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(54) Title: BACTERIAL EXPRESSION OF PHOSPHORYLATED HSP20 AND REAGENTS THEREFOR

(57) Abstract: The present invention provides recombinant host cells and methods for making phosphorylated HSP20 polypeptides or other targets of protein kinase A or protein kinase G.

Bacterial expression of phosphorylated HSP20 and reagents therefor

Cross Reference

This application claims priority from U.S. Provisional Patent Application Serial No. 60/808,488 filed May 25, 2006, the disclosure of which is incorporated by reference herein in its entirety.

Statement of Government Support

This invention was made with government support under National Institute of Health Grants RO1HL58027-01 and R01DK47936- 11. The government has certain rights in the invention.

Background of the invention

Smooth muscle relaxation can be induced by a variety of endogenous and pharmacologic agents such as nitric oxide (NO) through cyclic nucleotide signaling cascades. NO released from stimulated endothelial cells or provided by NO donors such as sodium nitroprusside (SNP) diffuses across cell membranes activating guanylyl cyclase, increasing intracellular cGMP concentrations, and activating cGMP-dependent protein kinase [1]. An alternate pathway for smooth muscle relaxation involves activation of adenylyl cyclase (by agonists such as forskolin), increasing intracellular cAMP concentrations, and activation of cAMP-dependent protein kinase [2].

One downstream target of both cyclic nucleotide signaling cascades is the heat shock-related protein 20, HSP20. HSP20 is highly and constitutively expressed in skeletal, cardiac, and smooth muscle and increases in the phosphorylation of HSP20 are associated with cyclic nucleotide-dependent relaxation of vascular smooth muscle [3-5]. Like other members of the heat shock protein family, HSP20 requires phosphorylation at a serine or threonine residue (S16 in HSP20) to render it functionally active [5,6]. HSP20 is a specific substrate protein of PKA and PKG *in vivo* and accumulating evidence suggests HSP20 has several physiological and biochemical roles including controlling muscle tone, regulating cell motility, modulating actin filament dynamics and protection against intimal hyperplasia and ischemia/reperfusion injury [7-14]. Although the molecular mechanisms of action for HSP20 have not been determined fully, it is an actin-associated protein and we have shown that HSP20 peptide treatment leads to loss of actin stress fibers and disruption of focal

adhesion complexes [9-10]. Treatment with a phosphopeptide HSP20 analogs leads to a dissociation and activation of cofilin resulting in depolymerization of the actin cytoskeleton. Collectively, these data suggest HSP20 involvement in thin filament regulatory mechanisms

Recently, we used a direct approach to establish the role of phosphoHSP20 in smooth muscle relaxation. We transduced phosphoproteins and peptides into tissues using protein transduction domains (PTDs) and in doing so prevented smooth muscle contraction [12,13]. The use of PTDs as tools for intracellular delivery is a relatively new approach that is receiving increased attention as new methods for its implementation are developed and evidence for its efficacy is established. PTDs, first discovered independently by the Green and Frankel laboratories, are positively charged amino acid sequences facilitating the entry of proteins into cells through macropinocytotic mechanisms [15-17]. Our laboratory has successfully implemented the use of these sequences to transduce different proteins and peptides into numerous tissues and cells [8-14].

One limitation of previous approaches was the requirement for *in vitro* phosphorylation of expressed, purified protein prior to physiological testing. In view of the potential shortcomings of this method for large scale preparations, an alternate phosphorylation scheme was undertaken. The bacterial co-expression of the catalytic subunit of cGMP dependent protein kinase (cPKG) and a recombinant transducible form of HSP20 (TAT-HSP20) was performed to determine if biologically active recombinant phospho-proteins could be produced. The results delineate possible strategies for the production of other proteins through co-expression of the enzyme responsible for post-translational modification.

Summary of the Invention

In one aspect, the present invention provides recombinant host cells, comprising:

(a) a recombinant expression vector operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and

(b) a recombinant expression vector operably linked to a nucleic acid sequence encoding an active HSP20 polypeptide.

In a second aspect, the present invention provides methods for making phosphorylated HSP20 polypeptide, comprising culturing the recombinant host cells of the first aspect of the invention under conditions to promote expression of the catalytic subunit

of the protein kinase and the active HSP20 polypeptide, and to promote phosphorylation of the active HSP20 polypeptide by the expressed protein kinase catalytic subunit.

In a third aspect, the present invention provides recombinant host cells, comprising:

(a) a recombinant expression vector operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and

(b) a recombinant expression vector operably linked to a nucleic acid sequence encoding a polypeptide target of protein kinase A or protein kinase G.

In a fourth aspect, the present invention provides methods for treating a condition susceptible to treatment with an active HSP20 polypeptide, comprising administering a recombinant host cell of the invention to a subject in need thereof, wherein the active HSP20 polypeptide produced by the host cell treats the condition.

Detailed Description of the Invention

Within this application, unless otherwise stated, the techniques utilized may be found in any of several well-known references such as: *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press), *Gene Expression Technology* (Methods in Enzymology, Vol. 185, edited by D. Goeddel, 1991. Academic Press, San Diego, CA), "Guide to Protein Purification" in *Methods in Enzymology* (M.P. Deutshcer, ed., (1990) Academic Press, Inc.); *PCR Protocols: A Guide to Methods and Applications* (Innis, et al. 1990. Academic Press, San Diego, CA), *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY), *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX).

In one aspect, the present invention provides recombinant host cells, comprising:

(a) a recombinant expression vector operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and

(b) a recombinant expression vector operably linked to a nucleic acid sequence encoding an active HSP20 polypeptide.

The recombinant host cells of this aspect of the invention can be used, for example, to produce active, phosphorylated HSP20 polypeptides, which can be used for a variety of therapeutic uses, as described below, and can also be used for gene therapy.

The recombinant host cells can be prokaryotic or eukaryotic, and can be transiently or stably transfected. Exemplary host cells are bacterial cells (such as *E. coli*), yeast, and human-derived cells. Such transfection of expression vectors into prokaryotic and eukaryotic cells can be accomplished via any technique known in the art, including but not limited to standard bacterial transformations, calcium phosphate co-precipitation, electroporation, or liposome mediated-, DEAE dextran mediated-, polycationic mediated-, or viral mediated transfection. (See, for example, *Molecular Cloning: A Laboratory Manual* (Sambrook, et al., 1989, Cold Spring Harbor Laboratory Press; *Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed.* (R.I. Freshney. 1987. Liss, Inc. New York, NY).

"Recombinant expression vector" includes vectors that operatively link so as to permit expression of a nucleic acid coding region or gene to any promoter capable of effecting expression of the gene product. The promoter sequence used to drive expression of the disclosed nucleic acid sequences in a mammalian system may be constitutive (driven by any of a variety of promoters, including but not limited to, CMV, SV40, RSV, actin, EF) or inducible (driven by any of a number of inducible promoters including, but not limited to, tetracycline, ecdysone, steroid-responsive). The construction of expression vectors for use in transfecting prokaryotic and eukaryotic cells is also well known in the art, and thus can be accomplished via standard techniques. (See, for example, Sambrook, Fritsch, and Maniatis, in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989; *Gene Transfer and Expression Protocols*, pp. 109-128, ed. E.J. Murray, The Humana Press Inc., Clifton, N.J.), and the Ambion 1998 Catalog (Ambion, Austin, TX); see also the examples below.

The expression vector must be replicable in the host organisms either as an episome or by integration into host chromosomal DNA. In a preferred embodiment, the expression vector comprises a plasmid. However, the invention is intended to include other expression vectors that serve equivalent functions, such as viral vectors.

The recombinant expression vector operably linked to the protein kinase catalytic subunit and to the active HSP20 peptide can be the same recombinant expression vector expressing both. In one embodiment, a first recombinant expression vector is operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected

from the group consisting of protein kinase A and protein kinase G, or catalytic subunits thereof; and a second recombinant expression vector operably linked to a nucleic acid sequence encoding an active HSP20 polypeptide

The nucleic acid sequences encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G can encode full length protein kinase A or G, or may encode only the catalytic subunit. In one embodiment, the protein kinase G is the human protein kinase G amino acid sequence comprising or consisting of **SEQ ID NO: 45**, or catalytic subunit thereof. In a further embodiment, the protein kinase G is encoded by the nucleic acid sequence comprising or consisting of **SEQ ID NO: 46** or **SEQ ID NO: 49**. In another embodiment, the protein kinase A is the human protein kinase A amino acid sequence comprising or consisting of **SEQ ID NO: 47**, or catalytic subunit thereof. In a further embodiment, the protein kinase G is encoded by the nucleic acid sequence comprising or consisting of **SEQ ID NO: 48**. The nucleic acid sequence encoding the protein kinase A or protein kinase C can be any known in the art, including but not limited to those encoding the protein kinases as shown in **Table 1**. In a preferred embodiment, a nucleic acid sequence encoding the catalytic subunit of protein kinase G is used.

As used herein, the term "active HSP20 polypeptide" refers to full length HSP20, or any polypeptide portion thereof that can be used to promote smooth muscle relaxation after appropriate phosphorylation (see, for example, the methods disclosed herein for demonstrating the ability to promote smooth muscle relaxation). In one embodiment, the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of general formula I:

X1-[X2-ASAPLP-X3-]$_u$-X4 (**SEQ ID NOs: 2-6**)

wherein X1 is absent or comprises a transduction domain;

X2 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (**SEQ ID NO:7**);

X3 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

Z2 is selected from the group consisting of L and K; and

Z3 is selected from the group consisting of S, K and T;

X4 is absent or comprises a transduction domain; and

wherein u is 1, 2, 3, 4, or 5.

In a preferred embodiment of general formula I, at least one of X1 and X4 comprises a transduction domain.

In another embodiment, the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of general formula II:

X1-X2-ASAPLP-X3-X4 **(SEQ ID NO: 8)**

wherein X1 is absent or comprises a cell transduction domain;

X2 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of **SEQ ID NO:1**;

X3 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of **SEQ ID NO:1**; and

X4 is absent or comprises a cell transduction domain.

In a preferred embodiment of general formula II, at least one of X1 and X4 comprises a transduction domain.

Full length HSP20

Met Glu Ile Pro Val Pro Val Gln Pro Ser Trp Leu Arg Arg Ala Ser Ala Pro Leu Pro Gly Leu Ser Ala Pro Gly Arg Leu Phe Asp Gln Arg Phe Gly Glu Gly Leu Leu Glu Ala Glu Leu Ala Ala Leu Cys Pro Thr Thr Leu Ala Pro Tyr Tyr Leu Arg Ala Pro Ser Val Ala Leu Pro Val Ala Gln Val Pro Thr Asp Pro Gly His Phe Ser Val Leu Leu Asp Val Lys His Phe Ser Pro Glu Glu Ile Ala Val Lys Val Val Gly Glu His Val Glu Val His Ala Arg His Glu Glu Arg Pro Asp Glu His Gly Phe Val Ala Arg Glu Phe His Arg Arg Tyr Arg Leu Pro Pro Gly Val Asp Pro Ala Ala Val Thr Ser Ala Leu Ser Pro Glu Gly Val Leu Ser Ile Gln Ala Ala Pro Ala Ser Ala Gln Ala Pro Pro Pro Ala Ala Ala Lys. **(SEQ ID NO:1)**.

In another embodiment the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of formula:

X1-**SEQ ID NO:10**-X4 **(SEQ ID NO: 9)**,

wherein X1 is absent or comprises a cell transduction domain;

wherein **(SEQ ID NO: 10)** is Trp Leu Arg Arg Ala Ser Ala Pro Leu Pro Gly Leu Lys; and wherein X4 is absent or comprises a cell transduction domain.

In another embodiment, the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of general formula III:

X1-[X2-X3-RRASAP]$_u$-X4 **(SEQ ID NOs: 11-15)**

wherein X1 is absent or comprises a transduction domain;

X2 is absent or is W;

X3 is absent or is L

X4 is absent or comprises a transduction domain; and

wherein u is 1, 2, 3, 4, or 5.

In a preferred embodiment of general formula III, at least one of X1 and X4 comprises a transduction domain.

As used herein, the term "transduction domain" means one or more amino acid sequence or any other molecule that can carry the active domain across cell membranes. These domains can be linked to other polypeptides to direct movement of the linked polypeptide across cell membranes. Transduction domains are known to those of skill in the art, and include, but are not limited to:

$(R)_{4-9}$ **(SEQ ID NO: 44)**

GRKKRRQRRRPPQ **(SEQ ID NO: 16)**;

AYARAAARQARA **(SEQ ID NO: 17)**;

DAATATRGRSAASRPTERPRAPARSASRPRRPVE **(SEQ ID NO: 18)**;

GWTLNSAGYLLGLINLKALAALAKKIL **(SEQ ID NO: 19)**;

PLSSIFSRIGDP **(SEQ ID NO: 20)**;

AAVALLPAVLLALLAP **(SEQ ID NO: 21)**;

AAVLLPVLLAAP **(SEQ ID NO: 22)**;

VTVLALGALAGVGVG **(SEQ ID NO: 23)**;

GALFLGWLGAAGSTMGAWSQP **(SEQ ID NO: 24)**;

GWTLNSAGYLLGLINLKALAALAKKIL **(SEQ ID NO: 25)**;

KLALKLALKALKAALKLA **(SEQ ID NO: 26)**;

KETWWETWWTEWSQPKKKRKV **(SEQ ID NO: 27)**;

KAFAKLAARLYRKAGC **(SEQ ID NO: 28)**;

KAFAKLAARLYRAAGC **(SEQ ID NO: 29)**;

AAFAKLAARLYRKAGC **(SEQ ID NO: 30)**;

KAFAALAARLYRKAGC **(SEQ ID NO: 31)**;

KAFAKLAAQLYRKAGC **(SEQ ID NO: 32)**;

AGGGGYGRKKRRQRRR **(SEQ ID NO: 33)**;

YGRKKRRQRRR (**SEQ ID NO: 34**);

YARAAARQARA (**SEQ ID NO: 35**);

GGGGYGRKKRRQRRR (**SEQ ID NO: 36**); and

YGRKKRRQRRR (**SEQ ID NO: 37**).

In a further embodiment, the transduction domain comprises or consists of a polypeptide of general formula IV:

$(J_1J_2B_1B_2J_3B_3J_4)_n$ (**SEQ ID NOs: 38-39**)

wherein **J** is any hydrophobic amino acid;

wherein B_1, B_2, and B_3 are independently any basic amino acid; and

wherein n is between 1 and 10.

Thus, "n" can be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In a preferred embodiment, n is 1, 2, or 3.

In a a further preferred embodiment of general formula IV, J_1-J_4 are independently any hydrophobic amino acid selected from the group consisting of Trp, Tyr, Leu, Ile, Phe, Val, Met, Cys, Pro, and Ala; and

B_1, B_2, and B_3 are independently arginine, histidine, or lysine.

In various preferred embodiments of general formula IV, both B_1 and B_2 are arginine or lysine and B_3 is either lysine or arginine but is not the same as B_1 and B_2. In a most preferred embodiment, B_1 and B_2 are arginine and B_3 is lysine.

In further preferred embodiments of general formula IV, J_1-J_4 are independently selected from the group consisting of Trp, Leu, Ile, and Ala. In various further preferred embodiments of general formula IV, J_1 is Trp, J_2 is Leu, J_3 is Ile, or J_4 is Ala, or any combination thereof.

In a further preferred embodiment of general formula IV, the transduction domains comprises or consists of the amino acid sequence $(WLRRIKA)_n$ (**SEQ ID NO: 40**), wherein n is 1-10. Thus, in this embodiment the polypeptide can comprise or consist of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies of WLRRIKA. In preferred embodiments of this most preferred embodiment, n is 1, 2, or 3. Non-limiting examples of such polypeptides include:

WLRRIKA (**SEQ ID NO: 41**);

WLRRIKAWLRRIKA (**SEQ ID NO: 42**); and

WLRRIKAWLRRIKAWLRRIKA (**SEQ ID NO: 43**).

In a second aspect, the present invention provides methods for making phosphorylated HSP20 polypeptide, comprising culturing the recombinant host cells of the

first aspect of the invention under conditions to promote expression of the catalytic subunit of the protein kinase and the active HSP20 polypeptide, and to promote phosphorylation of the active HSP20 polypeptide by the expressed protein kinase catalytic subunit. All of the various embodiments of the first aspect of the invention also apply to this second aspect of the invention. Determination of suitable conditions for expression of the catalytic subunit of the protein kinase and the active HSP20m polypeptide, and for phosphorylation of the active HSP20 polypeptide by the expressed protein kinase catalytic subunit are within the level of skill in the art based on the teachings herein.

In a third aspect, the present invention provides recombinant host cells, comprising:

(a) a recombinant expression vector operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and

(b) a recombinant expression vector operably linked to a nucleic acid sequence encoding a polypeptide target of protein kinase A or protein kinase G.

The various embodiments of recombinant host cells, recombinant expression vectors, and protein kinase A and G from the first aspect of the invention also apply to the third aspect of the invention. However, the recombinant expression vector operably linked to the protein kinase catalytic subunit in this third aspect does not encode the full length protein, but only the catalytic subunit. In a preferred embodiment, a first recombinant expression vector is operably linked to a nucleic acid sequence encoding the catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and a second recombinant expression vector operably linked to a nucleic acid sequence encoding a protein kinase A or protein kinase G target. In a preferred embodiment, a nucleic acid sequence encoding the catalytic subunit of protein kinase G is used.

As will be appreciated by those of skill in the art, any nucleic acid encoding a protein kinase A or G target containing the consensus sequence recongiton site RRAS (**SEQ ID NO: 50**) and capable of being phosphorylated, can be used in the recombinant host cells of the invention.

In a further preferred embodiment, the recombinant host cell comprises a bacterial host cell.

In a fourth aspect, the present invention provides methods for treating a condition susceptible to treatment with an active HSP20 polypeptide, comprising administering a

recombinant host cell of the invention to a subject in need thereof, wherein the active HSP20 polypeptide produced by the host cell treats the condition.

The main mechanism of protein transduction is an electrostatic interaction with the plasma membrane, penetration into cells by macropinocytosis, and a release to the cytoplasm by vesicle rupture. In many instances, the protein transduction domain leads to a nuclear localization of the attached cargo through retrograde vesicle transport. In some cell types such as activated lymphocytes, transduction domains (TAT specifically) can induce apoptosis and allow for protein exit from the cell. This is a destructive cellular process. A cell therapy application may be possible wherein host mammalial cells containing the TD expression element would serve as a "biodegradable" implantable/injectable protein production vehicle that would yield active TD-HSP20 upon programmed cell death and lysis.

The inventors have previously disclosed the use of active HSP20 polypeptides as therapeutic agents for the following: (a) inhibiting smooth muscle cell proliferation and/or migration; (b) promoting smooth muscle relaxation; (c) increasing the contractile rate in heart muscle; (d) increasing the rate of heart muscle relaxation; (e) promoting wound healing; (f) reducing scar formation; (g) disrupting focal adhesions; (h) regulating actin polymerization; and (i) treating or inhibiting one or more of intimal hyperplasia, stenosis, restenosis, atherosclerosis, smooth muscle cell tumors, smooth muscle spasm, angina, Prinzmetal's angina (coronary vasospasm), ischemia, stroke, bradycardia, hypertension, pulmonary (lung) hypertension, asthma (bronchospasm), toxemia of pregnancy, pre-term labor, pre-eclampsia/eclampsia, Raynaud's disease or phenomenon, hemolytic-uremia, non-occlusive mesenteric ischemia, anal fissure, achalasia, impotence, migraine, ischemic muscle injury associated with smooth muscle spasm, vasculopathy, such as transplant vasculopathy, bradyarrythmia, bradycardia, congestive heart failure, stunned myocardium, pulmonary hypertension, and diastolic dysfunction. (See, for example, US 20030060399 filed March 27, 2003; WO2004017912 published March 4, 2004; WO04/075914; WO03/018758; WO05/037236).

Thus, in preferred embodiments of this aspect, the invention provides cell therapy methods for one or more of the following therapeutic uses:

(a) inhibiting smooth muscle cell proliferation and/or migration; (b) promoting smooth muscle relaxation; (c) increasing the contractile rate in heart muscle; (d) increasing the rate of heart muscle relaxation; (e) promoting wound healing; (f) reducing scar formation; (g) disrupting focal adhesions; (h) regulating actin polymerization; and (i)

treating or inhibiting one or more of intimal hyperplasia, stenosis, restenosis, atherosclerosis, smooth muscle cell tumors, smooth muscle spasm, angina, Prinzmetal's angina (coronary vasospasm), ischemia, stroke, bradycardia, hypertension, pulmonary (lung) hypertension, asthma (bronchospasm), toxemia of pregnancy, pre-term labor, pre-eclampsia/eclampsia, Raynaud's disease or phenomenon, hemolytic-uremia, non-occlusive mesenteric ischemia, anal fissure, achalasia, impotence, migraine, ischemic muscle injury associated with smooth muscle spasm, vasculopathy, such as transplant vasculopathy, bradyarrythmia, bradycardia, congestive heart failure, stunned myocardium, pulmonary hypertension, and diastolic dysfunction; wherein the method comprises administering to an individual in need thereof an effective amount to carry out the one or more therapeutic uses of an active HSP20-polypepetide expressing recombinant host cell according to the present invention. In preferred embodiments, the methods comprise administering to the individual an active HSP20-polypeptide expressing recombinant host cell according to one of the preferred embodiments disclosed above.

In a preferred embodiment, the subject is a mammal and the recombinant expression vector is of mammalian origin (preferably from the same mammal being treated); in a more preferred embodiment, the individual is a human and the recombinant host cell comprises a recombinant human cell. In a preferred embodiment, the nucleic acid encoding the protein kinase A or protein kinase G catalytic subunit is derived from a mammal; preferably a human.

As used herein, "treat" or "treating" means accomplishing one or more of the following: (a) reducing the severity of the disorder; (b) limiting or preventing development of symptoms characteristic of the disorder(s) being treated; (c) inhibiting worsening of symptoms characteristic of the disorder(s) being treated; (d) limiting or preventing recurrence of the disorder(s) in patients that have previously had the disorder(s); and (e) limiting or preventing recurrence of symptoms in patients that were previously symptomatic for the disorder(s).

As used herein, the term "inhibit" or "inhibiting" means to limit the disorder in individuals at risk of developing the disorder.

Example 1: cDNA cloning and co-expression

A 1600 bp cDNA encoding the type I catalytic subunit of cGMP-dependent protein kinase from *Bos taurus* (encoded residues 334-670) was liberated from pBKS+ cPKG (gift from Dr.

Thomas Lincoln, University of South Alabama) by NcoI-XhoI digestion and cloned into a NcoI-XhoI-digested shuttle vector pET29a yielding pET29a PKG. pACYC Duet PKG was constructed by ligating the BglII-XhoI insert of pET29a PKG and the backbone of BglII-XhoI-digested pACYC Duet (Stratagene). Construction of pET14b TAT-HSP20 (full length HSP20 conjugated to YGRKKRRQRRR **(SEQ ID NO:37)** via a linker) has been described previously [13].

pACYC Duet PKG and pET14b TAT-HSP20 were co-transformed into the *E. coli* expression strain BL21(DE3) and a single colony resistant to 50 mg/L ampicillin and 30 mg/L chloramphenicol used to inoculate 400 mL of Luria Broth (LB) seed culture and grown overnight on a platform shaker (220 rpm at 37°C). The following morning, 4 liters of LB was inoculated with the seed culture and the culture was induced with 2 mM isopropyl-β-D-thiopyranogalactoside (IPTG - Research Organics, Cleveland, OH) at an OD of 0.5. Induced cells were grown overnight (14hr) and the next morning harvested from media by centrifugation at 6000 X g for 15 min at 4°C.

Example 2: TAT-HSP20 purification and refolding

Frozen cells were thawed and thoroughly resuspended in 100 mL 1X TNE buffer (50 mM NaCl, 1 mM EDTA, and 500 mM Tris-HCl, pH 8.0, 10nM Caliculin A, 50µl Sigma Phosphatase Inhibitor Cocktail II, 50µl Sigma Protease Inhibitor Cocktail and 200 µg deoxyribonuclease) and incubated at 4°C for 30 min. After sonication on ice, the inclusion bodies containing recombinant protein were harvested by centrifugation (19,000 *g*, 10 min). Metal chelation affinity chromatography was used to purify recombinant rTAT-pHSP20. Briefly, inclusion bodies were first resuspended in binding buffer (20 mM Na_2HPO_4, 0.5 M NaCl, 50 mM imidazole, pH 7.4, 8 M urea). The sample was then added to Ni^{2+}-charged Chelating Sepharose Fast Flow (Pharmacia Biotech, Peapack, NJ) and incubated for 30 min at room temperature. The resin was then loaded in a water-chilled XK-26 column and washed extensively with binding buffer on an AKTÄ fast-performance liquid chromatography system (PHARMACIA BIOTECH). Protein was refolded by using an overnight linear gradient of urea from 8 to 0 M and eluted with binding buffer containing 500 mM imidazole. The eluate was concentrated and the imidazole removed by repeatedly reducing eluate volume 50% using a stirred ultrafiltration cell (8200, Millipore, Bedford, MA) with a 10,000-nominal molecular weight limit filter under 75 p.s.i. nitrogen and bringing the volume back to 100% again with PSS (physiological salt solution – 140 mM

NaCl, 5 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 5.6 mM glucose, 2 mM MOPS, and 0.02 mM EDTA, pH 7.4).

Example 3: In vitro analysis of phosphorylation & Co-expression of cPKG and TAT-HSP20

The plasmids pET14b-TAT-HSP20 and pACYC-Duet cPKG were constructed as described in Example 1, sequenced and the proteins expressed in the *E. coli* strain BL21(DE3), predominantly in insoluble inclusion body forms. One and two dimensional SDS-PAGE, immunoblot analyses and quantification were performed as follows. Protein (75 µg) in a 120µl volume was applied to the entire length of one channel in an isoelectric focusing sample tray (Bio-Rad) to which an immobilized pH gradient strip (linear pH gradient range 3–10, 7-cm length) was applied gel side down. Samples were overlaid with ~2 ml of mineral oil and allowed to equilibrate for 16 h at room temperature before isoelectric focusing for 20,000 V h. Samples were subsequently equilibrated for 15 min first in an SDS equilibration buffer (50 mM TrisCl pH 8.8, 6 M urea, 30% glycerol, 2% lauryl sulfate, and 0.02% bromophenol blue) containing 1% DTT and second (15 min) in equilibration buffer containing 2.5% iodoacetamide. After equilibration, samples were separated in the second dimension on precast 4–20% polyacrylamide mini-gels in 1X electrophoresis buffer (25 mM TrisCl pH 8.3, 192 mM glycine, 0.1% wt/vol SDS) at 120 V for 1.5 h. Electrophoretic transfer of proteins from the gels onto polyvinylidene difluoride membranes was carried out in 1X electrophoresis buffer at 50 volts for 12 h at 4°C. The blot was subsequently incubated with the appropriate antibody. Membranes were scanned (Odyssey Infrared Imaging System, Li-Cor Biosciences, Lincoln, NE), and the intensities of selected bands were quantified by using software packaged with the instrument.

Both proteins were expressed at approximately 20 mg/L in four liter induction experiments. TAT-pHSP20 is expressed and phosphorylated in *E. coli.* Representative immunoblot analyses of BL21(DE3) bacterial extracts taken from a large scale (4L) co-expression experiment. Extracts were generated before or after induction with 2 mM IPTG and 20 µg protein was loaded per well. Lanes 1 and 2 were pET14b TAT-HSP20 vector only; lanes 3 and 4 were pACYC Duet PKG C subunit vector only; lanes 5 and 6 were pET14b TAT-HSP20 and pACYC Duet PKG C subunit vectors together. Blots were probed with monoclonal antibodies raised against human HSP20, anti-PKG polyclonal IgGs, and anti-phospho-HSP20 specific IgGs. As judged by SDS-PAGE, TAT-HSP20 migrated with

a molecular weight of ~25 kDa while cPKG migrated at approximately 39 kDa. Antibody detections of bacterial lysates expressing the TAT-HSP20 cDNA alone with or without IPTG induction revealed an immunoreactive polypeptide that was of the molecular weight predicted for TAT-HSP20 [13]. Anti-PKG antibodies or anti-phospho-HSP20 specific IgGs were not reactive. Bacterial lysates containing the cPKG cDNA alone revealed an anti-PKG reactive band only in the sample induced with IPTG. Immunoblots performed on bacterial samples transformed with both pET14b-TAT-HSP20 and pACYC-Duet cPKG plasmids before and after IPTG induction showed immunoreactive patterns with both anti-HSP20 and anti-PKG antibodies similar to those observed in lanes 1-4. In addition, a prominent polypeptide band immunoreactive with anti-phospho-HSP20 specific IgGs appeared that was absent in lanes 1-5. Pellets from four liter expression experiments where TAT-HSP20 cDNA was expressed alone or co-expressed with cPKG in the presence of IPTG (~20 g each) were used subsequently for TAT-HSP20 protein purification and physiological testing. Recombinant proteins from both *E. coli* strains were purified independently by urea-solubilization and metal chelation affinity binding then refolded by the gradual removal of urea during a linear urea-physiological salt solution (PSS) gradient. Proteins were dialyzed against PSS to remove imidazole and concentrated to ~ 10 μM.

To determine the extent to which TAT-pHSP20 was phosphorylated by co-expression with cPKG, purified phosphorylated and non-phosphorylated protein samples were separated by isoelectric focusing followed by SDS-PAGE, blotted to nitrocellulose and probed with anti-HSP20 antibodies. TAT-HSP20 co-expressed with cPKG yields two anti-HSP20 immunoreactive polypeptides. Two-dimensional gel electrophoresis and Western blot analyses of purified recombinant proteins, PTD-HSP27, TAT-HSP20, TAT-pHSP20 was performed. 75 μg of recombinant protein were subjected to isoelectric focusing (linear pH gradient range 3-10, 7 cm length) then separated in the second dimension (4-20% polyacrylamide). Proteins were transferred onto PVDF membranes and immunoblots were incubated with mouse anti-HSP20 and IRDye 700DX conjugated affinity purified goat anti-mouse secondary antibodies. A control protein of weight and composition similar to TAT-HSP20 (PTD-HSP27) did not react with anti-HSP20 antibodies demonstrating antibody specificity. Immunoblots of TAT-HSP20 purified from *E. coli* revealed a single spot at a pI of ~9.0 and an electrophoretic mobility of ~ 25 kD. Sequences comprising the amino-terminal PTD are basic in nature, can alter electrophoretic mobility and contribute to a higher pI (~9.0) compared to HSP20 without a PTD (pI ~ 5.9, mass ~17kD) [6, 13]. In contrast,

immunoblots of TAT-pHSP20 purified from *E. coli* co-expressing cPKG revealed two immunoreactive proteins. One spot exhibited a pI of ~9.0 and a mass of 25 kD while the second spot was more acidic by about 0.4 pI units. Sensitive fluorescence-based quantitative analyses of these spots revealed that the more acidic form had a relative intensity of 3.46 (arbitrary units) while the intensity of the more basic spot was 3.70 indicating that the more acidic or phosphorylated spot represented 48% of the total anti-HSP20 immunoreactive protein population isolated from *E. coli*. These results coupled with the phospho-HSP20-specific detections show that a distinct portion of TAT-HSP20 is phosphorylated in *E. coli* when co-expressed with cPKG.

Example 4: Physiological activity of TAT-pHSP20 analogs on in intact tissues

Sprague-Dawley rats (400g) were sacrificed via CO_2 asphyxiation and the aorta of each rat was dissected out, cleaned of excess fat and connective tissue, stored in PSS buffer and 1 hr later cut transversely into rings that were 1 mm in width. Arterial tissues were tied to 4.0 silk, fixed at one end to a stainless steel wire, and attached to a Kent Scientific (Litchfield, CT) force transducer (TRN001) interfaced with a Data Translation analog-to-digital board, DT2801 (Data Translation, Marlboro, MA). Data were acquired with Powerlab software (AD Instruments, Colorado Springs, CO). The rings were repeatedly contracted with 110 mM KCl (with equimolar replacement of NaCl in buffer), and the length was progressively adjusted until maximal tension was obtained. The tissue was washed with PSS buffer and equilibrated for at least 30 min between contractions. Contractile responses to serotonin (5-hydroxytryptamine, 5-HT) were subsequently tested by treating the tissue with increasing doses (0.01, 0.1, and 1μM) of 5-HT (not shown). Tissues were rinsed and treated with 0.5 μM 5-HT (submaximal dose) to determine pretreatment contraction. Any tissue failing to contract was considered nonviable and was not used in further experiments. After washing tissues and allowing a return to baseline tension, rTAT-pHSP20 (10μM) protein was added to the bath and the ring incubated for 10 min. The effectiveness of the pretreatment was assessed by addition of 0.5 μM 5-HT, and the contractile response recorded. Percent contraction was calculated relative to force generated with 5-HT prior to pretreatment with rTAT-pHSP20. Protein extracts of vascular rings were obtained by flash freezing tissues still connected to the transducer, then vortexing frozen vascular rings in UDC (9M urea, 2% CHAPS, 10 mM DTT) sample buffer for 1 hr. For treatments with synthesized PTD-HSP20

peptides, tissues were contracted with 0.5 μM 5-HT (submaximal dose) followed by increasing doses of peptide (100 μM, 500 μM and 1 mM, respectively).

The physiological activity of rTAT-pHSP20 from bacterial co-expression experiments was assessed in muscle bath experiments. Rings of male rat aorta were washed, equilibrated with PSS and treated with sub-maximal doses of 5-HT to determine tissue responsiveness. After washing in PSS and letting tension return to baseline levels, rings were incubated in PSS buffer alone or in PSS buffer containing rTAT-pHSP20 (10μM for 10 min. Tissues were subsequently challenged with the same sub-maximal doses of 5-HT (0.5μM) and the contractile responses recorded. There were no significant differences in the magnitude of contraction in response to serial treatments with 5HT. In contrast, pre-incubation with rTAT-pHSP20 inhibited 5-HT-induced contraction by 57 ± 4.5%. Similar results were observed with pig coronary artery where pre-incubation with rTAT-pHSP20 (10 μM) inhibited 5-HT contractions by 60% (data not shown). These results are similar to the inhibition observed using rTAT-HSP20 that was phosphorylated *in vitro* where pre-incubation with rTAT-pHSP20 inhibited contraction of human umbilical artery by 81.99 ± 6.83% [13].

The *in vivo* efficacy of molecules with putative therapeutic application is often established using peptide mimetics. Peptide mimetics are advantageous in determining full-length protein activity as they have fewer domains available for binding/interaction, are usually easier to synthesize in large quantities, and less susceptible to proteolytic activity. A synthesized HSP20 analog, PTD-pHSP20 (YARAAARQARA **(SEQ ID NO:35)** transduction domain conjugated to WLRRApSAPLPGLK **(SEQ ID NO: 10)** composed of an optimized protein transduction domain, fused to sequences encoding the active portion (phosphorylation site, serine 16 of HSP20) [6] has been shown to relax a wide array of smooth muscles including bovine carotid artery, porcine coronary artery, human saphenous vein and human umbilical artery [8,10,12,13]. To compare the magnitude of the physiological responses of the entire recombinant molecule to the peptide mimetics, rings of rat aorta contracted with 5-HT (0.5 μM) displayed steady contractions that were sustained for long periods of time (> 20 min), whereas treatment of aorta rings with increasing amounts of PTD-pHSP20 (100 μM, 500 μM and 1 mM respectively) led to a dose-dependent decrease in contraction that was maximal at a PTD-pHSP20 concentration of 1 mM (data not shown). Collectively, the data from physiological experiments indicate that the full-length, transducible, recombinant, and phosphorylated HSP20 (rTAT-pHSP20) obtained by co-

expressing TAT-HSP20 together with cPKG in *E. coli,* diminished rat aortic contractions at concentrations 100 times less than a peptide control (10 μM vs. 1 mM).

Example 5: NIH 3T3 cell culture and Effect of rTAT-pHSP20 on actin stress fibers

NIH 3T3 cells (ATCC CRL 1658) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) BCS, penicillin (100 U/ml), and 100μg/ml streptomycin (Gibco/BRL), L-glutamine (4 mM) in a CO_2 incubator (5% CO_2) at 37 °C. Serum free media supplemented with insulin-transferrin sodium selenite (5 ng/ml) and HEPES (pH 7.55 - 25mM) was used to quiesce and potentiate cells for stress fiber formation.

To visualize the effect of the rTAT-pHSP20 on the actin cytoskeleton, serum-starved cells seeded on coverslips in 6-well dishes (50-70% confluent) were rinsed briefly with phosphate buffered saline (PBS) then treated with recombinant TAT-pHSP20 (10 μM) in PSS for 1 h at 37°C. Following this incubation, cells were washed in PBS, fixed in 4% formaldehyde (w/v) for 1hr at RT and permeabilized with 0.1% Triton X-100 for 20 min. Washed cells were subsequently incubated with Alexa 568-conjugated phalloidin (0.2 U/mL) and DAPI (1 μg/mL) for 1 hr at RT, washed with PBS then viewed using a Zeiss Axiovert 200M fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY, USA) equipped with DAPI and Alexa 568 excitation/emission filter sets. Digital micrographs were acquired using an Axiocam MRM high-resolution camera then adjusted for contrast and assembled into figures using Adobe Photoshop (Mountain View, CA).

Purified recombinant TAT-pHSP20 induces actin rearrangements in 3T3 cells. Cells were then washed with PBS and processed for fluorescence microscopy. Immunofluorescence microscopic images of NIH 3T3 cells serum starved for 24 hrs – treated for one hour with physiological salt solution (PSS) or treated with 10μM recombinant TAT-pHSP20 recombinant protein in PSS were obtained. Cells were washed, fixed, detergent permeabilized, then probed with Alexa568-phalloidin and DAPI. Cells treated with TAT-pHSP20 lack stress fibers and central actin filaments. Actin filaments are organized evenly throughout each cell and prominent stress fibers are visible. In contrast, rTAT-pHSP20-treated cells exhibiting actin reorganizations, i.e., cells clearly lack central actin stress fibers and exhibit most actin staining at the cell periphery. Considering that cytoskeletal rearrangements involved in stress fiber deformation are identical in many respects to those events contributing to relaxation in muscle cells, it seems likely that the physiology results observed are mediated by thin filament altering mechanisms.

Example 6:

Several protein kinases and their subunits have been expressed in *E. coli* in both active and inactive forms, including cGMP- and cAMP dependent protein kinases, myosin light chain kinase, and casein kinase [18-25]. Full length bovine cGMP-dependent protein kinase has been expressed previously in *E. coli* using both T7 RNA polymerase and tac+ promoters [19]. Both experiments resulted in predominantly insoluble and catalytically inactive kinases with a small fraction of soluble PKG able to bind cGMP but not phosphorylate a GRTGRRNSI peptide substrate. Despite these findings, the catalytic subunit of bovine PKG (cPKG) was selected as the kinase of choice for this study as it is well able to autophosphorylate itself, it has been shown to phosphorylate HSP20 *in vitro* and *in vivo*, and it has been expressed successfully in *E. coli* before [5,13,26,27]. As shown here, at least partial activation of cPKG was achieved, even when another cDNA was coordinately expressed. The identity of an expressed protein with kinase activity was established by multiple criteria. First, bacterial extracts from cells co-expressing TAT-HSP20 and cPKG as well as refolded TAT-pHSP20 purified under denaturing conditions revealed proteins that were immunoreactive to anti-phospho-HSP20 IgGs. Secondly, two-dimensional gel electrophoresis of TAT-HSP20 protein obtained from cells co-expressing cPKG, but not TAT-HSP20 alone, resulted in the appearance of a second, more acidic, anti-HSP20-immunoreactive spot consistent with TAT-HSP20 phosphorylation. Third, biological assays wherein TAT-pHSP20 was examined for its effects in inhibiting smooth muscle contraction revealed tissue responses indicative of phosphorylation. Finally, cultured cells pretreated with TAT-pHSP20 displayed actin cytoskeleton rearrangements consistent with the activity reported for pHSP20[28]. Collectively, these results support the notion that at least a portion of cPKG is catalytically active when expressed in *E. coli* and can phosphorylate a co-expressed substrate protein, HSP20.

Our findings suggesting that a functionally active PKG catalytic subunit can be expressed in *E. coli* contrast previous reports [19, 27]. In the former report [19], the full length PKG encoding both catalytic and regulatory subunits was expressed and in the latter [27] a cPKG (residues 335-670)-maltose binding protein (MBP) fusion was expressed. Although the fusion protein was soluble no activity could be detected. Proteolytic cleavage of the MBP fragment with Factor Xa yielded a spontaneously aggregating catalytic subunit for which activity was not reported. It seems likely that the secondary structures imparted by

the attached MBP or the presence of a regulatory subunit abrogated detectable activity using the assays described. Our encouraging results suggest that bacteria may possess a rudimentary chaperone system potentiating activation and productive folding of at least a portion of cPKG.

Substitution of a negatively charged amino acid can mimic the conformational effects of phosphorylation on some proteins, particularly when the major function of phosphorylation is to add negative charge [13]. The functionality of phosphatase-resistant mimics is diminished however, when residue substitutions lead to protein instability or when the geometric positioning of the aspartyl or glutamyl sidechains is incompatible with the interacting protein counterpart. In the case of HSP20, the molecular structure has not yet been elucidated but a transducible phosphomimetic of HSP20 peptide bearing an S16E mutation inhibited 5-HT induced contractions of umbilical artery smooth muscle by 50% as opposed to the 80% inhibition observed with the S16 peptide counterpart [13]. The biological activity of constitutively active full-length mutants (S16E and S16D) mutants should lend further insight into the role of HSP20 phosphorylation.

The observation that treatment of serum-starved cultured cells with rTAT-pHSP20 resulted in the destruction of actin stress fibers is congruent with previously published studies on heat shock proteins and the actin cytoskeleton [8-10, 28-30]. In general, the presence of stress fibers is associated with a proliferative response whereas the absence of stress fibers is associated with a quiescent phenotype [31]. Recent data from our laboratory and others suggests that thin filament stability and dynamics are regulated by competition between molecules stabilizing actin filaments (HSP27) and the severing/depolymerizing activities of proteins such as cofilin. Interestingly, cofilin, HSP27 and HSP20 all possess binding motifs for the scaffolding protein 14-3-3. 14-3-3 proteins comprise a highly conserved family of dimeric and cytosolic proteins that bind other proteins in a phosphorylation-dependent manner and thereby modulate their activity or prevent interaction with other proteins. Recent observations from our group have shown that the intracellular receptor for the peptide analog of HSP20 (PTD-pHSP20) is the scaffolding protein 14-3-3 [10]. Consequently, we expected similarities in the cellular responses to full-length rTAT-pHSP20 (described here) and the peptide mimics of HSP20. After either treatment, stress fibers were markedly reduced with most actin redistributed to the cell periphery [10]. These data suggest that phosphorylated HSP20 may modulate actin cytoskeletal dynamics by

competing with phosphorylated cofilin for binding to 14-3-3. Once released, cofilin is then dephosphorylated, activated and available to sever actin filaments.

The substantial differences in effective concentration required to induce relaxation for transducible recombinant HSP20 fusion proteins compared to transducible HSP20 peptides (1μM vs. 10μM, respectively) has been observed previously [13]. This observation is not altogether surprising given the greater conformational structure and additional interactive binding sites present in the entire molecule. The approach of generating recombinant molecules linked to a transduction domain allows for assessment of the biological function of the protein, with or without modifications, prior to generating and optimizing peptide mimetics. The large scale expression and purification of proteins in bacteria is a cost effective and desirable means of protein production primarily when post-translational modifications of the resultant product are not required. The results reported here lend credence to the approach of activating proteins in bacteria through co-expression of kinase(s) when alternate means such as *in vitro* phosphorylation or site-directed mutagenesis introducing acidic residues mimicking the effects of phosphorylation are not feasible.

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Table 1

cGMP-dependent Protein Kinase Isoform Accession Numbers	
Accession Numbers	**Protein Description**
Q13976	cGMP-dependent protein kinase 1, alpha isozyme (CGK 1 alpha) (cGKI-alpha)
AAZ75707	cGMP-dependent protein kinase type I [Sus scrofa]
NP_001013855	protein kinase, cGMP-dependent, type I alpha isoform [Mus musculus]
077676	cGMP-dependent protein kinase 1, alpha isozyme (CGK 1 alpha) (cGKI-alpha)
P21136	cGMP-dependent protein kinase 1, beta isozyme (cGK 1 beta) (cGKI-beta)
BAD12117	cGMP-dependent protein kinase I alpha [Oryzias latipes]
AAQ02512	protein kinase, cGMP-dependent, type I [synthetic construct]
CAI40743	protein kinase, cGMP-dependent, type I [Homo sapiens]
NP_935290	protein kinase, cGMP-dependent, type I beta isoform [Mus musculus
NP_957324	protein kinase, cGMP-dependent, type I [Danio rerio]
BAD12118	cGMP-dependent protein kinase I beta [Oryzias latipes]
CAF98611	unnamed protein product [Tetraodon nigroviridis]
AAL76256	PKG-Ia [Bombyx mori]
XP_851997	similar to protein kinase, cGMP-dependent, type I [Canis familiaris]
AAL76257	PKG-II [Bombyx mori]
EAL33756	GA10020-PA [Drosophila pseudoobscura
CAG05402	unnamed protein product [Tetraodon nigroviridis]
NP_477488	foraging CG10033-PB, isoform B [Drosophila melanogaster]
Q03043	cGMP-dependent protein kinase, isozyme 2 forms cD4/T1/T3A/T3B (CGK)
AAA28455	cGMP-dependent protein kinase
AAA28459	cGMP-dependent protein kinase
NP_001011581	cGMP-dependent protein kinase foraging [Apis mellifera]
AAR06171	PKG [Aplysia californica]
XP_319605	ENSANGP00000006403 [Anopheles gambiae str. PEST]
XP_790011	cGMP-dependent, type I alpha isoform [Strongylocentrotus purpuratus]
NP_001014464	foraging CG10033-PJ, isoform J [Drosophila melanogaster] foraging CG10033-PJ, isoform J [Drosophila melanogaster]
AAA28454	cGMP-dependent protein kinase
AAA28457	cGMP-dependent protein kinase
NP_995629	foraging CG10033-PG, isoform G [Drosophila melanogaster]
AAA28458	cGMP-dependent protein kinase
XP_507794	cGMP-dependent protein kinase 1, beta isozyme (cGKI-beta) [Pan troglodytes]
XP_968718	PREDICTED: similar to CG3324-PA [Tribolium castaneum]

XP_684200	PREDICTED: similar to protein kinase, cGMP-dependent, type II [Danio rerio]
Q03042	cGMP-dependent protein kinase, isozyme 1 (CGK)
AAB03405	cGMP-dependent protein kinase [Drosophila melanogaster]
XP_314690	ENSANGP00000013014 [Anopheles gambiae str. PEST]
EAL34020	GA17377-PA [Drosophila pseudoobscura]
XP_612707	PREDICTED: similar to protein kinase, cGMP-dependent, type II [Bos taurus]
NP_500141	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
NP_741329	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
NP_500142	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
NP_001023223	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
AAC23588	cyclic GMP-dependent protein kinase [Hydra oligactis]
XP_544949	protein kinase, cGMP-dependent, type II [Canis familiaris]
AAI3206	Protein kinase, cGMP-dependent, type II [Mus musculus]
NP_037144	protein kinase, cGMP-dependent, type II [Rattus norvegicus]
AAI11598	Protein kinase, cGMP-dependent, type II [synthetic construct]
NP_032952	protein kinase, cGMP-dependent, type II [Mus musculus]
Q61410	cGMP-dependent protein kinase 2(Type II cGMP-dependent protein kinase)
CAE63845	Hypothetical protein CBG08401 [Caenorhabditis briggsae]
BAA18934	cGMP-dependent protein kinase II [Homo sapiens]
XP_686609	similar to cGMP-dependent protein kinase 2 [Danio rerio]
AAH72999	MGC82580 protein [Xenopus laevis]
XP_426309	similar to protein kinase, cGMP-dependent, type II; cGKII [Gallus gallus]
CAA76073	cGMP-dependant protein kinase [Homo sapiens]
CAF90993	unnamed protein product [Tetraodon nigroviridis]
CAI40744	protein kinase, cGMP-dependent, type I [Homo sapiens]
XP_394420	PREDICTED: similar to ENSANGP00000015733 [Apis mellifera]
D88640	protein F55A8.2 [imported] - Caenorhabditis elegans
XP_318891	ENSANGP00000015733 [Anopheles gambiae str. PEST]
NP_001023224	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
AAV65146	cGMP-protein kinase [Pogonomyrmex barbatus]
XP_517194	similar to protein kinase, cGMP-dependent, type II [Pan troglodytes]
XP_219805	similar to cGMP-dependent protein kinase 1, beta isozyme (CGK 1 beta) (cGKI-beta) [Rattus norvegicus]
CAI20607	novel protein similar to vertebrate protein kinase, cGMP-dependent, type I (PRKG1) [Danio rerio]
XP_974277	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
EAL33366	GA18468-PA [Drosophila pseudoobscura]
NP_741467	C09G4.2c [Caenorhabditis elegans]
NP_741468	C09G4.2a [Caenorhabditis elegans]

T29830	hypothetical protein C09G4.2 - Caenorhabditis elegans
NP_723525	CG4839-PB, isoform B [Drosophila melanogaster]
CAE69446	Hypothetical protein CBG15634 [Caenorhabditis briggsae]
XP_969044	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
CAG09161	unnamed protein product [Tetraodon nigroviridis]
XP_794193	PREDICTED: similar to protein kinase, cGMP-dependent, type I [Strongylocentrotus purpuratus]
XP_624106	PREDICTED: similar to ENSANGP00000015733 [Apis mellifera]
AAH62688	PRKG1 protein [Homo sapiens]
XP_701295	PREDICTED: similar to cGMP-dependent protein kinase II, partial [Danio rerio]
1511094B	cGMP dependent protein kinase I beta
CAF91740	unnamed protein product [Tetraodon nigroviridis]
XP_693317	PREDICTED: similar to cGMP-dependent protein kinase II, partial [Danio rerio]
XP_694793	PREDICTED: similar to protein kinase, cGMP-dependent, type I [Danio rerio]
CAI41306	protein kinase, cGMP-dependent, type I [Homo sapiens]
AAX26936	SJCHGC04415 protein [Schistosoma japonicum]
XP_968796	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
NP_702235	cGMP-dependent protein kinase 1, beta isozyme, putative [Plasmodium falciparum 3D7]
NP_741469	C09G4.2b [Caenorhabditis elegans]
XP_680323	cGMP-dependent protein kinase 1, beta isozyme [Plasmodium berghei strain ANKA]
XP_730176	cGMP-dependent protein kinase [Plasmodium yoelii yoelii str. 17XNL]
CAI39147	cAMP-dependent protein kinase, catalytic subunit 1-1 [Paramecium tetraurelia]
CAI39146	cAMP-dependent protein kinase, catalytic subunit 1-2 [Paramecium tetraurelia]
XP_666287	protein kinase (EC 2.7.1.37), cAMP-dependent, catalytic chain [Cryptosporidium hominis TU502]
AAX55640	cAMP-dependent protein kinase catalytic subunit isoform 2 [Toxoplasma gondii]
BAB18105	cyclic nucreotide dependent protein kinase II [Chlamydomonas reinhardtii]
BAB18104	cyclic nucleotide dependent protein kinase [Chlamydomonas reinhardtii]
AAH41073	Protein kinase, X-linked [Homo sapiens]
AAW26566	SJCHGC06207 protein [Schistosoma japonicum]
XP_763539	cGMP-dependent protein kinase [Theileria parva strain Muguga]
AAM27174	cGMP dependent protein kinase [Toxoplasma gondii]
AAM20901	cGMP-dependent protein kinase [Toxoplasma gondii]
EAS31296	hypothetical protein CIMG_06775 [Coccidioides immitis RS]
XP_955206	cGMP-dependent protein kinase [Theileria annulata strain Ankara]
XP_973065	PREDICTED: similar to CG6117-PA, isoform A [Tribolium castaneum]
AAC24243	cAMP-dependent protein kinase catalytic subunit [Ustilago maydis]

XP_757271	hypothetical protein UM01124.1 [Ustilago maydis 521]
CAJ30022	protein kinase A-like kinase [Trypanosoma brucei brucei]
AAU50669	PRKY [Pan troglodytes]
AAD16002	cAMP-dependent protein kinase catalytic subunit isoform 1 [Amblyomma americanum]
AAD16004	cAMP-dependent protein kinase catalytic subunit isoform 3 [Amblyomma americanum]
AAD16003	cAMP-dependent protein kinase catalytic subunit isoform 2 [Amblyomma americanum]
CAJ30026	protein kinase A-like kinase [Trypanosoma brucei brucei]
AAT81143	cGMP-dependent protein kinase [Chlamydomonas reinhardtii]
XP_690430	PREDICTED: similar to Serine/threonine-protein kinase PRKX (Protein kinase PKX1) [Danio rerio]
XP_582115	PREDICTED: similar to Serine/threonine-protein kinase PRKX (Protein kinase PKX1) [Bos taurus]
CAI38998	cAMP-dependent protein kinase, catalytic subunit 4-1 [Paramecium tetraurelia]
CAI38997	cAMP-dependent protein kinase, catalytic subunit 4-2 [Paramecium tetraurelia]
CAI38995	cAMP-dependent protein kinase, catalytic subunit 4-4 [Paramecium tetraurelia]
CAI38996	cAMP-dependent protein kinase, catalytic subunit 4-3 [Paramecium tetraurelia]
CAI39138	cAMP-dependent protein kinase, catalytic subunit 2-8 [Paramecium tetraurelia]
CAA45015	catalytic subunit of protein kinase A [Aplysia californica]
XP_821623	protein kinase A catalytic subunit isoform 1 [Trypanosoma cruzi]
XP_416852	PREDICTED: similar to Serine/threonine-protein kinase PRKX (Protein kinase PKX1) [Gallus gallus]
CAI39139	cAMP-dependent protein kinase, catalytic subunit 2-7 [Paramecium tetraurelia]
NP_001029135	protein kinase, X-linked [Rattus norvegicus]
XP_852513	PREDICTED: similar to Serine/threonine-protein kinase PRKX (Protein kinase PKX1) [Canis familiaris]
CAI39140	cAMP-dependent protein kinase, catalytic subunit 2-6 [Paramecium tetraurelia]
YP_054237	cAMP-dependent protein kinase catalytic subunit, putative [Paramecium tetraurelia]
CAI39144	cAMP-dependent protein kinase, catalytic subunit 2-2 [Paramecium tetraurelia]
CAG44453	cAMP-dependent protein kinase catalytic subunit [Amblyomma hebraeum]
CAG44454	cAMP-dependent protein kinase catalytic subunit [Amblyomma hebraeum]
BAE24043	unnamed protein product [Mus musculus]
CAG44455	cAMP-dependent protein kinase catalytic subunit [Amblyomma hebraeum]
CAI39000	cAMP-dependent protein kinase, catalytic subunit 3-2 [Paramecium tetraurelia]
CAI39145	cAMP-dependent protein kinase, catalytic subunit 2-1 [Paramecium tetraurelia]
AAH46697	Kin-1-prov protein [Xenopus laevis]

XP_867488	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 5 [Canis familiaris]
XP_867517	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 8 [Canis familiaris]
AAX26927	SJCHGC08677 protein [Schistosoma japonicum]
XP_867499	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 6 [Canis familiaris]
XP_867524	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 9 [Canis familiaris]
P49673	cAMP-dependent protein kinase catalytic subunit (PKA C)
CAI38999	cAMP-dependent protein kinase, catalytic subunit 3-3 [Paramecium tetraurelia]
CAH91423	hypothetical protein [Pongo pygmaeus]
XP_537099	PREDICTED: similar to cAMP-dependent protein kinase catalytic subunit beta isoform 1 isoform 1 [Canis familiaris]
XP_867534	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 10 [Canis familiaris]
XP_867543	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 11 [Canis familiaris]
XP_867507	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 7 [Canis familiaris]
XP_821620	protein kinase A catalytic subunit isoform 2 [Trypanosoma cruzi]
P05383	cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta)
XP_524752	similar to cAMP-dependent protein kinase catalytic subunit beta isoform 1; PKA C-beta [Pan troglodytes]
CAI39143	cAMP-dependent protein kinase, catalytic subunit 2-3 [Paramecium tetraurelia]
CAG03461	unnamed protein product [Tetraodon nigroviridis]
AAX41031	protein kinase cAMP-dependent catalytic beta [synthetic construct]
BAE29331	unnamed protein product [Mus musculus]
CAA29415	C-beta subunit (338 AA) [Sus scrofa]
CAI16845	protein kinase, cAMP-dependent, catalytic, beta [Homo sapiens]
XP_215070	similar to protein kinase, cAMP dependent, catalytic, beta [Rattus norvegicus]
CAI16846	protein kinase, cAMP-dependent, catalytic, beta [Homo sapiens]
BAE24005	unnamed protein product [Mus musculus]
CAH93444	hypothetical protein [Pongo pygmaeus]
AAA30424	cAMP-dependent protein kinase II-beta catalytic subunit
NP_777010	cAMP-dependent protein kinase catalytic subunit beta [Bos taurus]
NP_740960	protein KINase family member (kin-1) [Caenorhabditis elegans]
BAE28648	unnamed protein product [Mus musculus]
NP_957317	hypothetical protein LOC393998 [Danio rerio]
JC7968	cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase (EC 2.7.1.37), catalytic subunit - rainbow trout
1ATPE	Chain E, c-AMP-Dependent Protein Kinase (E.C.2.7.1.37) (cAPK) (Catalytic Subunit) Complex With The Peptide Inhibitor Pki(5-24) And Mnatp (A Ternary Complex Of Capk)
NP_740958	protein KINase family member (kin-1) [Caenorhabditis elegans]

AAH54834	Protein kinase, cAMP dependent, catalytic, alpha [Mus musculus]
CAG06638	unnamed protein product [Tetraodon nigroviridis]
1CTPE	Chain E, Camp-Dependent Protein Kinase (E.C.2.7.1.37) (Capk) (Catalytic Subunit)
1APME	Chain E, c-AMP-Dependent Protein Kinase (E.C.2.7.1.37) (cAPK) (Catalytic Subunit) "alpha" Isoenzyme Mutant With Ser 139 Replaced By Ala (S139A) Complex With The Peptide Inhibitor Pki(5-24) And The Detergent Mega-8
CAC88366	cAMP-dependent protein kinase catalytic subunit alpha [Xenopus laevis]
P36887	cAMP-dependent protein kinase, alpha-catalytic subunit (PKA C-alpha)
CAI39142	cAMP-dependent protein kinase, catalytic subunit 2-4 [Paramecium tetraurelia]
BAA08297	cGMP-dependent protein kinase type I alpha [Homo sapiens]
NP_001038039	protein kinase, cGMP-dependent, type I [Sus scrofa]
XP_001080425	PREDICTED: similar to protein kinase, cGMP-dependent, type I alpha isoform 1 [Rattus norvegicus]
NP_001013855	protein kinase, cGMP-dependent, type I alpha isoform [Mus musculus]
O77676	cGMP-dependent protein kinase 1, alpha isozyme (CGK 1 alpha) (cGKI-alpha)
XP_219805	PREDICTED: similar to protein kinase, cGMP-dependent, type I alpha isoform [Rattus norvegicus]
P21136	cGMP-dependent protein kinase 1, beta isozyme (cGK 1 beta) (cGKI-beta)
BAD12117	cGMP-dependent protein kinase I alpha [Oryzias latipes]
NP_006249	protein kinase, cGMP-dependent, type I [Homo sapiens]
AAQ02512	protein kinase, cGMP-dependent, type I [synthetic construct]
ABJ74168	cGMP-dependent protein kinase type I beta [Sus scrofa]
XP_001080430	PREDICTED: similar to cGMP-dependent protein kinase 1, beta isozyme (cGK 1 beta) (cGKI-beta) isoform 2 [Rattus norvegicus]
Q9Z0Z0	cGMP-dependent protein kinase 1, beta isozyme (cGK 1 beta) (cGKI-beta)
NP_957324	protein kinase, cGMP-dependent, type I [Danio rerio]
XP_001374685	PREDICTED: hypothetical protein [Monodelphis domestica]
BAD12118	cGMP-dependent protein kinase I beta [Oryzias latipes]
CAF98611	unnamed protein product [Tetraodon nigroviridis]
ABG56236	PKG/For protein [Lobesia botrana]
AAL76256	PKG-Ia [Bombyx mori]
XP_851997	PREDICTED: similar to protein kinase, cGMP-dependent, type I [Canis familiaris]
AAL76257	PKG-II [Bombyx mori]
EAL33756	[Drosophila pseudoobscura]
ABI97017	foraging [Diabrotica virgifera virgifera]
CAG05402	unnamed protein product [Tetraodon nigroviridis]
NP_477487	foraging CG10033-PA, isoform A [Drosophila melanogaster]
NP_477488	foraging CG10033-PB, isoform B [Drosophila melanogaster]
XP_973707	PREDICTED: similar to CG10033-PA, isoform A [Tribolium castaneum]
AAA28459	cGMP-dependent protein kinase
AAA28455	cGMP-dependent protein kinase
ABL74445	cGMP dependent protein kinase [Vespula vulgaris]
EAT40455	cgmp-dependent protein kinase [Aedes aegypti]
XP_694793	PREDICTED: similar to cGMP kinase type I alpha [Danio rerio]
AAL93136	cGMP-dependent protein kinase foraging [Apis mellifera]
AAR06171	PKG [Aplysia californica]
8XP_319605	ENSANGP00000006403 [Anopheles gambiae str. PEST]
AAA28454	cGMP-dependent protein kinase
AAG22254	isoform E [Drosophila melanogaster]

8NP_995627	foraging CG10033-PF, isoform F [Drosophila melanogaster]
AAA28457	cGMP-dependent protein kinase
AAA28458	cGMP-dependent protein kinase
XP_968718	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
XP_684200	PREDICTED: similar to Type II cGMP-dependent protein kinase [Danio rerio]
AAL13517	GH03852p [Drosophila melanogaster]
AAB03405	cGMP-dependent protein kinase [Drosophila melanogaster]
XP_314690	ENSANGP00000013014 [Anopheles gambiae str. PEST]
EAL34020	[Drosophila pseudoobscura]
EAT42737	cgmp-dependent protein kinase [Aedes aegypti]
XP_001364928	PREDICTED: similar to Type II cGMP-dependent protein kinase [Monodelphis domestica]
XP_612707	PREDICTED: similar to Type II cGMP-dependent protein kinase [Bos taurus]
NP_500141	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
AAM98010	Egg laying defective protein 4, isoform c [Caenorhabditis elegans]
AAD36953	Egg laying defective protein 4, isoform b [Caenorhabditis elegans]
AAU05567	Egg laying defective protein 4, isoform e [Caenorhabditis elegans]
AAC23588	cyclic GMP-dependent protein kinase [Hydra oligactis]
XP_544949	PREDICTED: similar to protein kinase, cGMP-dependent, type II [Canis familiaris]
XP_001195817	PREDICTED: similar to PKG [Strongylocentrotus purpuratus]
BAC30119	unnamed protein product [Mus musculus]
ABK42092	cGMP-dependent protein kinase, type 2 [synthetic construct]
Q64595	cGMP-dependent protein kinase 2 (CGK 2) (cGKII) (Type II cGMP-dependent protein kinase)
EAX05867	protein kinase, cGMP-dependent, type II [Homo sapiens]
NP_032952	protein kinase, cGMP-dependent, type II [Mus musculus]
Q61410	cGMP-dependent protein kinase 2 (CGK 2) (cGKII) (Type II cGMP-dependent protein kinase)
CAE63845	Hypothetical protein CBG08401 [Caenorhabditis briggsae]
BAA18934	cGMP-dependent protein kinase II [Homo sapiens]
CAM15194	novel protein similar to vertebrate protein kinase, cGMP-dependent, type II (PRKG2) [Danio rerio]
AAH72999	protein [Xenopus laevis]
XP_001084948	PREDICTED: similar to protein kinase, cGMP-dependent, type II [Macaca mulatta]
CAA76073	cGMP-dependant protein kinase [Homo sapiens]
CAF90993	unnamed protein product [Tetraodon nigroviridis]
XP_517194	PREDICTED: protein kinase, cGMP-dependent, type II [Pan troglodytes]
XP_001186191	PREDICTED: similar to cGMP kinase type I alpha [Strongylocentrotus purpuratus]
CAI40744	protein kinase, cGMP-dependent, type I [Homo sapiens]
XP_001122119	PREDICTED: similar to cGMP-dependent protein kinase 21D CG3324-PA [Apis mellifera]
D88640	protein F55A8.2 [imported] - Caenorhabditis elegans
EAT34556	cgmp-dependent protein kinase [Aedes aegypti]
EAA14287	ENSANGP00000015733 [Anopheles gambiae str. PEST]
NP_001023224	EGg Laying defective family member (egl-4) [Caenorhabditis elegans]
AAV65146	cGMP-protein kinase [Pogonomyrmex barbatus]
CAI20607	novel protein similar to vertebrate protein kinase, cGMP-dependent, type I (PRKG1) [Danio rerio]
XP_001340942	PREDICTED: hypothetical protein [Danio rerio]
XP_974277	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
EAL33366	[Drosophila pseudoobscura]
AAM75371	Hypothetical protein C09G4.2c [Caenorhabditis elegans]
AAM75369	Hypothetical protein C09G4.2a [Caenorhabditis elegans]

T29830	hypothetical protein C09G4.2 - Caenorhabditis elegans
AAF52864	CG4839-PA, isoform A [Drosophila melanogaster]
CAE69446	Hypothetical protein CBG15634 [Caenorhabditis briggsae]
XP_969044	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
CAG09161	unnamed protein product [Tetraodon nigroviridis]
XP_624106	PREDICTED: similar to cGMP-dependent protein kinase 21D CG3324-PA [Apis mellifera]
AAH62688	PRKG1 protein [Homo sapiens]
XP_001340942	PREDICTED: hypothetical protein isoform 1 [Pan troglodytes]
1511094B	cGMP dependent protein kinase I beta
CAF91740	unnamed protein product [Tetraodon nigroviridis]
CAI41306	protein kinase, cGMP-dependent, type I [Homo sapiens]
AAX26936	SJCHGC04415 protein [Schistosoma japonicum]
XP_968796	PREDICTED: similar to CG3324-PA [Tribolium castaneum]
CAL52451	cGMP-dependent protein kinase, putative (ISS) [Ostreococcus tauri]
XP_001348520	cGMP-dependent protein kinase 1, beta isozyme, putative [Plasmodium falciparum 3D7]
AAM75370	Hypothetical protein C09G4.2b [Caenorhabditis elegans]
CAH98038	cGMP-dependent protein kinase 1, beta isozyme, putative [Plasmodium berghei]
EAA21741	cGMP-dependent protein kinase-related [Plasmodium yoelii yoelii]
XP_001254388	PREDICTED: similar to cGMP-dependent protein kinase type I beta [Bos taurus]
CAI39147	cAMP-dependent protein kinase, catalytic subunit 39083 [Paramecium tetraurelia]
CAI39146	cAMP-dependent protein kinase, catalytic subunit 39084 [Paramecium tetraurelia]
EAL36055	protein kinase (EC 2.7.1.37), cAMP-dependent, catalytic chain [Cryptosporidium hominis]
AAX55640	cAMP-dependent protein kinase catalytic subunit isoform 2 [Toxoplasma gondii]
XP_001388119	protein kinase, cAMP-dependent, catalytic chain [Cryptosporidium parvum Iowa II]
BAB18105	cyclic nucreotide dependent protein kinase II [Chlamydomonas reinhardtii]
XP_690430	PREDICTED: hypothetical protein [Danio rerio]
XP_001086667	PREDICTED: protein kinase, X-linked [Macaca mulatta]
BAB18104	cyclic nucleotide dependent protein kinase [Chlamydomonas reinhardtii]
CAA59733	protein kinase [Homo sapiens]
XP_416852	PREDICTED: similar to protein kinase [Gallus gallus]
AAW26566	protein [Schistosoma japonicum]
XP_763539	cGMP-dependent protein kinase [Theileria parva strain Muguga]
AAM20901	cGMP-dependent protein kinase [Toxoplasma gondii]
AAM27174	cGMP dependent protein kinase [Toxoplasma gondii]
XP_955206	cGMP-dependent protein kinase [Theileria annulata strain Ankara]
XP_001242879	hypothetical protein CIMG_06775 [Coccidioides immitis RS]
XP_973065	PREDICTED: similar to CG6117-PA, isoform A [Tribolium castaneum]
AAC24242	cAMP-dependent protein kinase catalytic subunit [Ustilago maydis]
AAC24243	cAMP-dependent protein kinase catalytic subunit [Ustilago maydis]
XP_001274194	cAMP-dependent protein kinase catalytic subunit, putative [Aspergillus clavatus NRRL 1]
EAN77055	protein kinase A catalytic subunit isoform 1 [Trypanosoma brucei]
AAU50669	PRKY [Pan troglodytes]
XP_001...	PREDICTED: similar to PRKY [Pan troglodytes verus]
AAD16003	cAMP-dependent protein kinase catalytic subunit isoform 2 [Amblyomma americanum]
AAD16004	cAMP-dependent protein kinase catalytic subunit isoform 3 [Amblyomma americanum]

AAD16002	cAMP-dependent protein kinase catalytic subunit isoform 1 [Amblyomma americanum]
XP_827379	protein kinase A catalytic subunit isoform 2 [Trypanosoma brucei]
AAT81143	cGMP-dependent protein kinase [Chlamydomonas reinhardtii]
CAI38998	cAMP-dependent protein kinase, catalytic subunit 39173 [Paramecium tetraurelia]
CAI38997	cAMP-dependent protein kinase, catalytic subunit 39174 [Paramecium tetraurelia]
CAK63546	unnamed protein product [Paramecium tetraurelia]
CAK74108	unnamed protein product [Paramecium tetraurelia]
CAI39138	cAMP-dependent protein kinase, catalytic subunit 39121 [Paramecium tetraurelia]
CAA45015	catalytic subunit of protein kinase A [Aplysia californica]
XP_821623	protein kinase A catalytic subunit isoform 1 [Trypanosoma cruzi]
AAL17691	protein kinase-A catalytic subunit [Trypanosoma cruzi]
NP_001029135	protein kinase, X-linked [Rattus norvegicus]
CAI39139	cAMP-dependent protein kinase, catalytic subunit 39120 [Paramecium tetraurelia]
XP_001381342	PREDICTED: similar to cAMP-dependent protein kinase, alpha-catalytic subunit (PKA C-alpha) [Monodelphis domestica]
XP_001347133	cAMP-dependent protein kinase catalytic subunit, putative [Paramecium tetraurelia]
XP_852513	PREDICTED: similar to Serine/threonine-protein kinase PRKX (Protein kinase PKX1) [Canis familiaris]
CAI39144	cAMP-dependent protein kinase, catalytic subunit 39115 [Paramecium tetraurelia]
CAK59278	unnamed protein product [Paramecium tetraurelia]
Q922R0	Serine/threonine-protein kinase PRKX (PKA-related protein kinase)
CAG44454	cAMP-dependent protein kinase catalytic subunit [Amblyomma hebraeum]
CAG44453	cAMP-dependent protein kinase catalytic subunit [Amblyomma hebraeum]
CAG44455	cAMP-dependent protein kinase catalytic subunit [Amblyomma hebraeum]
XP_001136686	PREDICTED: cAMP-dependent protein kinase catalytic subunit beta isoform 15 [Pan troglodytes]
AAH46697	Kin-1-prov protein [Xenopus laevis]
CAI39145	cAMP-dependent protein kinase, catalytic subunit 39114 [Paramecium tetraurelia]
CAK76252	unnamed protein product [Paramecium tetraurelia]
XP_001105747	PREDICTED: protein kinase, cAMP-dependent, catalytic, beta isoform 5 [Macaca mulatta]
XP_582115	PREDICTED: similar to protein kinase [Bos taurus]
P49673	cAMP-dependent protein kinase catalytic subunit (PKA C)
CAH91423	hypothetical protein [Pongo pygmaeus]
XP_867488	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 5 [Canis familiaris]
XP_867499	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 6 [Canis familiaris]
XP_867517	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 8 [Canis familiaris]
XP_867524	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 9 [Canis familiaris]
CAK71602	unnamed protein product [Paramecium tetraurelia]
AAX26927	SJCHGC08677 protein [Schistosoma japonicum]
XP_537099	PREDICTED: similar to cAMP-dependent protein kinase catalytic subunit beta isoform 1 isoform 1 [Canis familiaris]
XP_821620	protein kinase A catalytic subunit isoform 2 [Trypanosoma cruzi]

XP_867543	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 11 [Canis familiaris]
XP_867507	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 7 [Canis familiaris]
XP_867534	PREDICTED: similar to cAMP-dependent protein kinase, beta-catalytic subunit (PKA C-beta) isoform 10 [Canis familiaris]

We claim:

1. A recombinant host cell, comprising:

(a) a recombinant expression vector operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and

(b) a recombinant expression vector operably linked to a nucleic acid sequence encoding an active HSP20 polypeptide.

2. The recombinant host cell of claim1 wherein the host cell is prokaryotic.

3. The recombinant host cell of claim1 wherein the host cell is eukaryotic.

4. The recombinant host cell of claim 1 wherein the host cell is selected from the group consisting of a bacterial cell, yeast, and a human-derived cell.

5. The recombinant host cell of claim 1 wherein the recombinant expression vector comprises a plasmid.

6. The recombinant host cell of claim 1 wherein a first recombinant expression vector is operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and a second recombinant expression vector is operably linked to a nucleic acid sequence encoding an active HSP20 polypeptide.

7. The recombinant host cell of claim 1 wherein the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of general formula I:

X1-[X2-ASAPLP-X3-]u-X4,

wherein X1 is absent or comprises a transduction domain;

X2 is 0, 1, 2, 3, or 4 amino acids of the sequence WLRR (SEQ ID NO: 7);

X3 is 0, 1, 2, or 3 amino acids of a sequence of genus Z1-Z2-Z3,

wherein Z1 is selected from the group consisting of G and D;

Z2 is selected from the group consisting of L and K; and

Z3 is selected from the group consisting of S, K and T;

X4 is absent or comprises a transduction domain; and

wherein u is 1, 2, 3, 4, or 5.

8. The recombinant host cell of claim 7, wherein at least one of X1 and X4 comprises a transduction domain.

9. The recombinant host cell of claim 1 wherein the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of general formula II:

X1-X2-ASAPLP-X3-X4 (SEQ ID NO: 8)

wherein X1 is absent or comprises a cell transduction domain;

X2 is 0-14 amino acids of the sequence of heat shock protein 20 between residues 1 and 14 of SEQ ID NO:1;

X3 is 0-140 amino acids of heat shock protein 20 between residues 21 and 160 of SEQ ID NO:1; and

X4 is absent or comprises a cell transduction domain.

10. The recombinant host cell of claim 9, wherein at least one of X1 and X4 comprises a transduction domain.

11. The recombinant host cell of claim 1 wherein the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of formula:

X1-(**SEQ ID NO:10**)-X4,

wherein X1 is absent or comprises a cell transduction domain;

wherein (SEQ ID NO: 10) is Trp Leu Arg Arg Ala Ser Ala Pro Leu Pro Gly Leu Lys; and

wherein X4 is absent or comprises a cell transduction domain.

12. The recombinant host cell of claim 1, wherein the nucleic acid sequence encoding an active HSP20 polypeptide encodes an active HSP20 polypeptide of general formula III:

X1-[X2-X3-RRASAP]u-X4,

wherein X1 is absent or comprises a transduction domain;

X2 is absent or is W;

X3 is absent or is L

X4 is absent or comprises a transduction domain; and

wherein u is 1, 2, 3, 4, or 5.

13. The recombinant host cell of claim 12, wherein at least one of X1 and X4 comprises a transduction domain.

14. The recombinant host cell of any one of claims 10-13 wherein the transduction domain comprises a polypeptide of formula

$(WLRRIKA)_n$,

wherein n is between 1 and 10.

15. The recombinant host cell of claim 14 wherein the transduction domain is YARAAARQARA (SEQ ID NO: 35).

16. The recombinant host cell of claim 14 wherein the transduction domain is YGRKKRRQRRR (SEQ ID NO: 34).

17. A method for making phosphorylated HSP20 polypeptide, comprising culturing the recombinant host cells of claim 1 under conditions to promote expression of the catalytic subunit of the protein kinase and the active HSP20 polypeptide, and to promote phosphorylation of the active HSP20 polypeptide by the expressed protein kinase catalytic subunit.

18. A recombinant host cell, comprising:

(a) a recombinant expression vector operably linked to a nucleic acid sequence encoding a catalytic subunit of a protein kinase selected from the group consisting of protein kinase A and protein kinase G; and

(b) a recombinant expression vector operably linked to a nucleic acid sequence encoding a polypeptide target of protein kinase A or protein kinase G.

19. A method for treating a condition susceptible to treatment with an active HSP20 polypeptide, comprising administering a recombinant host cell of any one of claims 1-16 to a subject in need thereof, wherein the active HSP20 polypeptide produced by the host cell treats the condition.

20. The method of claim 19 wherein the condition is selected from the group consisting of: intimal hyperplasia, stenosis, restenosis, atherosclerosis, smooth muscle cell tumors, smooth muscle spasm, angina, Prinzmetal's angina (coronary vasospasm), ischemia, stroke, bradycardia, hypertension, pulmonary (lung) hypertension, asthma (bronchospasm), toxemia of pregnancy, pre-term labor, pre-eclampsia/eclampsia, Raynaud's disease or phenomenon, hemolytic-uremia, non-occlusive mesenteric ischemia, anal fissure, achalasia, impotence, migraine, ischemic muscle injury associated with smooth muscle spasm, vasculopathy, such as transplant vasculopathy, bradyarrythmia, bradycardia, congestive heart failure, stunned myocardium, pulmonary hypertension, and diastolic dysfunction.