors.

[0096] As used herein, the term "expression vector" refers to a construct made up of genetic material (*i.e.*, nucleic acids). Typically, a expression vector contains an origin of replication which is functional in bacterial host cells, *e.g.*, *Escherichia coli*, and selectable markers for detecting bacterial host cells comprising the expression vector. Expression vectors of the present invention contain a promoter sequence and include genetic elements as described herein arranged such that an inserted coding sequence can be transcribed and translated in eukaryotic cells. In certain embodiments described herein, an expression vector is a closed circular DNA molecule.

[0097] The term "expression" refers to the biological production of a product encoded by a coding sequence. In most cases, a DNA sequence, including the coding sequence, is transcribed to form a messenger-RNA (mRNA). The messenger-RNA is then translated to form a polypeptide product which has a relevant biological activity. Also, the process of expression may involve further processing steps to the RNA product of transcription, such as splicing to remove introns, and/or post-translational processing of a polypeptide product.

[0098] The fusion proteins or chimeric proteins of this invention can be prepared by recombinant DNA methodology. In accordance with the present invention, a gene sequence coding for a desired protein is isolated, synthesized or otherwise obtained and operably linked to a DNA sequence coding for the PTD peptide. The hybrid gene containing the gene for a desired protein operably linked to a DNA sequence encoding a PTD peptide is referred to as a chimeric gene. Optionally, the gene sequence coding for a desired protein may be operably linked to the DNA sequence coding for the PTD peptide via a linker sequence.

[0100] The term "linker peptide" is intended to define any sequence of amino acid residues which preferably provide a hydrophilic region when contained in an expressed protein. Such a hydrophilic region may facilitate cleavage by an enzyme at the proteolytic cleavage site.

[0101] The chimeric gene is inserted into an expression vector which allows for the expression of the desired chimeric protein in a suitable transformed host. The expression

vector provides the inserted chimeric gene with the necessary regulatory sequences to control expression in the suitable transformed host.

[0102] The nucleic acid construct may be in the form of a vector, for example, an expression vector, and may include, among others, chromosomal, episomal and virus-derived vectors, for example, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculo-viruses, papova-viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. Generally, any vector suitable to maintain, propagate or express nucleic acid to express a polypeptide in a host, may be used for expression in this regard.

[0103] Regulatory elements that control expression of the fusion protein of the present invention include the promoter region, the 5' untranslated region, the signal sequence, the chimeric coding sequence, the 3' untranslated region, and the transcription termination site. Fusion proteins which are to be secreted from a host into the medium also contain the signal sequence.

[0104] Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These include, but are not limited to ribosome binding sites, and translation initiation and termination codons.

[0105] Methods and materials for preparing recombinant vectors and transforming host cells using the same, replicating the vectors in host cells and expressing biologically active foreign polypeptides and proteins are described in Principles of Gene Manipulation, by Old and Primrose, 2nd edition (1981), and Sambrook *et al.*, Molecular Cloning, 3rd edition, Cold Spring Harbor Laboratory (2001), both incorporated herein by reference.

[0106] As used herein, the term "DNA polynucleotide" may be a circular or linearized plasmid, or other linear DNA which may also be non-infectious and nonintegrating (*i.e.*, does not integrate into the genome of vertebrate cells). A linearized plasmid is a plasmid that was previously circular but has been linearized, for example, by digestion with a restriction endonuclease. Linear DNA may be advantageous in certain situations as discussed, *e.g.*, in Cherng, J.Y., *et al.*, *J. Control. Release 60*:343-53 (1999), and Chen,

Z.Y., *et al.*, *Mol. Ther.* *3*:403-10 (2001), both of which are incorporated herein by reference.

[0107] Further embodiments of the invention include vectors comprising chimeric genes, which comprise a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences of the vectors comprising chimeric genes described above.

[0108] Other embodiments of the invention include chimeric genes, which comprise a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences of the chimeric genes described above.

[0109] As a practical matter, whether any particular vector or chimeric gene is at least 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence according to the present invention, can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* *2*:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

Codon Optimization

[0110] "Codon optimization" is defined as modifying a nucleic acid sequence for enhanced expression in the cells of the subject of interest, *e.g.*, human, by replacing at least one, more than one, or a significant number, of codons of the native sequence with codons that are more frequently or most frequently used in the genes of that subject. Various species exhibit particular bias for certain codons of a particular amino acid.

[0111] In one aspect, the present invention relates to polynucleotide expression constructs or vectors, and host cells comprising nucleic acid fragments of codon-optimized coding

regions which encode therapeutic polypeptides, and fragments, variants, or derivatives thereof, and various methods of using the polynucleotide expression constructs, vectors, host cells to treat or prevent disease in a subject.

[0112] As used herein the term "codon-optimized coding region" means a nucleic acid coding region that has been adapted for expression in the cells of a given subject by replacing at least one, or more than one, or a significant number, of codons with one or more codons that are more frequently used in the genes of that subject.

[0113] Deviations in the nucleotide sequence that comprise the codons encoding the amino acids of any polypeptide chain allow for variations in the sequence coding for the gene. Since each codon consists of three nucleotides, and the nucleotides comprising DNA are restricted to four specific bases, there are 64 possible combinations of nucleotides, 61 of which encode amino acids (the remaining three codons encode signals ending translation). Many amino acids are designated by more than one codon. For example, the amino acids alanine and proline are coded for by four triplets, serine and arginine by six, whereas tryptophan and methionine are coded by just one triplet. This degeneracy allows for DNA base composition to vary over a wide range without altering the amino acid sequence of the proteins encoded by the DNA.

Consensus Sequences

[0114] The present invention is further directed to expression plasmids that contain chimeric genes which express therapeutic fusion proteins with specific consensus sequences, and fragments, derivatives and variants thereof. A "consensus sequence" is, *e.g.*, an idealized sequence that represents the amino acids most often present at each position of two or more sequences which have been compared to each other. A consensus sequence is a theoretical representative amino acid sequence in which each amino acid is the one which occurs most frequently at that site in the different sequences which occur in nature. The term also refers to an actual sequence which approximates the theoretical consensus. A consensus sequence can be derived from sequences which have, *e.g.*, shared functional or structural purposes. It can be defined by aligning as many known examples of a particular structural or functional domain as possible to maximize the homology. A sequence is generally accepted as a consensus when each particular amino acid is reasonably predominant at its position, and most of the sequences which form the

basis of the comparison are related to the consensus by rather few substitutions, *e.g.*, from 0 to about 100 substitutions. In general, the wild-type comparison sequences are at least about 50%, 75%, 80%, 90%, 95%, 96%, 97%, 98% or 99% identical to the consensus sequence. Accordingly, polypeptides of the invention are about 50%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the consensus sequence.

[0115] A "consensus amino acid" is an amino acid chosen to occupy a given position in the consensus protein. A system which is organized to select consensus amino acids can be a computer program, or a combination of one or more computer programs with "by hand" analysis and calculation. When a consensus amino acid is obtained for each position of the aligned amino acid sequences, then these consensus amino acids are "lined up" to obtain the amino acid sequence of the consensus protein.

Therapeutic Uses

[0116] Contemplated is the use of the therapeutic fusion proteins described above in the manufacture of a medicament for the alleviation and treatment of ischemic diseases or conditions and/or neural injury. Ischemic diseases or conditions leading to hypoxia in the heart and the brain can be effectively alleviated by administration of the PTD-PLC fusion protein.

[0117] Generally, the influx of calcium ions during an ischemic event results in the opening of mitochondrial pores thereby further increasing intracellular concentration of calcium and activation a number of cytoplasmic proteins, such as proteases and endonucleases. Calcium-activated proteases degrade proteins which normally regulate the intracellular calcium level, thereby reducing the reactivity of the proteins with calcium. This leads to myocardial hypertrophy, heart failure, apoptosis or necrosis. PLC-δ1 functions to inhibit the inflow of calcium, when an excess of calcium is present in the cytoplasm.

[0118] The therapeutic fusion proteins of the invention may be coadministered with one or more compounds or constructs. Other compounds include, but are not limited to, anti-platelet drugs, anti-coagulant drugs, or anti-thrombotic drugs. Other constructs include, but are not limited to, PTD-calpain or PTD-caspase inhibitors to prevent the degradation of PLC.

[0119] The therapeutic fusion proteins of the invention may be targeted to the following cells or cell types: cardiovascular cells, such as cardiac myocyte, ventricular myocyte, atrial myocyte, cardiac stem cell, endothelial cell, vascular smooth muscle cell, pacemaker cell, myofibroblast or fibroblast, and neural cells, such as neurons (also called nerve cell or neurocyte).

Methods and Administration

[0120] The present invention provides methods for delivery of a therapeutic fusion polypeptide, or a fragment, variant, or derivative thereof, in admixture with one or more pharmaceutically acceptable carriers or excipients. The therapeutic fusion polypeptide is provided as a recombinant protein, in particular, a fusion protein, or a purified subunit, which comprises administering to a subject one or more of the compositions described herein; such that upon administration of compositions such as those described herein, a therapeutic response is generated in a subject. The delivery can occur, for example, through the skin, nose, eye, into muscle, brain or heart, or by intravenous injection.

[0121] The term "subject" is intended to encompass living organisms such as humans, monkeys, cows, sheep, horses, pigs, cattle, goats, dogs, cats, mice, rats, cultured cells therefrom, and transgenic species thereof. In a preferred embodiment, the subject is a human.

[0122] The term "vertebrate" is intended to encompass a singular "vertebrate" as well as plural "vertebrates" and comprises mammals and birds, as well as fish, reptiles, and amphibians.

[0123] The term "mammal" is intended to encompass a singular "mammal" and plural "mammals," and includes, but is not limited to humans; primates such as apes, monkeys (*e.g.*, owl, squirrel, cebus, rhesus, African green, patas, cynomolgus, and cercopithecus), orangutans, baboons, gibbons, and chimpanzees; canids such as dogs and wolves; felids such as cats, lions, and tigers; equines such as horses, donkeys, and zebras, food animals such as cows, pigs, and sheep; ungulates such as deer and giraffes; ursids such as bears; and others such as rabbits, mice, ferrets, seals, whales. In particular, the mammal can be a human subject, a food animal or a companion animal.

[0124] The term "bird" is intended to encompass a singular "bird" and plural "birds," and includes, but is not limited to feral water birds such as ducks, geese, terns, shearwaters,

and gulls; as well as domestic avian species such as turkeys, chickens, quail, pheasants, geese, and ducks. The term "bird" also encompasses passerine birds such as starlings and budgerigars.

[0125] The present invention further provides a method for generating, enhancing or modulating a therapeutic response comprising administering to a human one or more of the compositions described herein. In this method, the compositions may include one or more polypeptides, or a fragment, variant, or derivative thereof, wherein the protein is provided as a recombinant protein, in particular, a fusion protein, or a purified subunit.

[0126] As used herein, a "therapeutic response" refers to the ability of a subject to elicit a positive reaction to a composition, as disclosed herein, when delivered to that subject.

[0127] As mentioned above, compositions of the present invention can be used to therapeutically treat and prevent disease or disease conditions. As defined herein, "treatment " refers to the use of one or more compositions of the present invention to prevent, cure, retard, or reduce the severity of a disease or disease symptoms in a subject, and/or result in no worsening of the disease.

[0128] The diseases or disease conditions caused by or leading to ischemia/hypoxia that are contemplated as part of this invention include, but are not limited to, calcium overload, cardiac hypoxia, cardiac hypoxia-reoxygenation, cardiac ischemia-reperfusion injury, ischemic heart disease, heart failure, heart hypertrophy, heart surgery, traumatic heart injury, coronary angioplasty, vascular defects or blockages (obstruction of blood flow), congenital heart disease, congestive heart failure, cardiac cell muscle regeneration, chemotherapeutic induced cardiomyophathy, myocardial infarction, cardiac arrest, cardiotoxicity, cardiac damage due to parasitic infection, fulminant cardiac amyloidosis, cardiac transplantation, or traumatic cardiac injury.

[0129] Additional diseases or disease conditions caused by or leading to ischemia/hypoxia that are contemplated as part of this invention include, but are not limited to, traumatic brain injury, neurological disease or injury, neural disease or injury (*e.g.*, spinal cord), frost damage, ischemic or hemorrhagic stroke, intracranial bleedings (subarachnoid hemorrhage, thrombolytica-induced etc.), blood clots, hypoxia-induced apoptosis, and tissue damage following ischemia-reperfusion.

[0130] The term "prevention" refers to the use of one or more compositions of the present invention to generate a therapeutic responses in a subject. It is not required that any composition of the present invention totally cure or eliminate all disease symptoms.

[0131] In certain embodiments, one or more compositions of the present invention are delivered to a subject by methods described herein, thereby achieving an effective therapeutic response. More specifically, the compositions of the present invention may be administered to any tissue of a subject, including, but not limited to, skin, muscle, brain tissue, lung tissue, liver tissue, spleen tissue, bone marrow tissue, thymus tissue, heart tissue, *e.g.*, myocardium, endocardium, and pericardium, lymph tissue, blood tissue, bone tissue, pancreas tissue, kidney tissue, gall bladder tissue, stomach tissue, intestinal tissue, testicular tissue, ovarian tissue, uterine tissue, vaginal tissue, rectal tissue, nervous system tissue, eye tissue, glandular tissue, tongue tissue, and connective tissue, *e.g.*, cartilage. The preferred tissues are heart and brain tissue.

[0132] Furthermore, the compositions of the present invention may be administered to any internal cavity of a subject, including, but not limited to, the lungs, the mouth, the nasal cavity, the stomach, the peritoneal cavity, the intestine, any heart chamber, veins, arteries, capillaries, lymphatic cavities, the uterine cavity, the vaginal cavity, the rectal cavity, joint cavities, ventricles in brain, spinal canal in spinal cord, the ocular cavities, the lumen of a duct of a salivary gland or a liver. When the compositions of the present invention is administered to the lumen of a duct of a salivary gland or liver, the desired polypeptide is expressed in the salivary gland and the liver such that the polypeptide is delivered into the blood stream of the subject from each of the salivary gland or the liver. Certain modes for administration to secretory organs of a gastrointestinal system using the salivary gland, liver and pancreas to release a desired polypeptide into the bloodstream is disclosed in U.S. Patent Nos. 5,837,693 and 6,004,944, both of which are incorporated herein by reference in their entireties.

[0133] According to the disclosed methods, compositions of the present invention

[0134] According to the disclosed methods, compositions of the present invention can be administered by injection, intravenous (i.v.), intramuscular (i.m.), subcutaneous (s.c.), or intrapulmonary routes. Other suitable routes of administration include, but are not limited to intratracheal instillation, transdermal, intraocular, intranasal, inhalation, intracavity, intraductal (*e.g.*, into the pancreas) and intraparenchymal (*i.e.*, into any tissue)

administration. For intravenous administration, appropriate pharmaceutically acceptable carriers can be used, such as phosphate buffered saline, saline, or other materials used for administration of drugs intravenously. Transdermal delivery includes, but is not limited to intradermal (*e.g.*, into the dermis or epidermis), transdermal (*e.g.*, percutaneous) and transmucosal administration (*i.e.*, into or through skin or mucosal tissue). Intracavity administration includes, but is not limited to administration into oral, vaginal, rectal, nasal, peritoneal, or intestinal cavities as well as, intrathecal (*i.e.*, into the spinal canal), intraventricular (*i.e.*, into the brain ventricles or the heart ventricles), intra-atrial (*i.e.*, into the heart atrium) and sub arachnoid (*i.e.*, into the sub arachnoid spaces of the brain) administration.

[0135] Any mode of administration can be used so long as the mode results in delivery or the expression of the desired peptide or protein, in the desired tissue, in an amount sufficient to generate a therapeutic response to a disease condition in a human in need of such a response.

[0136] Administration means of the present invention include needle injection (for example as a sterile aqueous dispersion, preferably isotonic), transdermal, catheter infusion, biolistic injectors, particle accelerators (*e.g.*, "gene guns" or pneumatic "needleless" injectors) Med-E-Jet (Vahlsing, H., *et al.*, *J. Immunol. Methods 171*:11-22 (1994)), Pigjet (Schrijver, R., *et al.*, *Vaccine 15*:1908-1916 (1997)), Biojector (Davis, H., *et al.*, *Vaccine 12*:1503-1509 (1994); Gramzinski, R., *et al.*, *Mol. Med. 4*:109-118 (1998)), AdvantaJet (Linmayer, I., *et al.*, *Diabetes Care 9*:294-297 (1986)), Medi-jector (Martins, J., and Roedl, E. J., *Occup. Med. 21*:821-824 (1979)), gelfoam sponge depots, other commercially available depot materials (*e.g.*, hydrogels), osmotic pumps (*e.g.*, Alza minipumps), oral or suppositorial solid pharmaceutical formulations, such as tablets, pills, soft and hard capsules, liquids, suspensions, syrups, granules and elixers, topical skin creams or gels, and decanting, use of polynucleotide coated suture (Qin, Y., *et al.*, *Life Sciences 65*:2193-2203 (1999)) or topical applications during surgery.

[0137] Certain modes of administration are intramuscular needle-based injection and pulmonary application via catheter infusion. Energy-assisted plasmid delivery (EAPD) methods may also be employed to administer the compositions of the invention. One such method involves the application of brief electrical pulses to injected tissues, a procedure commonly known as electroporation. *See generally* Mir, L.M., *et al.*, *Proc.*

Natl. Acad. Sci USA 96:4262-7 (1999); Hartikka, J., *et al.*, *Mol. Ther. 4*:407-15 (2001); Mathiesen, I., *Gene Ther. 6*:508-14(1999); Rizzuto G., *et al.*, *Hum. Gen. Ther. 11*:1891-900 (2000). Each of the references cited in this paragraph is incorporated herein by reference in its entirety.

[0138] Determining an effective amount of one or more compositions of the present invention depends upon a number of factors including, for example, the fusion polypeptide, variants, or derivatives thereof being expressed or administered directly, the age, weight and sex of the subject, the precise condition requiring treatment and its severity, the route of administration, the *in vivo* half-life of the fusion polypeptide, the efficiency of uptake, and the area to be treated. Treatment can be repeated as necessary, based on clinical judgment, in view of patient response.

[0139] A "pharmaceutically effective amount" or a "therapeutically effective amount" is an amount sufficient to generate a therapeutic or clinical response to a disease condition. The terms "pharmaceutically effective amount" or a "therapeutically effective amount are interchangeable. Based on the above factors, determining the precise amount, number of doses, and timing of doses are within the ordinary skill in the art and will be readily determined by the attending physician or veterinarian.

[0140] For administration to mammals, and particularly humans, it is expected that the daily dosage of the active agent will be from 0.01 mg/kg body weight, typically around 1 mg/kg. The above dosages are exemplary of the average case. There can, of course, be instances where higher or lower dosages are merited, including picomolar and nanomolar concentrations, and such are within the scope of this invention.

[0141] The present invention also relates to compositions comprising the fusion polypeptide(s), as disclosed herein, and an additional pharmaceutically active agent. The fusion polypeptide(s) and associated pharmaceutically active agent may be employed in combination with pharmaceutically acceptable one or more carriers or excipients. Such carriers may include, but are not limited to, diluents (*e.g.*, lactose, dextrose, sucrose, mannitol, sorbitol, cellulose and/or glycine), lubricants (*e.g.*, silica, talc, stearic acid and polyethylene glycol), binders (*e.g.*, magnesium aluminum silicate, starch paste, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose and/or polyvinylpyrrolidone), and disintegrants, such as starches, agar, alginic acid, or its sodium

salt, and/or absorbents, colorants, flavors, and sweeteners, saline, buffered saline, liposomes, water, glycerol, ethanol and combinations thereof.

[0142] Compositions of the present invention may be solubilized in any of various buffers. Suitable buffers include, for example, phosphate buffered saline (PBS), normal saline, Tris buffer, and sodium phosphate (*e.g.*, 150 mM sodium phosphate). Insoluble polynucleotides may be solubilized in a weak acid or weak base, and then diluted to the desired volume with a buffer. The pH of the buffer may be adjusted as appropriate. In addition, a pharmaceutically acceptable additive can be used to provide an appropriate osmolarity. Such additives are within the purview of one skilled in the art. For aqueous compositions used in vivo, sterile pyrogen-free water can be used. Such formulations will contain an effective amount of a polynucleotide together with a suitable amount of an aqueous solution in order to prepare pharmaceutically acceptable compositions suitable for administration to a human.

[0143] Compositions of the present invention can be formulated according to known methods. Suitable preparation methods are described, for example, in *Remington's Pharmaceutical Sciences*, 16th Edition, A. Osol, ed., Mack Publishing Co., Easton, PA (1980), and *Remington's Pharmaceutical Sciences*, 19th Edition, A.R. Gennaro, ed., Mack Publishing Co., Easton, PA (1995), both of which are incorporated herein by reference in their entireties. Although the composition may be administered as an aqueous solution, it can also be formulated as an emulsion, gel, solution, suspension, lyophilized form, or any other form known in the art.

[0144] The following examples are included for purposes of illustration only and are not intended to limit the scope of the present invention, which is defined by the appended claims. All references cited in the Examples are incorporated herein by reference in their entireties.

EXAMPLES

EXAMPLE 1

Preparation of expression vector containing PTD-PLC-δ1

[0145] In order to link a base sequence encoding HSP-70 with a base sequence encoding a peptide region from 858th amino acid tyrosine to 868th amino acid from the N-terminus

of human transcription factor Hph-1 (GenBank Accession No: U63386), the primers having the following base sequences were synthesized: a base sequence corresponding to restriction enzyme *Bam*HI for cloning into a pET28B(+) vector having a base sequence from 858th amino acid tyrosine to 868th amino acid arginine from the N-terminus of Hph-1; and a base sequence corresponding to restriction enzyme *Hind*III for cloning with sequences corresponding to the 5'-terminus of said base sequence and the 3'-terminus of PTD-PLC-δ1. PCR was performed using the above primers, a pRS vector (commercially available from Invitrogen) containing the whole gene of the PTD-PLC-δ1 protein, as a template, and *pfu* turbo DNA polymerase (Stratagene, cat.# 600252-51).

[0146] The PCR reaction product was cut with restriction enzymes *Eco*RI and *Hind*III, and purified with the Quiaquick PCR purification kit (QIAGEN, cat.# 28104). The purified product was cloned into the *Bam*HI and *Hind*III sites of pET28B (+) purified using a gel extraction method, to prepare a recombinant expression vector. The prepared recombinant vector was named "pPTD-PLC-δ1."

EXAMPLE 2

Preparation of *E. coli* transformants and expression and purification of fusion protein

[0147] *E. coli* BL21-DE3 (ATCC No. 53863) was transformed with the expression vector pPTD-PLC-δ1 prepared in Example 1, by heat shock transformation, and the transformed *E. coli* strain was inoculated into 4 ml of LB medium and pre-cultured at 37 ℃ for 14 hours through stirring. Then, the pre-culture medium was inoculated into 250 ml of LB medium (10 g casein pancreatic digest, 5 g yeast extract, 10 g sodium chloride), and cultured at 37 °C for 3 hours. Then, 1 mM IPTG (isopropyl β-D-thiogalactopyranoside; GibcoBRL cat.# 15529-019) was added to the culture medium, and the mixture was cultured at 22 °C for 8 hours to induce the expression of a fusion protein. The culture medium was centrifuged at 4 °C and 6,000 rpm for 20 minutes, and the supernatant was removed, leaving pellets. The pellets were dissolved in 10 ml of buffer solution 1 (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and sonicated with an ultrasonic processor (Heat systems, ultrasonic processor XL) on ice at an intensity of 300 W for 6 seconds and then cooled. The sonication and cooling steps were repeated such that the total sonication time reached 8 minutes. The lysate was centrifuged at 4 °C and

12,000 rpm for 10 minutes, and the disrupted *E. coli* cells were removed and only a pure lysate was collected. To the collected lysate, 0.5 ml of 50% Ni^{2+}-NTA agarose slurry (Qiagen, cat# 30230) was added, and the suspension was stirred at 4 °C at 200 rpm for 1 hour, such that the fusion protein and the Ni^{2+}-NTA agarose were bound to each other. The mixture was passed through a 0.8 x 4 cm chromatography column (BioRad, cat.# 731-1550). The resulting material was washed two times with 4 ml of buffer solution 2 (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.9), and treated with 1 ml of buffer solution 3 (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and 1 ml of buffer solution 4 (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0), thus obtaining a fusion protein fraction. The fraction was desalted with a PD-10 desalting column (Amersham-Pharmacia Biotech cat.# 17-0851-01). The isolated and purified PTD-PLC-δ1 fusion protein was subjected to SDS-PAGE, and then analyzed by Coomassie blue staining, and the results are shown in FIG. 1.

EXAMPLE 3

PTD-PLC-δ1 prevents ischemia in myocardial cells

[0148] The heart of each of 1-3-day-old white rats was separated and placed in PBS, and only the left ventricle was separated and then cut with micro dissecting scissors to a size of about 1 mm^3, to which 5 ml of collagenase II (0.8 mg/ml, 262 units/mg, Gibco BRL) was added. The suspension was left to stand in a 5% CO_2 incubator at 37 °C for 5 minutes, and the floated collagenase II was removed. Five ml of fresh collagenase II was added thereto and suspended, and the suspension was left to stand for an additional time of 5 minutes, and the supernatant was then transferred into a fresh tube. Five ml of a cell culture medium (10% FBS-containing α-MEM, Gibco BRL) was added thereto, the cell solution was centrifuged at 1200 rpm for 4 minutes, and the cells were collected. The above procedure was repeated 7-9 times until almost no tissue was remaining, and the cell suspensions separated as single cells were collected in one tube. The cells were cultured on a 100-mm tissue culture plate for 1-3 hours to allow only fibroblasts to adhere to the plate, and cells non-adhered to plate were collected, seeded at a concentration of 5×10^5 cells/ml and cultured. After 4-6 hours, it was replaced with fresh medium, 0.1 mM BrdU was then added thereto to inhibit the growth of fibroblasts and, at the same time, various

concentrations (0 μM for a control group, and 0.1 μM, 0.5 μM and 1.0 μM concentrations for test groups) of the PTD-PLC-δ1 protein were added to the cultured myocardial cells. The cells were then cultured in low-oxygen conditions for 12 hours. The culturing in low-oxygen conditions was carried out in an airtight humidified chamber (Anaerobic Environment, ThermoForma, Marietta, OH, USA), which was maintained at 37 °C and continuously supplied with a mixed gas of 10% CO_2, 5% H_2 and 85% N_2. As medium in the culture step, a medium containing only 1% bovine fetal serum was used. The cultured cells were analyzed by Western blot using an anti-PTD-PLC-δ1 antibody.

[0149] The results show that the delivery effect of the PTD-PLC-δ1 protein increased in a concentration-dependent manner. It can be seen that the PTD-PLC-δ1 protein in the myocardial cells cultured under low-oxygen conditions for 12 hours remained with a concentration gradient of 0-500 nM, and the cell viability increased according to the concentration gradient of the remaining PTD-PLC-δ1 protein (*see* FIG. 2A). Also, the cell viability increased according to the concentration gradient of the remaining PTD-PLC-δ1 protein (*see* FIG. 2B).

EXAMPLE 4

PTD-PLC-δ1 inhibits calcium overload in ischemic myocardial cells

[0150] The measurement of the concentration of free Ca^{2+} in the cytoplasm was performed by confocal microscope analysis. To myocardial cells under low-oxygen conditions, the purified PTD-PLC-δ1 was added at a concentration of 1 μM, and the cells were then cultured for 12 hours and measured for the concentration of calcium. For this purpose, on a glass thin section coated with laminin (5 g/cm^2), the myocardial cells of newly born white rats were cultured in 0.1 mM BrdU-containing cell culture medium (10% FBS-containing-MEM, Gibco BRL) for one day. After completion of the culture, the cells were washed with a modified Tyrode's solution consisting of 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1 g/l glucose, 0.05 g/l NaH_2PO_4, and 1.0 g/l $NaHCO_3$. The modified Tyrode's solution was loaded with 2 μM fluo-4 acetoxymethyl ester (Fluo-4 AM, Molecular Probes, Eugene, OR) in a dark room at room temperature for 20 minutes. The fluorescent images were acquired with an argon laser confocal microscope (Leica, Solms, Germany). The fluorochromes were excited using the argon

laser turned at 488 nm, and the emission of fluorescence was collected through a 510-560 nm band pass filter. Also, the relative change in intracellular free Ca^{2+} was determined by the measurement of fluorescent intensity.

[0151] The results show that an overload of calcium in the myocardial cells cultured under low-oxygen conditions for 12 hours was about 3 times higher than in a control group incubated under oxygen conditions. However, when the myocardial cells under low-oxygen conditions were treated with 100 nM of the PTD-PLC-δ1 protein, the intracellular calcium overload was restored to a level approximately equal to that of the control group (*see* FIG. 3).

EXAMPLE 5

Effect of PTD-PLC-δ1 on treatment of cardiac ischemic area in myocardial infarct animal model

[0152] Myocardial infarct in white rats was induced by reperfusion after left anterior descending coronary artery ligation. Under general anesthesia, 8-week-old Sprague-Dawley male rats (weighing about 205 g each) were intubated with an endotracheal tube and then ventilated with positive pressure (180 ml/min), and the ventilation was maintained with oxygen-containing (2 L/min) indoor air using a Harvard ventilator. The rat heart was exposed by left thoracotomy, and the left anterior descending coronary artery was ligated with 5-0 silk suture, followed by standing for 1 hour. After the left anterior descending coronary artery was ligated for 1 hour, 100 nM PTD-PLC-δ1 was intravenously injected into the rat and, at the same time, the ligation was released.

[0153] In one group (n=3), to determine the amount of PLC-δ1 in the cardiac ischemic area, the reperfusion was performed for 3 hours, and the residual amount of PLC-δ1 protein in the tissue was analyzed. In another group (n=3), to determine the effect of PTD-PLC-δ1 on the survival of myocardial cells in the ischemic area, the reperfusion was continued for 2 weeks following the release of the ligation. Upon release of the ligation, the animals were bred in a breeding room in its normal environment for 2 weeks, after which the heart was isolated.

[0154] The isolated heart was perfused with 10% (v/v) neutrally buffered formaldehyde, fixed in the formaldehyde, transversely cut into four sections having the same thickness, and then embedded in paraffin according to a general method. The 2-μm thick sections

were placed on a gelatin-coated glass slide to enable dyes to work on the continuous section of the transplanted tissue area. After paraffin removal and rehydration, the sections were stained with haematoxylin and eosin in order to observe cytological details, such as nuclei, cytoplasm, and connective tissues.

[0155] The analysis of the amount of PLC-δ1 revealed that in the kidney and liver, the test group injected with PTD-PLC-δ1 was no different than the control group. However, in the heart, the injected PTD-PLC-δ1 protein was present in a significant amount, and the phosphorylation of protein kinase C was greatly increased (*see* FIG. 4).

[0156] The analysis of the effect of PTD-PLC-δ1 on the survival of myocardial cells revealed that the survival rate of the myocardial cells in the group injected intravenously with PTD-PLC-δ1 was significantly higher than that of the control group (*see* FIG. 5).

[0157] It is to be appreciated that the Detailed Description section, and not the Summary and Abstract sections, is intended to be used to interpret the claims. The Summary and Abstract sections may set forth one or more but not all exemplary embodiments of the present invention as contemplated by the inventor(s), and thus, are not intended to limit the present invention and the appended claims in any way.

What Is Claimed Is:

1. A fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C polypeptide.

2. The fusion polypeptide of claim 1, wherein the phospholipase C polypeptide comprises the amino acid sequence of SEQ ID NO:11.

3. The fusion polypeptide of claim 1, wherein the phospholipase C polypeptide comprises the amino acid sequence of SEQ ID NO:13.

4. The fusion polypeptide of claim 1, wherein the phospholipase C polypeptide comprises the amino acid sequence of SEQ ID NO:15.

5. The fusion protein of claim 1, wherein the protein transduction domain (PTD) comprises an amino acid sequence selected from the group consisting of:

(i) SEQ ID NO:1;

(ii) SEQ ID NO:2;

(iii) SEQ ID NO:3;

(iv) SEQ ID NO:4;

(v) SEQ ID NO:5;

(vi) SEQ ID NO:6;

(vii) SEQ ID NO:7;

(viii) SEQ ID NO:8; and

(ix) SEQ ID NO:9.

6. The fusion protein of claim 1, wherein the PTD and the phospholipase C polypeptide are linked to each other by a direct covalent bond, a peptide bond, or a linker.

7. The fusion protein of claim 6, wherein the linker is a non-cleavage linker comprising 1 to 5 amino acids.

8. The fusion protein of claim 7, wherein the linker comprises Gly-Gly-Gly.

9. The fusion protein of claim 6, wherein the linker is a cleavage linker.

10. A pharmaceutical composition comprising the fusion protein of claim 1 in admixture with one or more pharmaceutically acceptable excipients.

11. The pharmaceutical composition of claim 10, further comprising at least one anti-platelet drug, anti-coagulant drug, or anti-thrombotic drug.

12. A fusion polypeptide comprising a protein transduction domain (PTD) and an amino acid sequence at least 90% identical to a phospholipase C polypeptide.

13. A method of reducing or preventing calcium overload in a subject, comprising administering to a subject in need thereof a therapeutically effective amount of a fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C polypeptide.

14. The method of claim 13, wherein the phospholipase C polypeptide is a phospholipase C-δ polypeptide.

15. The method of claim 13, wherein the calcium overload is caused by hypoxia, ischemia, cardiovascular disease, cardiovascular injury, neurological disease, or neurological injury.

16. The method of claim 15, wherein the hypoxia or ischemia is the result of a shortage in blood supply.

17. The method of claim 16, wherein the shortage in blood supply is the result of the obstruction of a blood vessel.

18. The method of claim 13, wherein the calcium overload is in the heart or the brain.

19. The method of claim 15, wherein the hypoxia is caused by a myocardial infarction.

20. The method of claim 13, wherein the method reduces or inhibits neural cell death.

21. The method of claim 20, wherein the neural cell is a neuron.

22. The method of claim 15, wherein the neurological disease is ischemic or hemorrhagic stroke.

23. The method of claim 15, wherein the method reduces or inhibits hypoxia-induced cell death of a cardiovascular cell.

24. The method of claim 23, wherein the cardiovascular cell is a cardiac myocyte, ventricular myocyte, atrial myocyte, cardiac stem cell, endothelial cell, vascular smooth muscle cell, pacemaker cell, myofibroblast or fibroblast.

25. The method of claim 15, wherein the cardiovascular disease or injury is selected from the group consisting of: cardiac hypoxia, cardiac hypoxia-reoxygenation, cardiac post-ischemic reperfusion, ischemic heart disease, heart failure, heart hypertrophy, heart surgery, coronary angioplasty, vascular defects, congenital heart defects, congestive heart failure, cardiac cell muscle regeneration, chemotherapy-induced cardiomyopathy, myocardial infarction, cardiac arrest, cardiotoxicity, cardiac damage due to parasitic infection, fulminant cardiac amyloidosis, cardiac transplantation, and traumatic cardiac injury.

26. The method of claim 25, wherein said fusion polypeptide is administered prior to post-ischemic reperfusion, at the onset of post-ischemic reperfusion, or during post-ischemic reperfusion.

27. The method of claim 25, wherein the cardiovascular injury is a myocardial infarction.

28. The method of claim 25, wherein the cardiotoxicity is caused by doxorubicin.

29. The method of claim 25, wherein the cardiac damage due to parasitic injection is from syphilis or chronic *Trypanosoma cruzi* infection.

30. The method of claim 25, wherein the traumatic cardiac injury is via penetrating or blunt cardiac injury, or aortic valve rupture.

31. The method of claim 25, wherein the onset of stroke is prevented in a subject suffering from heart failure.

32. The method of claim 13, wherein calcium overload is restricted to a body part of said subject.

33. The method of claim 32, wherein the body part is brain, spinal cord, heart, or transplanted organ or limb.

34. The method of claim 13, wherein the fusion polypeptide is applied in picomolar concentration.

35. The method of claim 13, wherein the fusion polypeptide is administered intravenously.

36. The method of claim 35, wherein the administration is targeted to the heart.

37. The method of claim 13, wherein the fusion polypeptide is administered by injection.

38. The method of claim 37, wherein the injection is directly into heart or muscle tissue.

39. The method of claim 13, wherein the fusion polypeptide is administered transdermally.

40. The method of claim 20, wherein the cell death is necrotic or apoptotic.

41. A method of preventing ischemia-reperfusion injury in a subject suffering from hypothermia, comprising pre-treating the subject with a therapeutically effective amount of a fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C polypeptide.

42. The method of claim 41, wherein the phospholipase C polypeptide is a phospholipase C-δ polypeptide.

43. A method of reducing the concentration of free calcium ions in a cell, comprising administering to a cell an effective amount of a fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C polypeptide.

44. The method of claim 43, wherein the phospholipase C polypeptide is a phospholipase C-δ polypeptide.

45. A method of preventing organ or tissue damage during organ or tissue transplantation, comprising contacting a fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C polypeptide with one or more organs or tissues before, during, or after reperfusion.

46. The method of claim 45, wherein the phospholipase C polypeptide is a phospholipase C-δ polypeptide.

47. The method of claim 45, wherein said contacting is by administering to an organ donor a fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C polypeptide prior to, or concurrent with, removal of the organ.

48. The method of claim 45, wherein the organ is the donor heart.

49. A method of reducing or inhibiting hypoxia-induced cell death of a cardiovascular cell, comprising intravenously administering to a subject in need thereof a therapeutically effective amount of a fusion polypeptide comprising a protein transduction domain (PTD) and a phospholipase C-δ1 polypeptide, wherein the hypoxia is caused by a myocardial infarction, and wherein said administration is prior to post-ischemic reperfusion, at the onset of post-ischemic reperfusion, or during post-ischemic reperfusion.

50. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:16.

51. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:17.

52. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:18.

53. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:19.

54. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:20.

55. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:21.

56. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:22.

57. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:23.

58. The fusion polypeptide of claim 1, wherein the phospholipase polypeptide comprises the amino acid sequence of SEQ ID NO:24.

59. A fusion polypeptide comprising a protein transduction domain (PTD) and a calcium-binding domain (C2) of a phospholipase C polypeptide.

60. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:25.

61. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:26.

62. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:27.

63. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:28.

64. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:29.

65. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:30.

66. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:31.

67. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:32.

68. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:33.

69. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:34.

70. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:35.

71. The fusion polypeptide of claim 59, wherein the calcium-binding domain comprises the amino acid sequence of SEQ ID NO:36.

72. The fusion polypeptide of claim 9, wherein the linker comprises the amino acid sequence of SEQ ID NO:37.

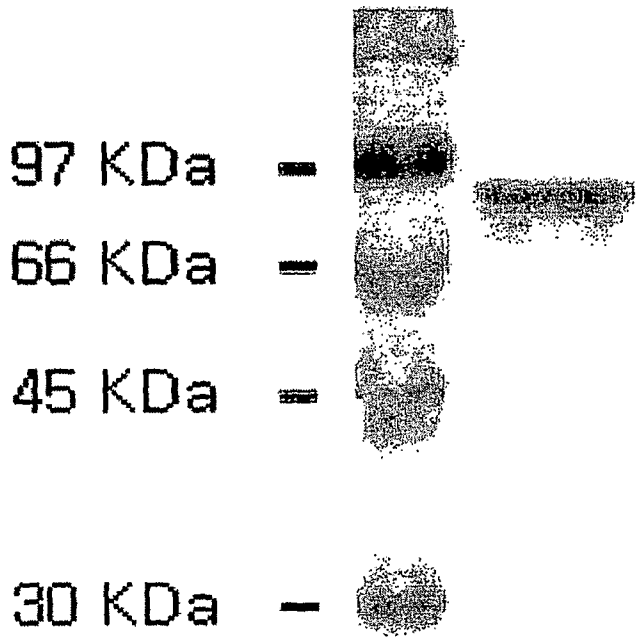

FIG. 1

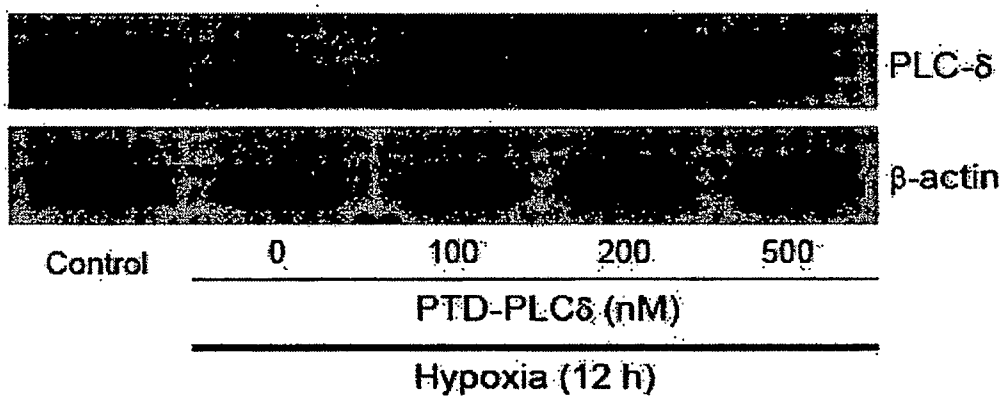

FIG. 2A

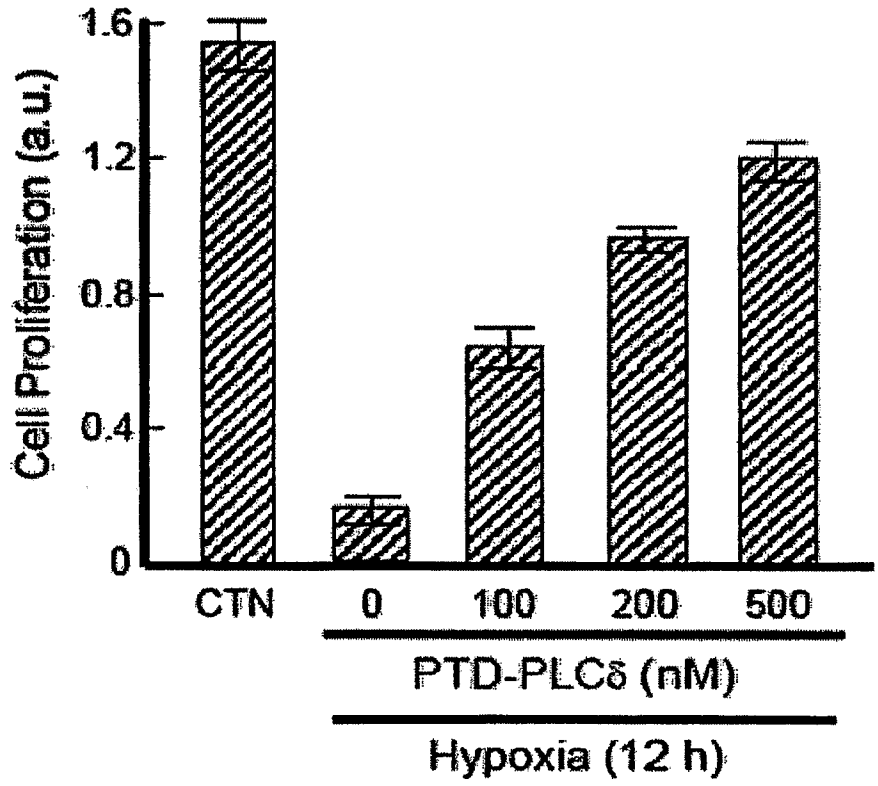
Cell Proliferation (a.u.)
1.6
1.2
0.8
0.4
0
CTN
0
100
200
500
PTD-PLCδ (nM)
Hypoxia (12 h)

FIG. 2B

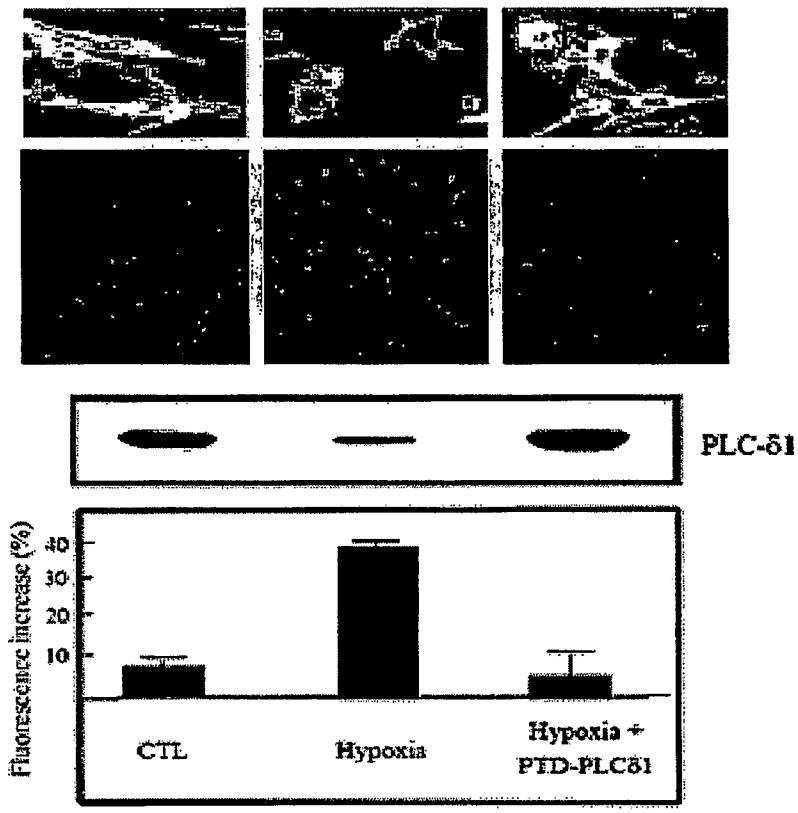
PLC-δ1
Fluorescence increase (%)
40
30
20
10
CTL
Hypoxia
Hypoxia +
PTD-PLCδ1

FIG. 3

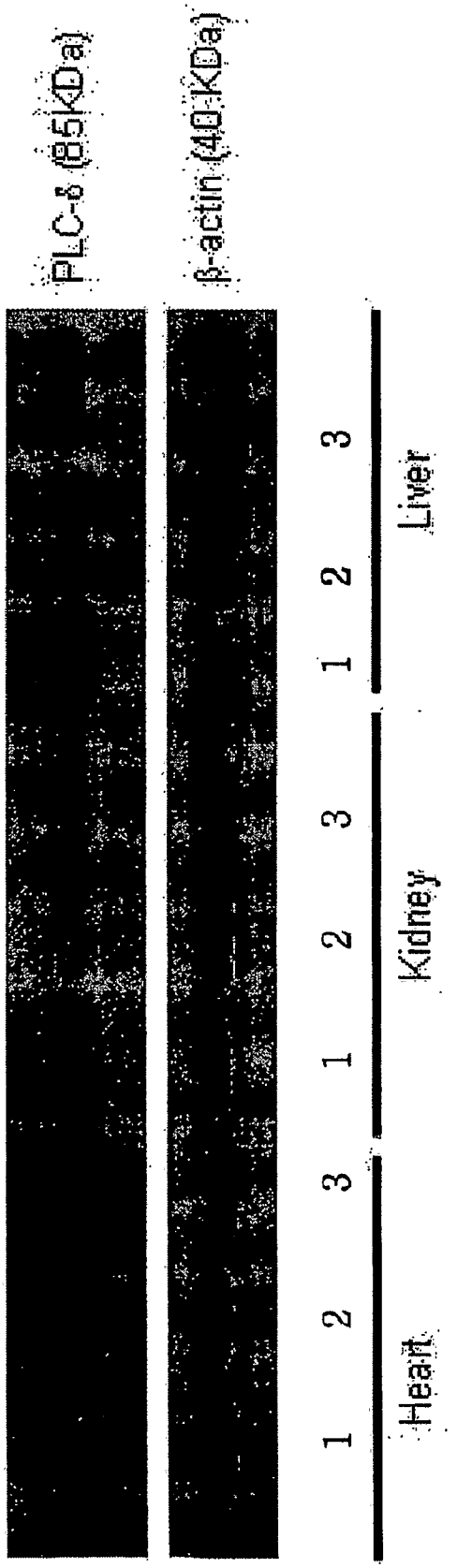
PLC-δ (85KDa)
β-actin (40 KDa)
1 2 3 1 2 3 1 2 3
Heart
Kidney
Liver
p-PKC (80 KDa)

FIG. 4

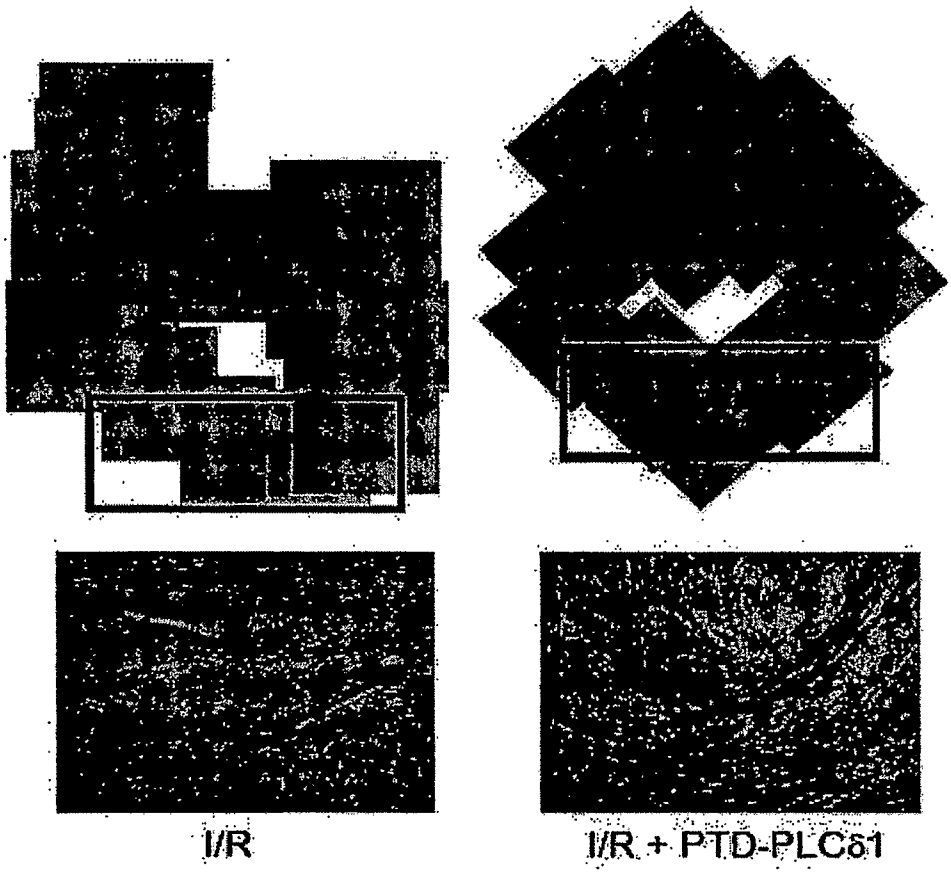

FIG. 5