KINASE ANCHOR PROTEIN MUTEINS, PEPTIDES THEREOF AND RELATED METHODS

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ABSTRACT
A-kinase anchor protein (AKAPs) muteins, peptides thereof, and nucleic acids encoding the peptides are provided herein. Also provided are transgenic animals, cells comprising transgenes and various methods employing such peptides.
PKA-Rla-GFP
L-amino acid substitution

PKA-IIla-GFP
L-amino acid substitution

valine variant peptide

isoleucine variant peptide

FIG. 1
This application is a continuation of allowed U.S. application Ser. No. 10/428,254, filed May 1, 2003. Benefit of priority under §119(e) is also claimed to U.S. Provisional Application Ser. No. 60/377,852, entitled “Kinase Anchor Protein Muteins, Peptides Thereof, and Related Methods”, filed May 3, 2002, attorney docket number 24736-P2065; and to U.S. Provisional Application Ser. No. 60/453,408, entitled “Kinase Anchor Protein Muteins, Peptides Thereof, and Related Methods”, filed Mar. 7, 2003, attorney docket number 24736-I2065B. The subject matter of each of these applications is incorporated in its entirety by reference thereto.

This application is also related to International PCT Application No. PCT/US03/13698, filed on May 1, 2003, entitled “Kinase Anchor Protein Muteins, Peptides Thereof, and Related Methods.” The disclosure of the PCT application is herein incorporated by reference in its entirety.

Work described herein was supported by NIH grants DK-54441 and 5T32 DK-07233. The government may have certain rights in subject matter provided herein.

A-kinase anchor protein (AKAPs) muteins, peptides thereof, and nucleic acids encoding the peptides are provided herein.

Protein phosphorylation is an important mechanism for enzyme regulation and the transduction of extracellular signals across the cell membrane in eukaryotic cells. A wide variety of cellular substrates, including enzymes, membrane receptors, ion channels and transcription factors, can be phosphorylated in response to extracellular signals that interact with cells. A key enzyme in the phosphorylation of cellular proteins in response to hormones and neurotransmitters is cyclic AMP (cAMP)-dependent protein kinase (PKA). Upon activation by cAMP, PKA thus mediates a variety of cellular responses to such extracellular signals.

An array of PKA isoforms are expressed in mammalian cells. The PKA holoenzymes usually exist as inactive tetramers containing a regulatory (R) subunit dimer and two catalytic (C) subunits. Genes encoding three C subunits (Cα, Cβ and Cγ) and four R subunits (RⅠα, RⅠβ, RⅠδ and RⅠγ) have been identified (see Takio et al. (1982) Proc. Natl. Acad. Sci. USA, 79:2544-2548; Lee et al. (1983) Proc. Natl. Acad. Sci. USA, 80:3608-3612; Jahnus et al. (1996) J. Biol. Chem., 261:12352-12361; Clegg et al. (1988) Proc. Natl. Acad. Sci. USA, 85:3703-3707; and Scott (1991) Pharmacol. Ther., 50:123-145). The type I (RⅠ)α and type II (RⅠ)α subunits are distributed ubiquitously, whereas RⅠβ and RⅠγ are present mainly in brain (see, e.g., Miki and Eddy (1999) J. Biol. Chem., 274:29057-29062). The type I PKA holoenzyme (RⅠα and RⅠγ) is predominantly cytoplasmic, whereas the majority of type II PKA (RⅠα and RⅠβ) associates with cellular structures and organelles (Scott (1991) Pharmacol. Ther., 50:123-145). Many hormones and other signals act through receptors to generate cAMP which binds to the R subunits of PKA and releases and activates the C subunits to phosphorylate proteins.


Up to 75% of type II PKA is localized to various intracellular sites through association of the regulatory subunit (RⅠ) with AKAPs (see, e.g., Hausken et al. (1996) J. Biol. Chem., 271:29016-29022). RⅠ subunits of PKA bind to AKAPs with nanomolar affinity (Carr et al. (1992) J. Biol. Chem., 267:13376-13382), and many AKAP-RⅠ complexes have been isolated from cell extracts. RⅠ subunits of PKA bind to AKAPs with only micromolar affinity (Burton et al. (1997) Proc. Natl. Acad. Sci. USA 94:11067-11102). Evidence of binding of a PKA RⅠ subunit to an AKAP has been reported (Miki and Eddy (1998) J. Biol. Chem., 273:34384-34390) in which RⅠα-specific and RⅠα/RⅠγ dual specificity PKA anchoring domains were identified on FSC1/AKAP82. Additional dual specific AKAPs, referred to as D-AKAP1 and D-AKAP2, which interact with the type I and type II regulatory subunits of PKA have also been reported (Huang et al. (1997) J. Biol. Chem., 272:8057-8064; Huang et al. (1997) Proc. Natl. Acad. Sci. USA, 94:11184-11189).

More than 20 AKAPs have been reported in different tissues and species. Complementary DNAs (cDNAs) encoding AKAPs have been isolated from diverse species, ranging from Caenorhabditis elegans and Drosophila to human (see, e.g., Collinge and Scott (1999) Trends Cell Biol., 9:216-221). Regions within AKAPs that mediate association with RⅠ subunits of PKA have been identified. These regions of approximately 10-18 amino acid residues vary substantially in primary sequence, but secondary structure predictions indicate that they are likely to form an amphipathic helix with hydrophobic residues aligned along one face of the helix and charged residues along the other (Carr et al. (1991) J. Biol. Chem., 266:14188-14192; Carr et al. (1992) J. Biol. Chem., 267:13376-13382). Hydrophobic amino acids with a long aliphatic side chain, e.g., valine, leucine or isoleucine, may participate in binding to RⅠ subunits (Glantz et al. (1993) J. Biol. Chem., 268:12796-12804).

Many AKAPs also have the ability to bind to multiple proteins, including other signalling enzymes. For example, AKAP79 binds to PKA, protein kinase C (PKC) and the protein phosphatase calcineurin (PP2B) (Coghlan et al. (1995) Science, 267:108-112 and Klunck et al. (1996) Science, 271:1589-1592). Therefore, the targeting of AKAP79 to neuronal postsynaptic membranes brings together enzymes with opposite catalytic activities in a single complex.
AKAPs thus serve as potential regulatory mechanisms that increase the selectivity and intensity of a cAMP-mediated response. There is a need, therefore, to identify and elucidate the structural and functional properties of AKAPs in order to gain a complete understanding of the important role these proteins play in the basic functioning of cells.

SUMMARY OF THE INVENTION

Provided herein are D- or N-terminal peptide epitopes that exhibit modified binding to a regulatory subunit of PKA compared to native D- or N-terminal peptide epitopes. Provided herein are transgenic animals and cells comprising the peptides provided herein. In one embodiment, the epitopes exhibit a preferred exclusive binding to PKA-Ria subunits relative to PKA-Rir subunits; or enhanced binding to both Ria and Rir subunits. In another embodiment, the epitopes exhibit a preferred exclusive binding to PKA-Ria subunits relative to PKA-Rir subunits.

The peptides provided herein are useful to disrupt, e.g., in vitro, the binding of particular isoforms of PKA, e.g., PKA-Ria or PKA-Rir, to D- or N-terminal peptide epitopes provided herein. For example, peptides provided herein have enhanced ability to bind to either one of PKA-Ria or PKA-Rir isoforms are useful to competitively bind to their target PKA isoform and so prevent binding of the particular PKA isoform to its target protein. In a particular embodiment, the enhanced binding for one PKA isoform that binds weakly to the other isoform (e.g., VQGNTDEQEEAEOLAKIMWSDLLVIMQQ; SEQ ID NO:54 and 101, which binds tightly to PKA-Ria and weakly to PKA-Rir; see Table 8) is employed to specifically knock out a specific function of one particular isoform mediated by D- or N-terminal peptide epitopes.

The peptides provided herein that have a combination of one, two or more specific amino acid changes relative to unmodified native protein sequences can also be used to specifically design peptide mimetics (peptidomimetics) or other small molecules to modulate the D- or N-terminal peptide epitope biological function within cells or organisms. This change of function is contemplated herein to treat medical conditions like heart failure, arrhythmias, or prevent sudden death syndrome. In addition, the binding properties of the peptides and the peptides provided herein are well suited to design and establish high-throughput assay systems to screen large chemical compound libraries for the purpose of drug discovery. Such assay systems are also useful to characterize chemically modified lead compounds after the initial high-throughput screening.

The PKA Ria specific binding differences for the D- or N-terminal peptide epitopes provided herein are contemplated as translating into one or more health risks. AKAPs in general coordinate signaling through PKA by bringing together effector molecules at specific subcellular locations (Edwards et al. (2000) Current Opinion in Cell Biology, 12:217-221). The Ile/Val(646) variant (SEQ ID NO:64), which is selected for in the healthy population, is contemplated herein to result in a reduction in signaling through the PKA Ria isoform. This signal reduction could have a beneficial effect on the cell. The narrow affinity differences observed for the binding of the variants to Ria suggest that local concentrations of Ria are tightly regulated. While AKAP specific anchoring of PKA through Ria has been well documented, little was previously known about anchoring PKA through Ria. As provided herein, however, anchoring PKA through the Ria subunit appears to be more dynamic. Ria is found diffuse in the cytoplasm of most cells, however

several examples of Ria localization have been reported. Ria is recruited to the plasma membrane upon antigen-mediated lymphocyte activation (Skalbegg et al. (1994) Science, 263:84-87), localized to the neuromuscular junction of skeletal muscle (Barradeau et al. (2001) Proc. Natl. Acad. Sci. USA, 264:250-265), and associated with microtubules during certain stages of the cell cycle (Imazumi et al. (2001) Experimental Cell Research, 264:250-265). Therefore, dynamic regulation between intracellular compartments seems to be key for Ria mediated signaling and might be different depending on the D- or N-terminal peptide epitopes provided herein.

In addition to altering the dynamic nature of the Ria signaling pathway, the Ile/Val(646) variant of D- or N-terminal peptide epitopes is contemplated herein to alter the PKA isoform distribution and change the signaling specificity of PKA. Dynamic regulation between intracellular compartments seems to be key for Ria mediated signaling and might be different depending on the D- or N-terminal peptide epitopes provided herein.

Increasing evidence indicates that the Ria and Rir isoforms of PKA have distinct functions. The Ria isoform is predominant in growing cells while the Rir isoform is predominant in differentiated cells (Cho et al. (1995) Critical Reviews in Oncology/Hematology, 21:33-61). The importance of Ria regulation for the cell is indicated by the fact that Ria knockout mice are embryonically lethal (Amieux et al. (1997) J. Biol. Chem., 272:3993). In Ria and Rir knockout mice, Ria is up-regulated and seems to compensate for loss of these isoforms (Amieux et al. (1997) J. Biol. Chem., 272; 3993-3998). However, novel phenotypes result since the mice are lean and have an increased tolerance to alcohol (Cummings et al. (1996) Nature, 382:622-626); Thiele et al. (2000) J. Neuroscience, 20 RC75:1-6). These observations suggest an implication for dynamic PKA isoform regulation in lipid metabolism, which is contemplated herein as being modulated by D- or N-terminal peptide epitopes.

Also provided herein are methods of disrupting the binding of an Ile/Val(646) variant of D- or N-terminal peptide epitopes corresponding to SEQ ID NO:64 and 65 to the Ria subunit of PKA comprising contacting the Ria subunit with a peptide provided herein, such as set forth in Examples 5-9 or in the claims. Also provided is a method for modulating the amount of PKA-Ria bound to D- or N-terminal peptide epitopes provided herein, and contacting the PKA-Ria subunit with a peptide provided herein, such as set forth in Examples 5-9 or in the claims.
the effective intracellular concentration of the Val(646) isoform of D-AKAP2 (corresponding to SEQ ID NO:65) in the cell. The change can be where the intracellular concentration of the Val(646) isoform is increased, thereby increasing the amount of PKA localized to the mitochondria; or where the intracellular concentration of the Val(646) isoform is decreased, thereby decreasing the amount of PKA localized to the mitochondria. Also provided is a method of treating a subject manifesting a disease or disorder of signal transduction wherein there is an increased mitochondrial localization of PKA, said method comprising administering a peptide provided herein, such as disclosed in Example 5-9 and in the claims, or peptidomimetic thereof.

Also provided is a method of increasing the longevity of a subject in need thereof, comprising identifying a subject having the Val(646) isoform of D-AKAP2 therein, and treating said subject with an agent that disrupts the binding of the Val(646) isoform of D-AKAP2 to Rlx subunit of PKA. The agent can be any peptide disclosed herein, such as in Examples 5-9 or any of the claims, or peptidomimetic thereof. Also provided are methods for altering the ratio of PKA-Rlx/PKA-Rlx bound to D-AKAP2 in a cell comprising increasing the concentration of a Val(646) variant of D-AKAP2, corresponding to SEQ ID NO:65, in a cell, thereby increasing the ratio of PKA-Rlx PKA/PKA-Rlx bound to D-AKAP2 in a cell. Also provided is a method of decreasing the concentration of cAMP required to stimulate a cAMP mediated signalling pathway, comprising increasing the concentration of a Val(646) variant of D-AKAP2, corresponding to SEQ ID NO:65, in a cell.

Also provided are methods of screening for agents that decrease or disrupt the binding of a Val(646) variant of D-AKAP2 with Rlx PKA, comprising combining a candidate agent with a cell comprising a nucleotide sequence which encodes a Val(646) variant D-AKAP2 protein corresponding to SEQ ID NO:65 or the complement thereof, operably linked to a promoter such that the nucleotide sequence is expressed as a D-AKAP2 protein in the cell; and determining the effect of the agent upon the localization of PKA to the mitochondria, wherein a decrease in localization to the mitochondria identifies an agent that decreases the binding of a Val(646) variant of D-AKAP2 with Rlx PKA.

Provided herein are methods of screening for agents that decrease or disrupt the binding of a Val(646) variant of D-AKAP2 to an Rlx subunit of PKA, comprising combining a candidate agent with a cell comprising a nucleotide sequence encoding a D-AKAP2 peptide sequence that binds to Rlx; and determining the effect of the agent upon the binding of the peptide to Rlx and/or the localization of PKA to the mitochondria. The candidate agent is combined with the admixture in a cell-free system or intracellularly. The peptide sequence can be obtained from any one of the Examples or claims provided herein.

Also provided are methods for identifying a molecule that modulates the biological activity of a D-AKAP2 protein, comprising

combining the candidate molecule with a cell comprising a nucleotide sequence encoding a D-AKAP2 mutein or portion thereof that retains a biological activity exhibited by a full length variant protein, operably linked to a promoter such that the nucleotide sequence is expressed as an D-AKAP2 mutein or portion thereof in the cell; and

[0025] determining the effect of the molecule upon a biological activity of the D-AKAP2 mutein or portion thereof. The biological activity of the D-AKAP2 mutein or portion thereof can be determined by examining signal transduction in the cell. The biological activity can be the binding of D-AKAP2 protein or portion thereof to protein kinase A. Also, the biological activity of the D-AKAP2 protein or portion thereof can be determined by examining protein phosphorylation in the cell. The screening methods provided herein can be high-throughput.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows the results of incubating membranes containing the peptide array of the single amino acid substitutions using L-amino acids set forth in Example 7.

FIGS. 2A and 2B show the binding of AKB (dual) (■), AKB (RI) (Q9F, V21W, M25F) (●), AKB (RI) (A13L) (△) and AKB (null) (♦) peptides to full-length Rlx (A) and Rlx (B). Each peptide was fluorescently labeled and incubated with the corresponding regulatory subunit for 1 hour in 10 mM Hepes, 150 mM NaCl, 3 mM EDTA pH 7.4. Fluorescence anisotropy was used to monitor bound peptide.

DETAILED DESCRIPTION

A. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. All patents, patent applications, published applications and publications, Genbank sequences, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. Where reference is made to a URL or other such identifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, the term “mutein” refers to a peptide variant having one, two or more amino acid residue substitutions compared to a reference polypeptide, which can be a naturally occurring peptide sequence. The phrase “one, two, or more” in the context of amino acid residue substitutions encompasses peptide variants provided herein having one or more amino acid substitutions, e.g., in SEQ ID NOs:1, 2, 55, 63, 64 or 65, including peptides having any combination of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more of the amino acid residue substitutions set forth herein, such as those described above and in Examples 5-9. In addition to these combinations of variants, conservative substitutions can be added at residues that have not yet been modified or at already modified residues within the peptides.

As used herein, the phrase “modified binding” refers to either an increase or decrease in binding affinity to the respective control peptide. Control peptides used herein can be either one or both of the naturally occurring full length Ile/Val646 isoforms of D-AKAP2. Control peptides used herein can also be either one or both of the Ile/Val 27-mer isoforms set forth in SEQ ID NOs:1 or 2; or full length proteins, such as SEQ ID NOs:55, 64 or 65.
As used herein, the phrase “normal binding” refers to a substantially equivalent binding affinity of a mutein compared to the respective control peptide.

As used herein, the term “segment” refers to a contiguous portion of the reference polypeptide or nucleic acid. For example, a segment of amino acids 12-23 of SEQ ID NO:1 refers to the contiguous stretch of amino acids 12-23 of SEQ ID NO:1. The phrase “at least amino acids 12-23 of SEQ ID NOs:1 or 2 up to amino acids 1-27 of SEQ ID NOs:1 or 2” refers to multiple segments of different sizes ranging from amino acids 12-23 of SEQ ID NOs:1 or 2; amino acids 12-24 of SEQ ID NOs:1 or 2; amino acids 11-23 of SEQ ID NOs:1 or 2; amino acids 10-25 of SEQ ID NOs:1 or 2; up to amino acids 1-27 of SEQ ID NOs:1 or 2.

As used herein, the phrase “a regulatory subunit of PKA” refers to one of the subunits of a PKA holoenzyme. Exemplary PKA subunits include RIIα and RIIβ subunits.

As used herein, the phrase “peptide exhibits a preferred or exclusive binding to PKA-RIIα subunits relative to PKA-RIIα subunits”, or grammatical variations thereof, refers to a peptide that either has a higher binding affinity for PKA-RIIα subunits than for PKA-RIIβ or has the ability to bind to PKA-RIIα and substantially no ability to bind PKA-RIIβ.

As used herein, the phrase “peptide exhibits enhanced binding to both RIIα and RIIβ subunits” refers to a peptide that has a higher binding affinity for both PKA-RIIα and PKA-RIIβ subunits compared to the binding affinity of the peptide of SEQ ID NOs:1 or 2 for both PKA-RIIα and PKA-RIIβ subunits.

As used herein, the phrase “peptide exhibits a preferred or exclusive binding to PKA-RIIα subunits relative to PKA-RIIβ subunits”, or grammatical variations thereof, refers to a peptide that either has a higher binding affinity for PKA-RIIα subunits than for PKA-RIIβ or has the ability to bind to PKA-RIIα and substantially no ability to bind PKA-RIIβ.

As used herein, “polymorphism” refers to the coexistence of more than one form of a gene or portion thereof. A portion of a gene of which there are at least two different forms, i.e., two different nucleotide sequences, is referred to as a “polymorphic region of a gene”. A polymorphic region can be a single nucleotide, the identity of which differs in different alleles. A polymorphic region can also be several nucleotides in length.

As used herein, “polymorphic gene” refers to a gene having at least one polymorphic region.

As used herein, “allele”, which is used interchangeably herein with “allelic variant”, refers to alternative forms of a gene or portions thereof. Alleles occupy the same locus or position on homologous chromosomes. When a subject has two identical alleles of a gene, the subject is said to be homozygous for the gene or allele. When a subject has two different alleles of a gene, the subject is said to be heterozygous for the gene. Alleles of a specific gene can differ from each other in a single nucleotide, or several nucleotides, and can include substitutions, deletions, and insertions of nucleotides. An allele of a gene can also be a form of a gene containing a mutation.

As used herein, “predominant allele” refers to an allele that is represented in the greatest frequency for a given population. The allele or alleles that are present in lesser frequency are referred to as allelic variants.

As used herein, “associated” refers to coincidence with the development or manifestation of a disease, condition or phenotype. Association may be due to, but is not limited to, genes responsible for housekeeping functions whose alteration can provide the foundation for a variety of diseases and conditions, those that are part of a pathway that is involved in a specific disease, condition or phenotype and those that indirectly contribute to the manifestation of a disease, condition or phenotype.

As used herein, the term “subject” refers to mammals and in particular human beings.

As used herein, the term “gene” or “recombinant gene” refers to a nucleic acid molecule comprising an open reading frame and including at least one exon and (optionally) an intron sequence. A gene can be either RNA or DNA. Genes may include regions preceding and following the coding region (leader and trailer).

As used herein, “intron” refers to a DNA sequence present in a given gene which is spliced out during mRNA maturation.

As used herein, the term “coding sequence” refers to that portion of a gene that encodes an amino acid sequence of a protein.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art (see, Table 1).

As used herein, “amino acid residue” refers to an amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the “L” isomeric form. However, residues in the “D” isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH₂ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxyl terminus of a polypeptide. In keeping with standard polypeptide nomenclature described in J. Biol. Chem., 243:3552-3559 (1969) and adopted at 37 C.F.R. §§ 1.821-1.822, abbreviations for amino acid residues are shown in the following Table:

<table>
<thead>
<tr>
<th>SYMBOL</th>
<th>1-Letter</th>
<th>3-Letter</th>
<th>AMINO ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Tyr</td>
<td>tyrosine</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>Gly</td>
<td>glycine</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Phe</td>
<td>phenylalanine</td>
<td></td>
</tr>
<tr>
<td>M</td>
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<td>leucine</td>
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<td>T</td>
<td>Thr</td>
<td>threonine</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Val</td>
<td>valine</td>
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</tr>
<tr>
<td>P</td>
<td>Pro</td>
<td>proline</td>
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</tr>
<tr>
<td>K</td>
<td>Lys</td>
<td>lysine</td>
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</tr>
<tr>
<td>H</td>
<td>His</td>
<td>histidine</td>
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</tr>
<tr>
<td>Q</td>
<td>Glu</td>
<td>glutamine</td>
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</tr>
<tr>
<td>E</td>
<td>Glu</td>
<td>glutamic acid</td>
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<tr>
<td>Z</td>
<td>Gln</td>
<td>glutamine</td>
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<tr>
<td>W</td>
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<tr>
<td>R</td>
<td>Arg</td>
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TABLE 1-continued

Table of Correspondence

<table>
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<tr>
<th>SYMBOL</th>
<th>1-Letter</th>
<th>3-Letter</th>
<th>AMINO ACID</th>
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</thead>
<tbody>
<tr>
<td>D</td>
<td>Asp</td>
<td>aspartic acid</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Asn</td>
<td>asparagine</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Asx</td>
<td>Asn and/or Asp</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Cys</td>
<td>cysteine</td>
<td></td>
</tr>
<tr>
<td>X</td>
<td>Xaa</td>
<td>Unknown or other</td>
<td></td>
</tr>
</tbody>
</table>

[0048] It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminal to carboxyl-terminal. In addition, the phrase “amino acid residue” is broadly defined to include the amino acids listed in the Table of Correspondence and modified and unusual amino acids, such as those referred to in 37 C.F.R. § 1.821-1.822, and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or to an amino-terminal group such as NH₂ or to a carboxyl-terminal group such as COOH.

[0049] In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. (1987) Molecular Biology of the Gene, 4th Edition, The Benjamin/Cummings Pub. Co., p. 224).

[0050] Such substitutions are preferably made in accordance with those set forth in TABLE 2 as follows:

<table>
<thead>
<tr>
<th>Original residue (A)</th>
<th>Conservative substitution (G)</th>
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<tbody>
<tr>
<td>Ala (A)</td>
<td>Gly; Ser</td>
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<tr>
<td>Arg (R)</td>
<td>Lys</td>
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<tr>
<td>Asn (N)</td>
<td>Glu; His</td>
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<td>Ser</td>
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<td>Arg; Gin</td>
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<td>Leu; Val</td>
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<tr>
<td>Lys (K)</td>
<td>Arg; Glu; Glu</td>
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<tr>
<td>Met (M)</td>
<td>Leu; Tyr; Ile</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>Met; Leu; Tyr</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>Thr</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>Ser</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>Tyr</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>Trp; Phe</td>
</tr>
<tr>
<td>Val (V)</td>
<td>Ile; Leu</td>
</tr>
</tbody>
</table>

[0051] Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

[0052] As used herein, a DNA or nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a sequence encoding a therapeutic polypeptide. By the term “substantially homologous” is meant having at least 80%, preferably at least 90%, most preferably at least 95% homology therewith or a less percentage of homology or identity and conserved biological activity or function.

[0053] The terms “homology” and “identity” are often used interchangeably. In this regard, percent homology or identity may be determined, for example, by comparing sequence information using a GAP computer program. The GAP program uses the alignment method of Needleman and Wunsch (1970) J. Mol. Biol., 48:443, as revised by Smith and Waterman (1981) Adv. Appl. Math., 2:482. Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program may include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribkov and Burgess (1986) Nucl. Acids Res., 14:6745, as described by Schwartz and Dayhoff, eds., ATLAS OF PROTEIN SEQUENCE AND STRUCTURE, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

[0054] Whether any two nucleic acid molecules have nucleotide sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% “identical” can be determined using known computer algorithms such as the “FASTA” program, using, for example, the default parameters as in Pearson and Lipman (1988) Proc. Natl. Acad. Sci. USA, 85:2444. Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine identity.


[0056] Therefore, as used herein, the term “identity” represents a comparison between a test and a reference polypep-
tide or polynucleotide. For example, a test polypeptide may be defined as any polypeptide that is 90% or more identical to a reference polypeptide.

[0057] As used herein, the term at least “90% identical to” refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polypeptide length of 100 amino acids are compared, no more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons may be made between a test and reference polynucleotides. Such differences may be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they may be clustered in one or more locations of varying length up to the maximum allowable, e.g., 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deleions.

[0058] As used herein, stringency conditions refer to the washing conditions for removing the non-specific probes and conditions that are equivalent to either high, medium, or low stringency as described below:

[0059] 1) high stringency: 0.1xSSPE, 0.1% SDS, 65°C.
[0060] 2) medium stringency: 0.2xSSPE, 0.1% SDS, 50°C.
[0061] 3) low stringency: 1.0xSSPE, 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

[0062] As used herein, “heterologous DNA” is DNA that encodes RNA and proteins that are not normally produced in vivo by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes or is not present in the exact orientation or position as the homologous DNA in a wildtype cell. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

[0063] As used herein, “isolated” in reference to a nucleic acid molecule or polypeptide or other biomolecule means that the nucleic acid or polypeptide has been separated from the genetic environment from which the polypeptide or nucleic acid was obtained. It may also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not “isolated,” but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is “isolated”, as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as an “isolated polypeptide” or an “isolated polynucleotide” are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source.

For example, a recombinantly produced version of a compound can be substantially purified by the one-step method described in Smith and Johnson (1988) *Gene*, 67:31-40. The terms isolated and purified are sometimes used interchangeably.

[0064] Thus, by “isolated” is meant that the nucleic acid is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) immediately flank the gene encoding the nucleic acid of interest. Isolated DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a native DNA sequence, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

[0065] Isolated or purified, as it refers to preparations made from biological cells or hosts, means any cell extract containing the indicated DNA or protein, including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the protein of interest can be present at various degrees of purity in these preparations. The procedures may include for example, but are not limited to, ammonium sulfate fractionation, gel filtration, ion exchange change chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

[0066] As used herein, “production by recombinant DNA methods” refers to the use of the well known methods of molecular biology for expressing proteins encoded by cloned DNA, and may include methods such as gene shuffling and plasmid display with screening for desired specificities.

[0067] As used herein, a composition refers to any mixture of two or more products or compounds. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof.

[0068] As used herein, a combination refers to any association between two or more items.

[0069] As used herein, “substantially identical to a product” means sufficiently similar so that the property of interest is sufficiently unchanged so that the substantially identical product can be used in place of the product.

[0070] As used herein, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as “expression vectors”. In general, expression vectors of utility in recombinant DNA techniques are often in the form of “plasmids” which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. “Plasmid” and “vector” are used interchangeably as the plasmid is the most commonly used form of vector. Other such forms of expression vectors that serve equivalent functions and that become known in the art subsequently may be used.

[0071] As used herein, “predisposition to develop a disease or disorder” means that a subject having a particular genotype and/or haplotype has a higher likelihood than one not having such a genotype and/or haplotype for developing a particular disease or disorder.
As used herein, “morbidity” refers to conditions, such as diseases or disorders, that compromise the health and well-being of an organism, such as an animal. Morbidity susceptibility or morbidity-associated genes are genes that, when altered, for example, by a variation in nucleotide sequence, facilitate the expression of a specific disease clinical phenotype. Thus, morbidity susceptibility genes have the potential, upon alteration, of increasing the likelihood or general risk that an organism will develop a specific disease, which may decrease the longevity of the organism.

As used herein, “mortality” refers to the statistical likelihood that an organism, particularly an animal, will not survive a full predicted lifespan. Hence, a trait or a marker, such as a polymorphism, associated with increased mortality is observed at a lower frequency in older than younger segments of a population.

As used herein, the phrase “increasing the longevity of a subject in need thereof” refers to prolonging the life and/or health of a subject or organism. The methods provided herein are contemplated to increase the longevity or improve the health of a subject or organism by the administration of agents that disrupt the binding of the Val(646) isoform of D-AKAP2 to RIIa subunit of PKA. For example, protein phosphorylation is an important mechanism for enzyme regulation and signal transduction in eukaryotic cells, and therefore is an important mechanism to the longevity of cells and organisms as a whole. cAMP dependent protein kinase (PKA) mediates a variety of hormonal and neurotransmitter responses by phosphorylating a wide variety of substrates including enzymes, membrane receptors, ion channels and transcription factors. AKAPs direct the subcellular localization of cAMP-dependent protein kinase by binding to its regulatory subunits and therefore play a role in G-protein mediated receptor-signalling pathways (see, e.g., Huang et al. (1997) Proc. Natl. Acad. Sci., USA 94:11184). As set forth herein, AKAPs, such as D-AKAP2, have a PKA binding region therein.

In addition, the main way to rapidly regulate contractility in the mammalian heart is through the β-adrenergic receptor (β-AR) pathway. This mechanism is important as a means of responding to neurotransmitter (norepinephrine or hormone (epinephrine)) release. β-ARs belong to the large family of G protein-coupled receptors characterized by a typical structure having seven transmembrane domains. These receptors contain phosphorylation sites, which serve as targets for protein kinase A (PKA), protein kinase C, and β-adrenergic receptor kinases to desensitize the receptor in order to prevent an excessive β-adrenergic stimulation. The combination of the β-receptor, the G-protein complex, and adenyl cyclase is termed the β-adrenergic system that enhances activity of adenylate cyclase increasing cAMP levels. CAMP then activates PKA which initiates a cascade of events eventually leading to an increase in heart rate and contractility. Therefore, PKA, and thus D-AKAP2, are involved in both the signal transduction after β-adrenergic stimulation and the receptor desensitization through its phosphorylation.

The D-AKAP2 variants at amino acid 646 of SEQ ID NO:64 and 65 described herein map to the conserved AKB domain of D-AKAP2, which was previously shown to interact with the regulatory subunit of PKA. In accordance with the methods provided herein, it has been demonstrated that this variation in D-AKAP2 impacts the binding to PKA in an isoform specific manner both in vitro and in vivo. The Val(646) variant at amino acid 646 of SEQ ID NO:65, which has previously been identified as a deleterious allele associated with morbidity in the age-stratified approach (see, e.g., US Patent Application US20020040130A1 and WO 02/04489), binds three-fold tighter to the RIIa isoform when compared to the Ile(646) variant. At the cellular level, this affinity difference resulted in a decrease in mitochondrial localization of the Ile (646) variant. Accordingly, agents that disrupt the binding of the Val(646) isoform of D-AKAP2 to RIIa subunit of PKA are contemplated herein as having the ability to increase the longevity of a particular subject or organism.

As used herein, “transgenic animal” refers to any animal, preferably a non-human animal, e.g., a mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extra-chromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of a protein. However, transgenic animals in which the recombinant gene is silent are also contemplated, as for example, using the FLI or CRE recombinase dependent constructs. Moreover, “transgenic animal” also includes those recombinant animals in which gene disruption of one or more genes is caused by human intervention, including recombination and antisense techniques.

As used herein, “signal moiety” refers to any moiety that allows for the detection of a nucleic acid molecule. Included are moieties covalently attached to nucleic acids and those that are not.

As used herein, “molecule that modulates or effects the biological activity of an D-AKAP2 protein” refers to any drug, small molecule, nucleic acid (sense and antisense), ribozyme, protein, peptide, lipid, carbohydrate etc., or combination thereof, that directly or indirectly changes, alters, abolishes, increases or decreases a biological activity attributed to D-AKAP2 protein.

As used herein, “biological activity of an D-AKAP2 protein” refers to, but is not limited to: binding of D-AKAP2 to protein kinase A or its subunits (such as RI-α and/or RI-β); localization of D-AKAP2 protein to a subcellular site, e.g., the mitochondria; localization of protein kinase A to the mitochondria and/or binding of D-AKAP2 protein to other proteins including other signalling enzymes.

As used herein, “combining” refers to contacting the biologically active agent with a cell or animal such that the agent is introduced into the cell or animal. For a cell, any method that results in an agent traversing the plasma membrane is useful. For an animal, any of the standard routes of administration of an agent, e.g., oral, rectal, transmucosal, intravenous, intraperitoneal, intraventricular, subcutaneous, intramuscular, etc., can be used.

As used herein, “solid support” refers to a support substrate or matrix, such as silica, polymeric materials or glass. At least one surface of the support can be partially planar. Regions of the support may be physically separated, for example with trenches, grooves, wells or the like. Some examples of solid supports include slides and beads. Supports
are of such composition so as to allow for the immobilization or attachment of nucleic acids and other molecules such that these molecules retain their binding activity and/or biological activity.

[0083] As used herein, “array” refers to a collection of elements, such as nucleic acids, containing three or more members. An addressable array is one in which the members of the array are identifiable, typically by position on a solid support. Hence, in general, the members of the array will be immobilized to discrete identifiable loci on the surface of a solid phase.

[0084] As used herein, “specifically hybridizes” refers to hybridization of a probe or primer preferentially to a target sequence versus a non-target sequence. Those of skill in the art are familiar with parameters that affect hybridization; such as temperature, probe or primer length and composition, buffer composition and salt concentration and can readily adjust these parameters to achieve specific hybridization of a nucleic acid to a target sequence.

[0085] As used herein “nucleic acid” refers to polynucleotides such as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The terms should also be understood to include, as equivalents, derivatives, variants and analogs of either RNA or DNA made from nucleotide analogs, single-stranded (sense or antisense) and double-stranded polynucleotides. Deoxyribonucleotides include deoxyadenosine, deoxythymidine, deoxyguanosine and deoxyribonucleotides for RNA, the uracil base is uridine.

[0086] As used herein, “at a position corresponding to” refers to a position of interest (i.e., base number or residue number) in a nucleic acid molecule or protein relative to the position in another reference nucleic acid molecule or protein. Corresponding positions can be determined by comparing and aligning sequences to maximize the number of matching nucleotides or residues, for example, such that identity between the sequences is greater than 95%, preferably greater than 96%, more preferably greater than 97%, even more preferably greater than 98% and most preferably greater than 99%. The position of interest is then given the number assigned in the reference nucleic acid molecule. For example, it is shown herein that a particular polymorphism in D-AKAP2 occurs at nucleotide 2073 of SEQ ID NO:63. To identify the corresponding nucleotide in another allele or isolate, the sequences are aligned and then the position that lines up with 2073 is identified. Since various alleles may be of different length, the position designate 2073 may not be nucleotide 2073, but instead is at a position that corresponds to the position in the reference sequence.

[0087] As used herein, “primer” and “probe” refer to a nucleic acid molecule including DNA, RNA and analogs thereof, including protein nucleic acids (PNA), and mixtures thereof. Such molecules are typically of a length such that they are statistically unique (i.e., occur only once) in the genome of interest. Generally, for a probe or primer to be unique in the human genome, it contains at least 14, 16 or contiguous nucleotides of a sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleic acids long.

[0088] As used herein, “antisense nucleic acid molecule” refers to a molecule encoding a sequence complementary to at least a portion of an RNA molecule. The sequence is sufficiently complementary to be able to hybridize with the RNA, preferably under moderate or high stringency conditions to form a stable duplex. The ability to hybridize depends on the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it can contain and still form a stable duplex. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

[0089] As used herein, a “variant protein” or “variant peptide” refers to a protein or peptide encoded by a mutein variant of a D-AKAP2 gene which results in a change of an amino acid residue at a particular position relative to that position in the protein encoded by the predominant allele. Accordingly, a mutein protein or peptide refers to a polypeptide sequence that differs or varies from a respective naturally occurring sequence by one, two or more amino acid residues. For example, in the peptides provided herein, combinations of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more residue substitutions compared to a particular reference sequence, such as SEQ ID NOs:1 or 2, are contemplated herein in a single peptide. In a particular embodiment, the residue substitutions can be any combination of one or more of the single amino acid substitutions set forth herein, such as in Examples 5-9. In addition, other amino acid residue substitutions can be added to those disclosed herein, both conservative and non-conservative.

[0090] As used herein, “signal transduction” refers to the propagation of a signal. In general, an extracellular signal is transmitted through the cell membrane to become an intracellular signal. This signal can then stimulate a cellular response. The term also encompasses signals that are propagated entirely within a cell. The polypeptide molecules involved in signal transduction processes are typically receptor and non-receptor protein kinases, receptor and non-receptor protein phosphatases, nucleotide exchange factors, transcription factors, G-coupled protein receptors, G-proteins, and GTPase regulators. One of the key biochemical mechanisms involved in signal transduction is protein phosphorylation. D-AKAP2 proteins are involved in signal transduction as they bind to protein kinase A (PKA) and are thought to anchor the kinase at a location, e.g., the mitochondria, where PKA acts to phosphorylate a specific substrate, either at the mitochondria or at an ion channel. Thus, an alteration in D-AKAP2 binding to PKA, localization to the mitochondria, or phosphorylation by PKA, among other steps, will result in an alteration in signal transduction. Assays including those that determine phosphorylation by PKA, association of PKA and D-AKAP2 and localization of D-AKAP2 can be used to monitor the state of signal transduction.

[0091] As used herein, “binding to PKA”, refers to the interaction of the PKA binding domain (also referred to herein as the A-Kinase binding (AKB) domain) of an D-AKAP2 protein and the regulatory subunits RI and/or RII of the protein kinase A holoenzyme. For example, the AKB domain of human D-AKAP2 corresponds to amino acids 623-649 of SEQ ID NOs:63-65.

B. Polymorphic AKAPs

[0092] Polymorphisms of the genome can lead to altered gene function, protein function or mRNA instability. AKAPs provide a mechanism for regulating ubiquitous cAMP-dependent kinase (PKA) activity by tethering PKA to specific subcellular locations thereby segregating it with particular components in a given signaling pathway and contributing to specificity in cellular responses to extracellular signals. AKAPs thus play a fundamental role in the basic functioning
of cells, the response of cells to their environment and ultimately in the coordination of vital systems within an organism. Therefore, polymorphisms in AKAP gene sequences may significantly affect the proper functioning of cells and systems within organisms and could be directly linked with certain disorders or could predispose an organism to a variety of diseases and disorders, especially those involving alterations in cellular protein phosphorylation and/or signal transduction. Among such disorders and diseases are: neurodegenerative diseases, such as Alzheimer’s Disease, cardiovascular disorders, cardiac disorders, particularly disorders associated with altered left ventricular function, cardiomyopathies, proliferative disorders, bipolar disorder and other neurological disorders, obesity, neoplastic disease, diabetes, certain peripheral retinopathies, such as retinitis pigmentosa, and autoimmune disorders, such as Lupus erythematosus. The discovery of AKAP gene polymorphisms, such as those described herein, provides for the identification and development of diagnostic and prognostic methods, also provided herein, and the development of drug therapies and treatment regimens. Furthermore, polymorphisms of AKAP genes aid in the study of AKAP protein structure and function, which also contributes to the development of diagnostic methods and therapies.

AL-1. D-AKAP2

Although the mechanisms for targeting of D-AKAP2 are not known, the D-AKAP2 protein can be found associated with mitochondria. The sequence of a human D-AKAP2 cDNA (also referred to as D-AKAP2) is available in the GenBank database, at accession numbers AF037439 and NM 007202, and is provided in SEQ ID NO:63. The D-AKAP2 gene is located on chromosome 17.

The sequence of a mouse D-AKAP2 cDNA is also available in the GenBank database (see accession number AF021833). The mouse D-AKAP2 protein contains two RGS domains near the amino terminus that is characteristic of proteins that interact with Gtα subunits and possess GTPase activating protein-like activity (Huang et al. (1997) PNAS, USA, 94:11184-11189; and Wang et al. (2001) PNAS, USA, 98(6):3220-3225). The human D-AKAP2 protein has sequences homologous to two RGS domains. The carboxy-terminal 40 residues of the mouse D-AKAP2 protein are responsible for the interaction with the regulatory subunits of PKA. This sequence is fairly well conserved between the mouse D-AKAP2 and human D-AKAP2 proteins.

2. Polymorphisms of the Human D-AKAP2 Gene and Polymorphic D-AKAP2 Proteins

Polymorphisms of AKAP genes that alter gene expression, regulation, protein structure and/or protein function are more likely to have a significant effect on the regulation of enzyme (particularly PKA) activity, cellular transduction of signals and responses thereto and on the basic functioning of cells than polymorphisms that do not alter gene and/or protein function. Included in the polymorphic AKAPs herein are human D-AKAP2 proteins containing differing amino acid residues at position number 646 of SEQ ID NO:64.

Amino acid 646 of the human D-AKAP2 protein (SEQ ID NO:64) is located in the carboxy-terminal region of the protein within a segment that participates in the binding of R-subunits of PKAs. This segment includes the carboxy-terminal 40 amino acids.

The amino acid residue reported for position 646 of the human D-AKAP2 protein is an isoleucine. However, an allelic variant of the human D-AKAP2 gene is at the polymorphic site at position 2073 of the coding sequence (see SEQ ID NO:63) and encodes a valine at position 646 of the D-AKAP2 protein. This allelic variant has been found to vary in frequency in DNA samples from younger and older segments of a healthy population. This allele has the A at position 2073 of the D-AKAP2 gene coding sequence of SEQ ID NO:63 changed to a G. Consequently, the codon for amino acid 646 changes from ATT, coding for isoleucine, to GTT, coding for valine, as set forth in SEQ ID NO:65.

C. Peptides, Polypeptides and Peptide Mimetics

Provided herein are D-AKAP2 matein proteins, matein peptides thereof, and methods for identifying molecules (agents) that bind to and modulate the activity of PKA proteins. Included among mateins that bind to PKAs, particularly the regulatory subunits RIα and RIβ, are peptides derived from the AK/β binding domain of the Ile(Val)(464) variants of human D-AKAP2 corresponding to amino acids 623-649 of SEQ ID NO:64 and 65; polypeptides and peptide mimetics thereof, including cyclic peptides. In one embodiment, exemplary D-AKAP2-derived peptides provided herein comprise between 12 and 39 amino acid residues, wherein the peptide comprises amino acids 7-21 of SEQ ID NO:s1 or 2. In another embodiment, exemplary peptides comprise between 12 and 39 amino acid residues, wherein the peptide comprises a segment of SEQ ID NO:s1 or 2, wherein the length of the segment ranges from at least amino acids 12-23 of SEQ ID NO:s1 or 2, up to amino acids 1-27 of SEQ ID NO:s1 or 2. Accordingly, peptides having a length of 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39 amino acids are provided herein.

In another embodiment, exemplary D-AKAP2-derived peptides provided herein comprise between 15 and 39 amino acid residues, wherein the peptide comprises amino acids 7-21 of SEQ ID NO:s1 or 2. In one embodiment, the peptide binds to a regulatory subunit of PKA. In one embodiment the peptide binds to RIα. In another embodiment, the peptide comprises at least amino acids 11-24 of SEQ ID NO:s1 or 2 and binds to RIα. In a particular embodiment, the peptides provided herein bind to both RIα and RIβ subunits.

In one embodiment, the peptide exhibits a preferred or exclusive binding to PKA-RIα subunits relative to PKA-RIβ subunits; or enhanced binding to both RIα and RIβ subunits. In this particular embodiment, the peptide can be selected from the group consisting of peptides that correspond to the substitution in SEQ ID NO:s2 of one, two or more: of F at residue 9 with I, L, V, H, M, R, T, W or Y; of L at residue 12 with F, W or Y; of V at residue 21 with I, L or W; and of M at residue 25 with F, I, L, T, V, W or Y. In a particular embodiment, the peptide is 27 amino acids in length. In another embodiment, V at residue 21 is substituted with W. In yet another embodiment, V at residue 21 is substituted with W, and the peptide further comprises the substitution of either one or both of Q at residue 9 with F, and of M at residue 25 with F.

In another embodiment, the peptide exhibits a preferred or exclusive binding to PKA-RIα subunits relative to PKA-RIβ subunits. In this particular embodiment, the peptide can be selected from the group consisting of peptides that correspond to the substitution in SEQ ID NO:s of one, two or more: of L at residue 12 with A, C, or K; of A at residue 13 with F, H, I, K, L, M or N; of W at residue 14 with C; of K at residue 15 with C; of K at residue 18 with C; of M at residue
In a particular embodiment, the peptide is 27 amino acids in length.

In yet another embodiment, the peptide is 27 amino acids in length and exhibits preferred or exclusive binding to PKA-R1α subunits relative to PKA-R1β subunits. In this particular embodiment, the peptide can be selected from the group consisting of peptides that correspond to the substitution in SEQ ID NO: 1 of one, two, or more: of A at residue 13 with F, I, L, M, and S; of W at residue 14 with C; of K at residue 15 with C; of K at residue 18 with C; of M at residue 19 with C; of S at residue 22 with C; and of D at residue 23 with C.

In a particular embodiment, the peptide is 27 amino acids in length and exhibits preferred or exclusive binding to PKA-R1α subunits relative to PKA-R1β subunits. In this particular embodiment, the peptide can be selected from the group consisting of peptides that correspond to the substitution in SEQ ID NO: 1 of one, two, or more: of A at residue 13 with F, I, L, M, and S; of W at residue 14 with C; of K at residue 15 with C; of K at residue 18 with C; of M at residue 19 with C; of S at residue 22 with C; and of D at residue 23 with C.

In yet another embodiment, the D-AKAP2-derived peptide has enhanced binding to both PKA-R1α subunit and PKA-R1β subunit, compared to PKA R-subunit binding ability of the peptide of SEQ ID NOs: 1 or 2. In this particular embodiment, the peptide can be selected from the group consisting of:

\[
\begin{align*}
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 44}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 45}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 46}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 47}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 48}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 49}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 50})
\end{align*}
\]

In yet another embodiment, the D-AKAP peptide has a D-amino acid at the position indicated in bold, and an enhanced ability to bind to PKA-R1α subunit, while substantially no ability to bind to PKA-R1β subunit, compared to the PKA R-subunit binding ability of the peptide of SEQ ID NOs: 1 or 2. In this particular embodiment, the peptide can be selected from the group consisting of:

\[
\begin{align*}
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 51}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 52}) \\
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 53})
\end{align*}
\]

In yet another embodiment, the D-AKAP peptide has a D-amino acid at the position indicated in bold, and an enhanced ability to bind to PKA-R1α subunit, and a reduced ability to bind to PKA-R1β subunit, compared to the PKA R-subunit binding ability of the peptide of SEQ ID NOs: 1 or 2. In this particular embodiment, the peptide can be selected from the group consisting of:

\[
\begin{align*}
VQHTDEAEELANKIAKMIWSDI/VMQQ &; (\text{SEQ ID NO: 54})
\end{align*}
\]
In another embodiment, the D-AKAP peptide has enhanced ability to bind to PKA-Rlct subunit, and a reduced ability to bind to PKA-Rlla subunit, compared to the peptide of SEQ ID NOs:1 or 2, wherein the peptide is selected from the group consisting of:

`VQGNTDEAQEELAWKIAKMIVLDI/VMQQ` (SEQ ID NO:25) and `VQGNTDEAQEELAWKIAKMIVSDI/VFQQ` (SEQ ID NO:26)

In another embodiment, the D-AKAP peptide has enhanced ability to bind to PKA-Rlct subunit, while maintaining a normal ability to bind to PKA-Rlla subunit, compared to the peptide of SEQ ID NOs:1 or 2, wherein the peptide is:

`FEELAWKIAKMIVSDVFOOC` (SEQ ID NO:104; PV-37)
`FEELAWKIAKMIVSDVFOOC` (SEQ ID NO:103; PV-36)
`QEEFAWKIAKMIVSDVFOOC` (SEQ ID NO:105; PV-47)
`QEEFAWKIAKMIVSDVFOOC` (SEQ ID NO:106; PV-48)

In another embodiment, the peptide has enhanced ability to bind to PKA-Rlct subunit, compared to the peptide of SEQ ID NOs:1 or 2, wherein the peptide is:

`FEELAWKIAKMIVSDVFOOC` (SEQ ID NO:107; PV-49)

In another embodiment, peptide variants provided herein having one or more amino acid substitutions in SEQ ID NOs:1 or 2, include peptides having any combination of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more of the amino acid residue substitutions set forth herein, such as those described above and in Examples 5-9. In addition to these combinations of variants, conservative substitutions can be added at residues that have not yet been modified or at already modified residues within the peptides.

Also provided herein, based on the particular effect that a specific amino acid residue substitution has on the binding of a substituted peptide variant to either one or both of Rlct or Rlla, one or more amino acid residue substitutions can be selected to either selectively increase or decrease (i.e., disrupt) the binding affinity, and thereby increase the selectivity of a particular peptide for either Rlct or Rlla. The residues can be selected based on the effect of the residue substitutions provided herein as set forth in Examples 5-9 and Fig. 1. Accordingly, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more residues that enhance binding of a peptide to either one of both Rl or Rlla subunits can be combined. Accordingly, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more residues that decrease binding of a peptide to either one of both Rl or Rlla subunits can be combined. In certain embodiments, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more residues that either increase or decrease binding of a peptide to either one or both of Rl or Rlla subunits can be combined (e.g., residues substituted to increase binding to one R subunit and residues substituted to decrease binding to the other R subunit), and the like.

Accordingly, combinations of two or more amino acid substitutions that increase binding affinity for Rlct and/or decrease binding affinity for Rlla are contemplated herein. In another embodiment, combinations of two or more amino acid substitutions that decrease binding affinity for Rlct and/or increase binding affinity for Rlla are contemplated herein.

For example, in one embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 2 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 3 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 4 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 5 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 6 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 7 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 8 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 9 amino acid residue substitutions set forth herein. In another embodiment, peptides are provided having in a region corresponding to SEQ ID NOs:1 or 2, a combination of any 10 amino acid residue substitutions set forth herein.

Accordingly, provided herein are polypeptides that are muteins of a D-AKAP2 polypeptide, wherein the mutein exhibits modified binding to a regulatory subunit of PKA compared to a native D-AKAP2. The native D-AKAP2 can comprise a sequence of amino acids set forth as SEQ ID NOs:1 or 2. Also provided are polypeptides that are muteins of a D-AKAP2 polypeptide, wherein the mutein exhibits modified binding to a regulatory subunit of PKA compared to a control. The control is a polypeptide that consists essentially of the sequence of amino acids set forth as SEQ ID NOs:1 or 2. In one embodiment, the polypeptides exhibit enhanced binding to PKA-Rlla subunits. These peptides can further exhibit normal or reduced binding to PKA-Rlct subunits.

In another embodiment, the polypeptides exhibit enhanced binding to PKA-Rlla subunits relative to PKA-Rlct subunits. The peptides can further exhibit normal or reduced binding to PKA-Rlct subunits. In another embodiment, the peptides exhibit enhanced binding to both Rlct and Rlla subunits. In another embodiment, the peptides exhibit reduced binding to PKA-Rlct subunits. These peptides can further exhibit normal or increased binding to PKA-Rlla subunits. In another embodiment, the polypeptides exhibit reduced binding to PKA-Rlct. The peptides can further exhibit normal or increased binding to PKA-Rlla subunits. In yet another embodiment, the peptides exhibit reduced binding to both Rlct and Rlla subunits.

Also provided herein are variant D-AKAP2 peptides comprising one, two or more amino acid residue substitutions at positions corresponding to 1-27 of SEQ ID NOs:1 or 2, wherein the peptide further comprises a region of one or more amino acids, up to all of the amino acids, selected from amino acids 1-622 and 650-662 of SEQ ID NOs:64 or 65, wherein the one or more selected amino acids are contiguous with amino acid positions corresponding to 623-649 of SEQ ID NOs:64 or 65. In other words, polypeptides are provided herein that are at least 28 amino acids in length up to 662 amino acids that comprise the region corresponding to 623-649 of SEQ ID NOs:64 or 65, wherein the region correspond-
ing to 623-649 of SEQ ID NO:s:64 or 65 contains any combination of one, two or more of the amino acid residue substitutions provided herein, such as in the Examples or in the claims. Accordingly, in addition to containing single amino acid substitutions, D-AKAP2 proteins are provided having in a region corresponding to SEQ ID NO:s:1 or 2 (e.g., the region corresponding to 623-649 of SEQ ID NO:s:64 or 65), a combination of any 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acid residue substitutions set forth herein, such as in the Examples and in the claims.

[0119] In other embodiments, the additional amino acid residues obtained from amino acids 1-622 and 650-662 of SEQ ID NO:s:64 or 65 can contain conservative substitutions therein that do not alter the desired effect obtained by substituting one or more residues in the peptide region corresponding to 623-649 of SEQ ID NO:s:64 or 65. In addition, these polypeptides can further comprise additional contiguous amino acids at either end of the D-AKAP2 variant protein, where the additional amino acid sequence can be used for a variety of purposes, such as protein targeting, to facilitate protein purification.

[0120] Peptide mimetics are molecules or compounds that mimic the necessary molecular conformation of a ligand or polypeptide for specific binding to a target molecule such as a PKA holoenzyme. In an exemplary embodiment, the peptides, polypeptides or peptide mimetics bind to the RI and/or RII regulatory subunits of the PKA holoenzyme. Such peptides and peptide mimetics include those of antibodies that specifically bind to a PKA holoenzyme and, typically, bind to the RI and/or RII regulatory subunits of a PKA holoenzyme. The peptides, polypeptides and peptide mimetics identified by methods provided herein can be agonists or antagonists of PKA holoenzymes.

[0121] Such peptides, polypeptides and peptide mimetics are useful for diagnosing, treating, preventing, and screening for a disease or disorder associated with PKA holoenzyme activity in a mammal. In addition, the peptides and peptide mimetics are useful for identifying, isolating, and purifying molecules or compounds that modulate the activity of a PKA holoenzyme, or specifically bind to a PKA holoenzyme, generally the RI and/or RII regulatory subunits of a PKA holoenzyme. Low molecular weight peptides and peptide mimetics can have strong binding properties to a target molecule, e.g., a PKA holoenzyme or the RI and/or RII regulatory subunits of a PKA holoenzyme.

[0122] Peptides, polypeptides and peptide mimetics that bind to PKA holoenzymes as described herein can be administered to mammals, including humans, to modulate PKA holoenzyme activity. Thus, methods for therapeutic treatment and prevention of neurodegenerative diseases, such as Alzheimer’s Disease, cardiovascular disorders, cardiac disorders, particularly disorders associated with altered left ventricular function, cardiomyopathies, proliferative disorders, bipolar disorder and other neurologial disorders, lipid-metabolism disorders, such as obesity, neoplastic disease, diabetes and certain peripheral retinopathies, such as retinitis pigmentosa, and autoimmune disorders, such as Lupus erythematosus, comprise administering a peptide, polypeptide or peptide mimetic compound in an amount sufficient to modulate such activity are provided. Also provided herein are methods for treating a subject having such a disease or disorder in which a peptide, polypeptide or peptide mimetic compound is administered to the subject in a therapeutically effective dose or amount.

[0123] Compositions containing the peptides, polypeptides or peptide mimetics provided herein can be administered for prophylactic and/or therapeutic treatments. In therapeutic applications, compositions can be administered to a patient already suffering from a disease, as described above, in an therapeutically effective amount sufficient to cure or at least partially arrest the symptoms of the disease and its complications. Amounts effective for this use will depend on the severity of the disease and the weight and general state of the patient and can be empirically determined.

[0124] In prophylactic applications, compositions containing the peptides, polypeptides and peptide mimetics are administered to a patient susceptible to or otherwise at risk of a particular disease. Such an amount is defined to be a “prophylactically effective dose”. In this use, the precise amounts again depend on the patient’s state of health and weight.

[0125] Accordingly, the peptides, polypeptides and peptide mimetics that bind to a PKA holoenzyme can be used to prepare pharmaceutical compositions containing, as an active ingredient, at least one of the peptides or peptide mimetics in association with a pharmaceutical carrier or diluent. The compounds can be administered, for example, by oral, pulmonary, parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), inhalation (via a fine powder formulation), transdermal, nasal, vaginal, rectal, or sublingual routes of administration and can be formulated in dosage forms appropriate for each route of administration (see, e.g., International PCT Application Nos. WO93/25221 and WO94/17784; and European Patent Application 613,683).

[0126] Peptides, polypeptides and peptide mimetics that bind to PKA holoenzymes are useful in vitro as unique tools for understanding the biological role of PKA holoenzymes, including the evaluation of the many factors thought to influence, and be influenced by, the production of PKA holoenzyme. Such peptides, polypeptides and peptide mimetics are also useful in the development of other compounds that bind to and modulate the activity of a PKA holoenzyme, because such compounds provide important information on the relationship between structure and activity that should facilitate such development.

[0127] The peptides, polypeptides and peptide mimetics are also useful as competitive binders in assays to screen for new PKA holoenzymes or PKA holoenzyme agonists. In such assay embodiments, the compounds can be used without modification or can be modified in a variety of ways; for example, by labeling, such as covalently or non-covalently joining a moiety which directly or indirectly provides a detectable signal. In any of these assays, the materials thereto can be labeled either directly or indirectly. Exemplary labels for direct labeling include label groups such as: radionuclides such as 123I enzymes (U.S. Pat. No. 3,645,090), peroxidase and alkaline phosphate, and fluorescent labels (U.S. Pat. No. 3,940,475) capable of monitoring the change in fluorescence intensity, wavelength shift, or fluorescence polarization. Exemplary labels for indirect labeling include biotinylation of one constituent followed by binding to avidin coupled to one of the above label groups. The compounds can also include spacers or linkers in cases where the compounds are to be attached to a solid support.

[0128] In addition, based on their ability to bind to a PKA holoenzyme, the peptides, polypeptides and peptide mimetics can be used as reagents for detecting PKA holoenzymes in living cells, fixed cells, in biological fluids, in tissue homo-
gennates and in purified, natural biological materials. For example, by labelling such peptides, polypeptides and peptide mimetics, cells having PKA holoenzymes can be identified. In addition, based on their ability to bind to a PKA holoenzyme, the peptides, polypeptides and peptide mimetics can be used in situ staining, FACS (fluorescence-activated cell sorting), Western blotting, ELISA and other analytical protocols. Based on their ability to bind to a PKA holoenzyme, the peptides, polypeptides and peptide mimetics can be used in purification of PKA holoenzymes or in purifying cells expressing the PKA holoenzymes, e.g., a polypeptide encoding the RI and/or RII regulatory subunits of a PKA holoenzyme.

[0129] The peptides, polypeptides and peptide mimetics can also be used as commercial reagents for various medical research and diagnostic uses. The activity of the peptides and peptide mimetics can be evaluated either in vitro or in vivo in one of the numerous models described in McDonald (1992) Am. J. of Pediatric Hematology/Oncology, 14:8-21.

D. Peptide, Polypeptide and Peptide Mimetic Therapy


[0131] Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides containing a consensus sequence or a substantially identical consensus sequence variation can be generated by methods known in the art (Rizo et al. (1992) An. Rev. Biochem., 61:387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

[0132] Those skilled in the art appreciate that modifications can be made to the peptides and mimetics without deleteriously effecting the biological or functional activity of the peptide. Further, the skilled artisan would know how to design non-peptide structures in three dimensional terms, that mimic the peptides that bind to a target molecule, e.g., a PKA holoenzyme or, generally, the RI and/or RII subunit of PKA holoenzymes (see, e.g., Eck and Sprang (1989) J. Biol. Chem., 264:17605-18795).

[0133] When used for diagnostic purposes, the peptides and peptide mimetics can be labeled with a detectable label and, accordingly, the peptides and peptide mimetics without such a label can serve as intermediates in the preparation of labeled peptides and peptide mimetics. Detectable labels can be molecules or compounds, which when covalently attached to the peptides and peptide mimetics, permit detection of the peptide and peptide mimetics in vivo, for example, in a patient to whom the peptide or peptide mimetic has been administered, or in vitro, e.g., in a sample or cells. Suitable detectable labels are well known in the art and include, by way of example, radioisotopes, fluorescent labels (e.g., fluorescein), and the like. The particular detectable label employed is not critical and is selected to be detectable at non-toxic levels. Selection of the such labels is well within the skill of the art.

[0134] Covalent attachment of a detectable label to the peptide or peptide mimetic is accomplished by conventional methods well known in the art. For example, when the $^{125}$I radioisotope is employed as the detectable label, covalent attachment of $^{125}$I to the peptide or the peptide mimetic can be achieved by incorporating the amino acid tyrosine into the peptide or peptide mimetic and then iodinating the peptide (see, e.g., Weaver et al. (1994) Synthesis and Applications of Isotopically Labelled Compounds, pp. 137-140). If tyrosine is not present in the peptide or peptide mimetic, incorporation of tyrosine to the N or C terminus of the peptide or peptide mimetic can be achieved by well known chemistry. Likewise, $^{32}$P can be incorporated onto the peptide or peptide mimetic as a phosphate moiety through, for example, a hydroxyl group on the peptide or peptide mimetic using conventional chemistry.

[0135] Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecule(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivationization (e.g., labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

[0136] Peptides, polypeptides and peptide mimetics that can bind to a PKA holoenzyme or the RI and/or RII subunit of PKA holoenzymes and/or modulate the activity thereof, can be used for treatment of neurodegenerative diseases, such as Alzheimer’s Disease, cardiovascular disorders, cardiac disorders, particularly disorders associated with altered left ventricular function, cardiomyopathies, proliferative disorders, bipolar disorder and other neurological disorders, lipid-metabolism disorders, such as obesity, neoplastic disease, diabetes, certain peripheral neuropathies, such as retinitis pigmentosa, and autoimmune disorders, such as Lupus erythematosus. The peptides, polypeptides and peptide mimetics can be delivered, in vivo or ex vivo, to the cells of a subject in need of treatment. Further, peptides which have PKA holoenzyme activity can be delivered, in vivo or ex vivo, to cells which carry mutant or missing alleles encoding the PKA holoenzyme gene. Any of the techniques described herein or known to the skilled artisan can be used for preparation and in vivo or ex vivo delivery of such peptides, polypeptides and peptide mimetics that are substantially free of other human proteins. For example, the peptides, polypeptides and peptide mimetics can be readily prepared by expression in a microorganism or synthesis in vitro.

[0137] In particular embodiments, the peptides, polypeptides, and peptide mimetics provided herein are able to permeate cell membranes and thus affect binding of PKA to a D-AKAP2. For example, a peptide or mimetic may be modified to include a fatty-acid moiety by conventional methods, attached to either the amino terminus or the carboxy terminus of the peptide. Any fatty acid used in the art to achieve

These peptides, polypeptides, and peptide mimetics may be introduced into cells by any conventional means. For example, a peptide may be incorporated into liposomes. Alternatively, the peptide can be formulated in a composition that includes an amphipathic lipid, e.g., a head-to-tail amphiphile such as Lipofectin® or a cationic facial amphiphile (CFA) (a conjugate of polyamines and bile-acid-based amphiphiles).

The peptides or peptide mimetics can be introduced into cells, in vivo or ex vivo, by microinjection or by use of liposomes, for example. Alternatively, the peptides, polypeptides or peptide mimetics can be taken up by cells, in vivo or ex vivo, actively or by diffusion. In addition, extracellular application of the peptide, polypeptide or peptide mimetic can be sufficient to effect treatment of neurodegenerative diseases, such as Alzheimer’s Disease, cardiovascular disorders, cardiac disorders, particularly disorders associated with altered left ventricular function, cardiomyopathies, proliferative disorders, bipolar disorder and other neurological disorders, lipid-metabolism disorders, such as obesity, neoplastic disease, diabetes, certain peripheral retinopathies, such as retinitis pigmentosus, and autoimmune disorders, such as lupus erythematosus. Other molecules, such as drugs or organic compounds, that: 1) bind to a PKA holoenzyme or RII and/or RII subunit thereof; or 2) have a similar function or activity to D-AKAP2 or a D-AKAP2 peptide or mimetic capable of binding a PKA holoenzyme or RII and/or RII subunit thereof, can be used in methods for treatment.

E. Rational Drug Design

The goal of rational drug design is to produce structural analogs of biologically active polypeptides or peptides of interest or of small molecules or peptide mimetics with which they interact (e.g., agonists and antagonists) in order to fashion drugs which are, e.g., more active or stable forms thereof; or which, for example, enhance or interfere with the function of a polypeptide in vivo (e.g., a PKA holoenzyme). In one approach, one first determines the three-dimensional structure of a protein of interest (e.g., a PKA holoenzyme or polypeptide having a RII and/or RII subunit) or, for example, of a PKA holoenzyme-D-AKAP2 complex, by X-ray crystallography, by computer modeling or most typically, by a combination of approaches. Also, useful information regarding the structure of a polypeptide can be gained by modeling based on the structure of homologous proteins. In addition, peptides can be analyzed by an alanine scan. In this technique, an amino acid residue is replaced by Ala, and its effect on the peptide’s activity is determined. Each of the amino acid residues of the peptide is analyzed in this manner to determine the important regions of the peptide.

Also, a polypeptide or peptide that binds to a PKA holoenzyme or, generally, the RII and/or RII subunit of a PKA holoenzyme, can be selected by a functional assay, and then the crystal structure of this polypeptide or peptide can be determined. This approach can yield a pharmacophore upon which subsequent drug design can be based. Further, it is possible to bypass the crystallography altogether by generating anti-idotypic polypeptides or peptides, (anti-ids) to a functional, pharmacologically active polypeptide or peptide that binds to a PKA holoenzyme or RII and/or RII subunit of a PKA holoenzyme. As a mirror image of a mirror image, the binding site of the anti-ids is expected to be an analog of the original target molecule, e.g., a PKA holoenzyme or polypeptide having a PKA holoenzyme. The anti-id can then be used to identify and isolate peptides from banks of chemically or biologically produced peptides. Selected peptides would then act as the pharmacophore.

Thus, one can design drugs which have, for example, improved activity or stability or which act as modulators (e.g., inhibitors, agonists or antagonists) of PKA holoenzyme activity, and are useful in the methods, particularly the methods for diagnosis, treatment, prevention, and screening of neurodegenerative diseases, such as Alzheimer’s Disease, cardiovascular disorders, cardiac disorders, particularly disorders associated with altered left ventricular function, cardiomyopathies, proliferative disorders, bipolar disorder and other neurological disorders, lipid-metabolism disorders, such as obesity, neoplastic disease, diabetes, certain peripheral retinopathies, such as retinitis pigmentosus, and autoimmune disorders, such as lupus erythematosus. By virtue of the availability of nucleic acid that encodes PKA holoenzymes, sufficient amounts of the PKA holoenzyme can be made available to perform such analytical studies as X-ray crystallography. In addition, the knowledge of the amino acid sequence of a PKA holoenzyme or the RII and/or RII subunit thereof, e.g., the RII and/or RII subunit, can provide guidance on computer modeling techniques in place of, or in addition to, X-ray crystallography.

1. Methods of Identifying Additional Peptides and Peptide Mimetics that Bind to PKA Holoenzymes

In addition to the D-AKAP2 derived peptides provided herein, other peptides having a differential binding affinity to the RII and/or RII subunits of PKA holoenzyme can be readily identified, by example, by random peptide diversity generating systems coupled with an affinity enrichment process. Specifically, random peptide diversity generating systems include the “peptides on plasmids” system (see, e.g., U.S. Pat. Nos. 5,270,170 and 5,338,663); the “peptides on phage” system (see, e.g., U.S. Pat. No. 6,121,238 and Dower et al. (1990) Proc. Natl. Acad. Sci. USA, 87:6378-6382); the “polysome system”; the “encoded synthetic library (ESL)” system; and the “very large scale immobilized polymer synthesis” system (see, e.g., U.S. Pat. No. 6,121,238; and Dower et al. (1991) An. Rep. Med. Chem., 26:271-280).

For example, using the procedures described above, random peptides can generally be designed to have a defined number of amino acid residues in length (e.g., 12). To generate the collection of oligonucleotides encoding the random peptides, the codon motif (NNK)x, where N is nucleotide A, C, G, or T (equimolar; depending on the methodology employed, other nucleotides can be employed), K is G or T (equimolar), and x is an integer corresponding to the number of amino acids in the peptide (e.g., 12) can be used to specify any one of the 32 possible codons resulting from the NNK motif: 1 for each of 12 amino acids, 2 for each of 5 amino acids, 3 for each of 3 amino acids, and only one of the three stop codons. Thus, the NNK motif encodes all of the amino acids, encodes only one stop codon, and reduces codon bias.
The random peptides can be presented, for example, either on the surface of a phage particle, as part of a fusion protein containing either the pIII coat protein of a phage f1 derivative (peptides on phage) or as a fusion protein with the LacI peptide fusion protein bound to a plasmid (peptides on plasmids). The phage or plasmids, including the DNA encoding the peptides, can be identified and isolated by an affinity enrichment process using immobilized PKA holoenzyme having a RI and/or RII subunit. The affinity enrichment process, sometimes called “panning,” typically involves multiple rounds of incubating the phage, plasmids, or polysomes with the immobilized PKA holoenzyme or RI and/or RII subunits thereof, collecting the phage, plasmids, or polysomes that bind to the PKA holoenzyme (along with the accompanying DNA or mRNA), and producing more of the phage or plasmids (along with the accompanying LacI-peptide fusion protein) collected.

2. Characteristics of Peptides and Peptide Mimetics
Among the peptides, polypeptides and peptide mimetics for therapeutic application are those that have molecular weights from about 250 to about 8,000 daltons. If such peptides are oligomerized, dimerized and/or derivatized with a hydrophilic polymer (e.g., to increase the affinity and/or activity of the compounds), the molecular weights of such peptides can be substantially greater and can range anywhere from about 500 to about 120,000 daltons, generally from about 8,000 to about 80,000 daltons. Such peptides can contain 9 or more amino acids that are naturally occurring or synthetic (non-naturally occurring) amino acids. One skilled in the art can determine the affinity and molecular weight of the peptides and peptide mimetics suitable for therapeutic and/or diagnostic purposes (e.g., see Dower et al., U.S. Pat. No. 6,121,238).

The peptides can be covalently attached to one or more of a variety of hydrophilic polymers. Suitable hydrophilic polymers include, but are not limited to, polyalkylethers as exemplified by polyethylene glycol and polypropylene glycol, polyalactic acid, polyglycolic acid, polyoxyalkylenes, polyvinylalcohol, polyvinylpyridilone, cellulose and cellulose derivatives, dextrin and dextrin derivatives. When the peptide compounds are derivatized with such polymers, their solubility and circulation half-lives can be increased with little, if any, diminishment in their binding activity. The peptide compounds can be dimerized and each of the dimeric subunits can be covalently attached to a hydrophilic polymer. The peptide compounds can be PEGylated, i.e., covalently attached to polyethylene glycol (PEG).

Methods of Preparing Peptides and Peptide Mimetics
D-AKAP2 based peptides provided herein that bind to PKA holoenzymes can be prepared by classical methods known in the art, for example, by using standard solid phase techniques. The standard methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis, and even by recombinant DNA technology (see, e.g., Merrifield (1963) J. Am. Chem. Soc., 85:2149, incorporated herein by reference.)

Using the “encoded synthetic library” or “very large scale immobilized polymer synthesis” systems (see, e.g., U.S. Pat. Nos. 5,925,525, and 5,902,723), the minimum size of a peptide with the activity of interest can be determined. In addition, all peptides that form the group of peptides that differ from the desired motif (or the minimum size of that motif) in one, two, or more residues can be prepared. This collection of peptides then can be screened for the ability to bind to the target molecule, e.g., PKA holoenzyme or, generally, the RI and/or RII subunit of a PKA holoenzyme. This immobilized polymer synthesis system or other peptide synthesis methods can also be used to synthesize truncation analogs and deletion analogs and combinations of truncation and deletion analogs of the peptide compounds.

These procedures can also be used to synthesize peptides in which amino acids other than the 20 naturally occurring, genetically encoded amino acids are substituted at one, two, or more positions of the peptide. For instance, naphthylalanine can be substituted for tryptophan, facilitating synthesis. Other synthetic amino acids that can be substituted into the peptides include L-hydroxypropyl, L-3,4-dihydroxy-phenylalanly, D-amino acids such as L-d-hydroxylylysyl and D-d-methylalany, L-a-methylalany, L-b amino acids, and isoquinolinyl. D-amino acids and non-naturally occurring synthetic amino acids can also be incorporated into the peptides (see, e.g., Roberts et al. (1983) Unusual Amino Acids in Peptide Synthesis, 5(6):341-449).

The peptides can also be modified by phosphorylation (see, e.g., W. Banwart et al. (1996) Biorganic and Medicinal Chemistry Letters, 6(17):2141-2146), and other methods for making peptide derivatives (see, e.g., Hruby et al. (1990) Biochem. J., 268(2):245-262). Thus, peptide compounds also serve as a basis to prepare peptide mimetics with similar or improved biological activity.

Those of skill in the art recognize that a variety of techniques are available for constructing peptide mimetics with the same or similar desired biological activity as the corresponding peptide compound but with more favorable activity than the peptide with respect to solubility, stability, and susceptibility to hydrolysis and proteolysis (see, e.g., Morgan et al. (1989) An. Rep. Med. Chem., 24:243-252). Methods for preparing peptide mimetics modified at the N-terminal amino group, the C-terminal carboxyl group, and/or changing one or more of the amido linkages in the peptide to a non-amido linkage are known to those of skill in the art.

Amino terminus modifications include, but are not limited to, alkylation, acetylation and adding a carbobenzoxy group, forming a succinimide group (see, e.g., Murray et al. (1993) Burger’s Medicinal Chemistry and Drug Discovery, 5th ed., Vol. 1, Manfred E. Wolf, ed., John Wiley and Sons, Inc.). C-terminal modifications include mimetics wherein the C-terminal carboxyl group is replaced by an ester, an amide or modifications to form a cyclic peptide.

In addition to N-terminal and C-terminal modifications, the peptide compounds, including peptide mimetics, can advantageously be modified with or covalently coupled to one or more of a variety of hydrophilic polymers. It has been found that when peptide compounds are derivatized with a hydrophilic polymer, their solubility and circulation half-lives can be increased and their immunogenicity is masked, with little, if any, diminishment in their binding activity. Suitable nonproteinaceous polymers include, but are not limited to, polyalkylethers as exemplified by polyethylene glycol and polypropylene glycol, polyalactic acid, polyglycolic acid, polyoxyalkylenes, polyvinylalcohol, polyvinylpyridilone, cellulose and cellulose derivatives, dextrin and dextrin derivatives. Generally, such hydrophilic polymers have an average molecular weight ranging from about 500 to about 100,000 daltons, including from about 2,000 to about 40,000 daltons and, from about 5,000 to about 20,000 daltons. The
hydrophilic polymers also can have average molecular weights of about 5,000 daltons, 10,000 daltons and 20,000 daltons.

Methods for derivatizing peptide compounds or for coupling peptides to such polymers have been described (see, e.g., Zallipsky (1995) *Bioconjugate Chem.*, 6:150-165; Monfardini et al. (1995) *Bioconjugate Chem.*, 6:62-69; U.S. Pat. No. 4,640,835; U.S. Pat. No. 4,496,689; U.S. Pat. No. 4,301,144; U.S. Pat. No. 4,670,417; U.S. Pat. No. 4,751,192; U.S. Pat. No. 4,179,337 and WO95/34325, all of which are incorporated by reference in their entirety herein).

Other methods for making peptide derivatives are described, for example, in Hruby et al. (1990) *Biochem J.*, 268(2):249-262, which is incorporated herein by reference. Thus, the peptide compounds also serve as structural models for non-peptidic compounds with similar biological activity. Those of skill in the art recognize that a variety of techniques are available for constructing compounds with the same or similar desired biological activity as a particular peptide compound but with more favorable activity with respect to solubility, stability, and susceptibility to hydrolysis and proteolysis (see, e.g., Morgan et al. (1989) *An. Rep. Med. Chem.*, 24:243-252, incorporated herein by reference). These techniques include replacing the peptide backbone with a backbone composed of phosphonates, amidates, carbamates, sulfonamides, secondary amines, and N-methylamino acids.

Peptide compounds can exist in a cyclized form with an intramolecular disulfide bond between the thiol groups of the cysteines. Alternatively, an intermolecular disulfide bond between the thiol groups of the cysteines can be produced to yield a dimeric (or higher oligomeric) compound. One or more of the cysteine residues can also be substituted with a homocysteine.

G. Transgenic Animals


The term “transgene” is used herein to describe genetic material that has been or is about to be artificially inserted into the genome of a mammalian cell, particularly a mammalian cell of a living animal. The transgene is used to transform a cell, meaning that a permanent or transient genetic change, preferably a permanent genetic change, is induced in a cell following incorporation of exogenous DNA. A permanent genetic change is generally achieved by introduction of the DNA into the genome of the cell. Vectors for stable integration include, but are not limited to, plasmids, retroviruses and other animal viruses and YACS. Of interest are transgenic mammals, including, but are not limited to, cows, pigs, goats, horses and others, and particularly rodents, including rats and mice. Preferably, the transgenic-animals are mice.

Transgenic animals contain an exogenous nucleic acid sequence present as an extrachromosomal element or stably integrated in all or a portion of its cells, especially germ cells. Unless otherwise indicated, it will be assumed that a transgenic animal comprises stable changes to the germline sequence. During the initial construction of the animal, “chimeras” or “chimeric animals” are generated, in which only a subset of cells have the altered genome. Chimeras are primarily used for breeding purposes in order to generate the desired transgenic animal. Animals having a heterozygous alteration are generated by breeding of chimeras. Male and female heterozygotes are typically bred to generate homozygous transgenic animals.

The exogenous gene is usually either from a different species than the animal host, or is otherwise altered in its coding or non-coding sequence. The introduced gene may be a wild-type gene, naturally occurring polymorphism or a genetically manipulated sequence, for example having deletions, substitutions or insertions in the coding or non-coding regions. When the introduced gene is a coding sequence, it is usually operably linked to a promoter, which may be constitutive or inducible, and other regulatory sequences required for expression in the host animal.

Transgenic animals can comprise other genetic alterations in addition to the presence of alleles of AKAP genes. For example, the genome can be altered to affect the function of the endogenous genes, contain marker genes, or contain other genetic alterations (e.g., alleles of genes associated with cardiovascular disease).

A “knock-out” of a gene means an alteration in the sequence of the gene that results in a decrease of function of the target gene, preferably such that target gene expression is undetectable or insignificant. A knock-out of an endogenous AKAP gene means that function of the gene has been substantially decreased so that expression is not detectable or only present at insignificant levels. “Knock-out” transgenics can be transgenic animals having a heterozygous knock-out of an AKAP gene or a homozygous knock-out. “Knock-outs” also include conditional knock-outs. As used herein, “conditional” in reference to “knock-outs” and “knock-ins” means alteration of the target gene can occur upon, for example, exposure of the animal to a substance that promotes target gene alteration, introduction of an enzyme that promotes recombination at the target gene site (e.g., Cre in the Cre-lox system), or other method for directing the target gene alteration postnatally.

A “knock-in” transgenic animal refers to an animal that has had a modified gene introduced into its genome, wherein the modified gene can be of exogenous or endogenous origin (see, e.g., Roemer et al. (1991) *New Biol.*, 3:331). Accordingly, a “knock-in” of a target gene means an alteration in a host cell genome that results in either expression of an altered target gene; or altered expression (e.g., increased (including ectopic)) of the target gene, e.g., by introduction of an additional copy of the target gene, or by operatively inserting a regulatory sequence that provides for enhanced expression of an endogenous copy of the target gene. “Knock-in” transgenics of interest can be transgenic animals having a knock-in of an AKAP gene. Such transgenics can be heterozygous or homozygous for the knock-in gene. “Knock-ins” also encompass conditional knock-ins. As used herein, “knock-in” transgenic animals also encompasses animals in which an animal gene is replaced by the human equivalent within the genome of that animal. These transgenic knock-in animals are useful for drug discovery, for target validation, where the compound is specific for the human target. Transgenic knock-ins can be produced using...
homologous recombination, using transposons (e.g., Westphal et al. (1997) Curr. Biol., 7:530, and the like), using mutant recombination sites (e.g., Araki et al. (1997) NAR, 25:868, and the like), using PCR (e.g., Zhang et al. Biotechniques, 25:784, and the like), and the like.

[0167] For example, the binding data generated herein related to certain amino acid positions within the 27-mer AKB binding domain is used to knock-in specific amino acids at specific positions in the genome of cells and organisms (e.g., animals, cells) to change the binding ratio of both PKA-R and PKA-RIα subunits to its target protein D-AKAP2 in vivo. Such a changed binding ratio is contemplated herein to produce a changed phenotype useful to elucidate the biological function of D-AKAP2 and PKA subunits and their contribution to the manifestation of diseases set forth herein. Likewise, the binding data generated herein related to certain amino acid positions within the 27-mer AKB binding domain is used to knock-in specific amino acids at specific positions in the genome of cells and organisms (e.g., animals, cells) to disrupt the binding of one particular PKA-R subunit isofrom to D-AKAP2 in vivo. Such a binding disruption is contemplated herein to cause a specific loss of function of D-AKAP2 resulting in a changed phenotype useful to elucidate the biological function of D-AKAP2 and PKA subunits and their contribution to the manifestation of diseases set forth herein.

[0168] The transgenic knock-in cellular systems or animals provided herein are useful to screen chemical compound libraries for the purpose of drug discovery. In addition, these transgenic knock-in cellular systems or animals are useful in assays along the entire drug development process.

[0169] A construct is suitable for use in the generation of transgenic animals if it allows the desired level of expression of an AKAP encoding sequence. Methods of isolating and cloning a desired sequence, as well as suitable constructs for expression of a selected sequence in a host animal, are well known in the art and are described below.

[0170] For the introduction of a gene into the subject animal, it is generally advantageous to use the gene as a gene construct wherein the gene is ligated downstream of a promoter capable of and operably linked to expressing the gene in the subject animal cells. Specifically, a transgenic non-human mammal showing high expression of the desired gene can be created by microinjecting a vector ligated with said gene into a fertilized egg of the subject non-human mammal (e.g., rat fertilized egg) downstream of various promoters capable of expressing the protein and/or the corresponding protein derived from various mammals (rabbis, dogs, cats, guinea pigs, hamsters, rats, mice, etc.), preferably rats etc.)

[0171] Useful vectors include Escherichia coli-derived plasmids, Bacillus subtilis-derived plasmids, yeast-derived plasmids, bacteriophages such as lambda, phage, retroviruses such as Moloney leukemia virus, and animal viruses such as vaccinia virus or baculovirus.

[0172] Useful promoters for such gene expression regulation include, for example, promoters for genes derived from viruses (cytomegalovirus, Moloney leukemia virus, JC virus, breast cancer virus, etc.), and promoters for genes derived from various mammals (humans, rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, etc.), and birds (chickens, etc.) (e.g., genes for albumin, insulin II, erythropoietin, endothelin, osteocalcin, muscular creatine kinase, platelet-derived growth factor beta, keratins K1, K10 and K14, collagen types I and II, atrial natriuretic factor, dopamine beta-hydroxylase, endothelial receptor tyrosine kinase (generally abbreviated Tie2), sodium-potassium adenosine triphosphorylase (generally abbreviated Na,K-ATPase), neurofilament light chain, metallothioneins I and IIa, metalloproteinase I tissue inhibitor, MHC class 1 antigen (generally abbreviated H-2L), smooth muscle alpha actin, polypeptide chain elongation factor 1 alpha (EF-1 alpha), beta actin, alpha and beta myosin heavy chains, myosin light chains I and 2, myelin basic protein, serum amyloid component, myoglobin, renin, etc.).

[0173] It is preferable that the above-mentioned vectors have a sequence for terminating the transcription of the desired messenger RNA in the transgenic animal (generally referred to as terminator); for example, gene expression can be manipulated using a sequence with such function contained in various genes derived from viruses, mammals and birds. Preferably, the simian virus SV40 terminator, etc., are commonly used. Additionally, for the purpose of increasing the expression of the desired gene, the splicing signal and enhancer region of each gene, a portion of the intron of a eukaryotic organism gene may be ligated 5’ upstream of the promoter region, or between the promoter region and the translational region, or 3’ downstream of the translational region as desired.

[0174] A translational region for a protein of interest can be obtained using the entire or portion of genomic DNA of blood, kidney or fibroblast origin from various mammals (humans, rabbits, dogs, cats, guinea pigs, hamsters, rats, mice, etc.), or of various commercially available genomic DNA libraries, or using complementary DNA prepared by a known method from RNA of blood, kidney or fibroblast origin as a starting material. Also, an exogenous gene can be obtained using complementary DNA prepared by a known method from RNA of human fibroblast origin as a starting material. All these translational regions can be used in transgenic animals.

[0175] To obtain the translational region, it is possible to prepare DNA incorporating an exogenous gene encoding the protein of interest in which the gene is ligated downstream of the above-mentioned promoter (preferably upstream of the translation termination site) as a gene construct capable of being expressed in the transgenic animal.

[0176] DNA constructs for random integration need not include regions of homology to mediate recombination. Where homologous recombination is desired, the DNA constructs will comprise at least a portion of the target gene with the desired genetic modification, and will include regions of homology to the target locus. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown et al. (1990) Meth. Enzymol., 185:527-537.

[0177] The transgenic animal can be created by introducing an AKAP gene construct into, for example, an unfertilized egg, a fertilized egg, a spermatozoon or a germinal cell containing a primordial germinal cell thereof, preferably in the embryogenic stage in the development of a non-human mammal (more preferably in the single-cell or fertilized cell stage and generally before the 8-cell phase), by standard means, such as the calcium phosphate method, the electric pulse method, the lipofection method, the agglutination method, the microinjection method, the particle gun method, the DEAE-dextran method and other such method. Also, it is possible to introduce a desired AKAP gene into a somatic cell, a living organ, a tissue cell or other cell, by gene trans-
formation methods, and use it for cell culture, tissue culture and any other method of propagation. Furthermore, these cells may be fused with the above-described germline cell by a commonly known cell fusion method to create a transgenic animal.

[0178] For embryonic stem (ES) cells, an ES cell line may be employed, or embryonic cells may be obtained freshly from a host, e.g., mouse, rat, guinea pig, etc. Such cells are grown on an appropriate fibroblast-feeder layer or grown in the presence of appropriate growth factors, such as leukemia inhibiting factor (LIF). When ES cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer in an appropriate medium. Cells containing the construct may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination or integration of the construct. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 weeks old superovulated females. The ES cells are trypsinized, and the modified cells are injected into the blastocoele of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected. The chimeric animals are screened for the presence of the modified gene and males and females having the modification are mated to produce homozygous progeny. If the gene alterations cause lethality at some point in development, tissues or organs can be maintained as allogenic or xenogenic grafts or transplants, or in vitro culture.

[0179] Animals containing more than one transgene, such as allelic variants of AKAP genes and/or other genes associated with morbidity and/or mortality can be made by sequentially introducing individual alleles into an animal in order to produce the desired phenotype (manifestation of morbidity and/or predisposition to early mortality). In addition, animals containing one or more amino acid substitutions with that same transgene, such as D-AKAP2 can be made by sequentially introducing individual amino acid substitutions, or introducing one or more substitutions in a single construct, into an animal in order to produce the desired genotypic and/or phenotype (manifestation of morbidity and/or predisposition to early mortality). Animals having one or more amino acid substitutions in the D-AKAP2 gene, include transgenic animals having a combination of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more of the amino acid residue substitutions set forth herein, such as, but not limited to, those described above and in the Examples, or in the claims.

[0180] Accordingly, provided herein are transgenic nonhuman knock-in animals comprising a gene encoding the animal homolog of human D-AKAP2, wherein said gene has been modified to encode any combination of one or more amino acid substitutions in a 27-mer PKA binding region of the amino acid homolog of human D-AKAP2 corresponding to amino acids 623-649 of human D-AKAP2 set forth in SEQ ID NOs:63-65; or SEQ ID NOs:1 or 2. In one embodiment, the amino acid substitution in the animal homolog of human D-AKAP2 is selected from the group consisting of substitutions that correspond to the substitution in SEQ ID NOs:63-65 of one, two or more of Q at residue 631 with F, I, L, V, H, M, R, T, W or Y; of L at residue 634 with F, I, L, T, V, W or Y; of W at residue 636 with F, I, L, T, V, W or Y; of V at residue 643 with I, L or W; and of M at residue 647 with F, I, L, T, V, W or Y. In this embodiment, the modified animal protein homolog of human D-AKAP2 can exhibit a preferred or exclusive binding to PKA-R1α subunits relative to PKA-R1β subunits, or enhanced binding to both R1α and R1β subunits, compared to the native unmodified animal protein homolog of human D-AKAP2.

[0181] In another embodiment, the amino acid substitution in the animal homolog of human D-AKAP2 is selected from the group consisting of substitutions that correspond to the substitution in SEQ ID NOs:63-65 of one, two or more of L at residue 634 with A, C, or K; of A at residue 635 with F, H, I, K, L, M, N or S; of W at residue 636 with C; of K at residue 637 with C; of K at residue 640 with C; of M at residue 641 with C; of S at residue 644 with C; and of D at residue 645 with C. In this embodiment, the modified animal protein homolog of human D-AKAP2 can exhibit a preferred or exclusive binding to PKA-R1α subunits relative to PKA-R1β subunits, compared to the native unmodified animal protein homolog of human D-AKAP2.

[0182] In another embodiment, the animal is a mouse, and wherein the amino acid substitution in the mouse D-AKAP2 is selected from the group consisting of substitutions that correspond to the substitution in SEQ ID NOs:55 or 56 of one, two or more of Q at residue 341 with F, I, L, T, V, H, M, R, T, W or Y; of L at residue 344 with F, W or Y; of W at residue 355 with I, L or W; and of M at residue 357 with F, I, L, T, V, W or Y. In this embodiment, the modified animal protein homolog of human D-AKAP2 is mouse D-AKAP2 that exhibits a preferred or exclusive binding to PKA-R1α subunits relative to PKA-R1β subunits, or enhanced binding to both R1α and R1β subunits, compared to the native unmodified mouse D-AKAP2. In a particular embodiment, the amino acid substitution in the mouse D-AKAP2 corresponds to a single amino acid substitution in SEQ ID NOs:55 or 56 of Q at residue 341 with 1, and wherein the modified mouse D-AKAP2 exhibits an enhanced binding to PKA-R1α subunits, and normal binding to PKA-R1β subunits compared to the native unmodified mouse D-AKAP2. In another embodiment, the amino acid substitution in the mouse D-AKAP2 corresponds to a single amino acid substitution in SEQ ID NOs:55 or 56 of V at residue 353 with W, and wherein the modified mouse D-AKAP2 exhibits a normal binding to PKA-R1α subunits, and disrupted or decreased binding to PKA-R1β subunits compared to the native unmodified mouse D-AKAP2. In another embodiment, the animal protein homolog of human D-AKAP2 in the mouse D-AKAP2 corresponds to a single amino acid substitution in SEQ ID NOs:55 or 56 of Q at residue 341 with F, of V at residue 353 with W, and of M at residue 357 with F, and wherein the modified mouse D-AKAP2 exhibits increased binding to R1α and decreased binding affinity for R1β. In a particular embodiment, the modified mouse D-AKAP2 having the triple amino acid substitution exhibits approximately 10-fold increased binding to R1α and approximately 220-fold decreased binding affinity for R1β.

[0183] In another embodiment, the animal is a mouse, and wherein the modified animal protein homolog of human D-AKAP2 is mouse D-AKAP2 that exhibits a preferred or exclusive binding to PKA-R1β subunits relative to PKA-R1α subunits, compared to the native unmodified mouse D-AKAP2; and wherein the amino acid substitution in the mouse D-AKAP2 is selected from the group consisting of substitutions that correspond to the substitution in SEQ ID
amino acid residues, wherein the amino acid sequence of the D-AKAP2 mutein additional to AKB domain peptide region, is 1 to 635 amino acids in length and corresponds to a contiguous region from amino acids 1-622 and/or 650-662 of SEQ ID NOs:64 or 65. In another embodiment, the D-AKAP2 mutein can be 662 amino acids in length, and further comprises amino acids 1-622 and 650-662 of SEQ ID NOs:64 or 65. Also provided are isolated nucleic acid molecules, vectors, and cells containing these vectors, comprising a sequence of nucleotides that encodes the D-AKAP2 mutein provided herein. Also provided herein are methods of producing a D-AKAP2 mutein by growing the cells comprising a vector under conditions whereby the D-AKAP2 mutein is expressed, and isolating the mutein. The cell can be any animal cell (e.g., mammalian or human), yeast cell, insect cell or bacterial cell.

[0190] The isolated nucleic acid encoding a full-length mammalian (e.g., human, mouse, and the like) D-AKAP2 protein, mutein provided herein, or a portion thereof, such as a peptide fragment containing one or more of the biologically significant variant sites set forth herein in Examples 5-9, may be introduced into a vector for transfer into host cells. Fragments of the polymorphic mammalian, e.g., human, D-AKAP2 proteins can be produced by those skilled in the art, without undue experimentation, by eliminating portions of the coding sequence from the isolated nucleic acids encoding the full-length proteins.

[0191] The isolated nucleic acid encoding a full length D-AKAP2 protein, mutein or portion thereof can be modified to use a preferred codon bias to increase the expression level of the AKAP protein. The codon usage of the target organism or cell for expression can be determined by methods such as described in U.S. Pat. Nos. 5,082,767 and 4,562,639 (incorporated herein by reference). The isolated nucleic acid can then be modified by mutagenesis, recombination, or produced by synthetic DNA synthesis or other techniques known in the art such that the modified nucleic acid encoding the D-AKAP2 protein, mutein or portion thereof has at least one codon optimized for expression in the target organism or host cell. Using such methods, the expression of D-AKAP2 protein, mutein or portion thereof can be increased above the expression of the unmodified sequences. Conversely, if lower expression is desired, the codon usage of the nucleic acid encoding the D-AKAP2 protein, mutein or portion thereof can be modified to select for non-preferred or less preferred codons of the target organism or cell, for expression such as by methods described in U.S. Pat. Nos. 5,786,464 and 6,114,148 (incorporated herein by reference).

[0192] Expression vectors are used to express the protein in the desired host cell. An expression vector includes vectors capable of expressing nucleic acids that are operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such nucleic acids. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome. Such plasmids for expression of polymorphic mammalian, e.g., human, D-AKAP2-encoding nucleic acids in eukaryotic host cells, particularly mammalian cells, include cytomegalovirus
(CMV) promoter-containing vectors, such as PCMV5, the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, and MMTV promoter-based vectors.

Expression vectors can be constructed which up-regulate or down-regulate expression of D-AKAP2, a munei or portion thereof in a host cell or transgenic animal. Methods for down-regulation include antisense expression, RNAi constructs, ribozyme expression and other methods well known in the art. Such expression vectors may include the full length nucleotide sequence of D-AKAP2 sequence or munei provided herein or a portion thereof. Vectors can be designed that are specific for down-regulating expression a specific allele of D-AKAP2, for example down-regulate a Val(465) variant of D-AKAP2. Vectors can also be designed to down-regulate expression of all or most of alleles of D-AKAP2. Such vectors can also be designed to down-regulate D-AKAP2 homologs. Similarly, expression vectors can be designed which up-regulate D-AKAP2 expression or which express a high amount of a particular D-AKAP2 munei or portion thereof. For example, promoters can be used which are known to regulate high levels of expression, for example viral promoters and other promoters such as described herein and known in the art. Codon optimization, as described above, can also be used to increase expression of full length D-AKAP2, a munei or portion thereof. Another method of up-regulation is ectopic expression, the expression of D-AKAP2, a munei or portion thereof in a cell-type or tissue that does not normally express D-AKAP2. Such ectopic expression can be accomplished by using tissue-specific or regulatable promoters. One example of a regulatable promoter is the Tet-on/Tet-off system (available from Clontech, BD Biosciences, Palo Alto Calif.) in which gene expression is regulated by the administration of tetracycline or related analogs.

The nucleic acids encoding polymorphic human D-AKAP2 proteins, and vectors and cells containing the nucleic acids as provided herein permit production of the polymorphic protein variants, as well as antibodies to the proteins. This provides a means to prepare synthetic or recombinant polymorphic human D-AKAP2 proteins and fragments thereof that are substantially free of contamination from other AKAPs and proteins in general, the presence of which can interfere with analysis of the polymorphic proteins. In addition, the polymorphic proteins may be expressed in combination with selected other proteins that D-AKAP2 may associate with in cells. The ability to select polyclonally express the polymorphic D-AKAP2 proteins alone or in combination with other selected proteins makes it possible to observe the functioning of the recombinant polymorphic proteins with the environment of a cell. The expression of isolated nucleic acids encoding an AKAP protein will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or regulatable), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. One of skill in the art would recognize that modifications can be made to an D-AKAP2 protein without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (e.g., poly His) placed on either terminus to create conveniently located purification sequences. Restriction sites or termination codons can also be introduced. There are expression vectors that specifically allow the expression of functional proteins. One such vector, Plasmid 577, described in U.S. Pat. No. 6,020,122 and incorporated by reference, has been constructed for the expression of secreted antigens in a permanent cell line. This plasmid contains the following DNA segments: (a) a fragment of pBR322 containing bacterial beta-lactamase and origin of DNA replication; (b) a cassette directing expression of a neomycin resistance gene under control of HSV-1 thymidine kinase promoter and poly-A addition signals; (c) a cassette directing expression of a dihydrofolate reductase gene under the control of a SV-40 promoter and poly-A addition signals; (d) a cassette directing expression of a rabbit immunoglobulin heavy chain signal sequence fused to a modified hepatitis C virus (HCV) E2 protein under the control of the Simian Virus 40 T-Ag promoter and transcription enhancer, the hepatitis B virus surface antigen (HBsAg) enhancer I followed by a fragment of Herpes Simplex Virus-1 (HSV-1) genome providing poly-A addition signals; and (e) a fragment of Simian Virus 40 genome late region of no function in this plasmid. All of the segments of the vector were assembled by standard methods known to those skilled in the art of molecular biology. Plasmids for the expression of secreted AKAP proteins can be constructed by replacing the hepatitis C virus E2 protein coding sequence in plasmid 577 with an AKAP sequence of SEQ ID NO:63 or a fragment thereof. The resulting plasmid is transfected into CHO/dhfr-cells (DHB-111) (Uricchio et al. (1980) PNAS, 77:4451-4466); these cells are available from the ATCC, 13356 Parklawn Drive, Rockville, Md. 20852, under Accession No. CRL 9096, using the cationic liposome-mediated procedure (P. L. Felgner et al. (1987) PNAS 84:7413-7417). Proteins are secreted into the cell culture media.

Incorporation of cloned DNA into a suitable expression vector, transfection of cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct proteins, or with linear DNA, and selection of transfected cells are well known in the art (see, e.g., Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press). Heterologous nucleic acid may be introduced into host cell by any method known to those of skill in the art, such as transfection with a vector encoding the heterologous nucleic acid by calcium phosphate precipitation (see, e.g., Wigler et al. (1979) Proc. Natl. Acad. Sci. USA, 76:1373-1376) or lipofectamine (GIBCO BRL #18324-012). Recombinant cells can then be cultured under conditions whereby the polymorphic human D-AKAP2 protein encoded by the nucleic acid is expressed. Suitable host cells include mammalian cells (e.g., HEK293, including but are not limited to, those described in U.S. Pat. No. 5,024,393 to Gorman (see, also, Stillman et al. (1985) Mol. Cell. Biol., 5:2051-2060); also, HEK293 cells available from ATCC under accession #CRL 1573, CHO, COS,
BIKBI and Ltk\(^{-}\) cells, mouse monocyte macrophage P388D1 and J774A-1 cells (available from ATCC, Rockville, Md.) and others known to those of skill in this art), yeast cells, including, but are not limited to, Pichia pastoris, Saccharomyces cerevisiae, Candida tropicalis, Hansenula polymorpha, human cells, bacterial cells, including, but are not limited to, Escherichia coli, and insect cells. Xenopus oocytes may also be used for expression of in vitro RNA transcripts of the DNA.

[0196] Heterologous nucleic acid may be stably incorporated into cells or may be transiently expressed using methods known in the art. Stably transfected mammalian cells may be prepared by transfecting cells with an expression vector having a selectable marker gene (such as, for example, the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance, and the like), and growing the transfected cells under conditions selective for cells expressing the marker gene. To prepare transient transfectants, mammalian cells are transfected with a reporter gene (such as the E. coli β-galactosidase gene) to monitor transfection efficiency. Selectable marker genes are not included in the transient transfections because the transfectants are typically not grown under selective conditions, and are usually analyzed within a few days after transfection.

[0197] Heterologous nucleic acid may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing recombinant cells are known to the skilled artisan. Similarly, the polymorphic human D-AKAP2 proteins or fragments thereof may be purified using protein purification methods known to those of skill in the art. For example, antibodies or other ligands that specifically bind to the proteins may be used for affinity purification and immunoprecipitation of the proteins.

[0198] 3. Protein Purification

[0199] The D-AKAP2 proteins may be purified by standard techniques well known to those of skill in the art. Recombinantly produced proteins can be directly expressed or expressed as a fusion protein. The recombinant protein is purified by a combination of cell lysis (e.g., sonication, French press) and affinity chromatography. The proteins, recombinant or synthetic, may be purified to substantial purity by standard techniques well known in the art, including detergent solubilization, selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. (See, for example, R. Scopes, Protein Purification: Principles and Practice, Springer-Verlag: New York (1982); Deutscher, Guide to Protein Purification, Academic Press (1990)). For example, antibodies may be raised to the proteins as described herein. Purification from E. Coli can be achieved following procedures described in U.S. Pat. No. 4,511,503. The protein may then be isolated from cells expressing the protein and further purified by standard protein chemistry techniques as described herein. Detection of the expressed protein is achieved by methods known in the art and include, for example, radioimmunoassays, Western blotting techniques or immunoprecipitation.

1. Immuno detection of Variant D-AKAP2 Protein Species

[0200] Generally, the variant regions of the variant AKAP protein species provided herein, when presented as an immunogen, should elicit production of a specifically reactive antibody. Immunoassays for determining binding are well known to those of skill in the art, as are methods of making and assaying for antibody binding specificity/affinity. Exemplary immunoassay formats include ELISA, competitive immunoassays, radioimmunoassays, Western blots, indirect immunofluorescent assays, in vivo expression or immunization protocols with purified protein preparations. In general, the detection of immunocomplex formation is well known in the art and may be achieved by methods generally based upon the detection of a label or marker, such as any of the radioactive, fluorescent, biological or enzymatic tags. Labels are well known to those skilled in the art (see U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference). Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody or a biotin/avidin ligand binding arrangement, as is known in the art.

[0201] 1. Production of Polyclonal Antisera Against Specific Variant AKAPs

[0202] Antibodies can be raised to the variant AKAP protein species provided herein, including fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies are raised to these proteins in either their native configurations or in non-native configurations. Anti-idiotypic antibodies can also be generated. A variety of analytic methods are available to generate a hydrophilic profile of proteins. Such methods can be used to guide the artisan in the selection of peptides for use in the generation or selection of antibodies which are specifically reactive, under immunogenic conditions. See, e.g., J. Janin, (1979) Nature, 277:491-492; Wolfdend et al. (1981) Biochemistry 20:849-855; Kyte and Doolite (1982) J. Mol. Biol., 157:105-132; Rose et al. (1985) Science, 229:834-838.

[0203] A number of immunogens can be used to produce antibodies specifically reactive with a particular variant AKAP protein species. Isolated recombinant, synthetic, or native polypeptides are the preferred immunogens (antigen) for the production of monoclonal or polyclonal antibodies. Polypeptides are typically denatured, and optionally reduced, prior to formation of antibodies for screening expression libraries or other assays in which a putative AKAP protein is expressed or denatured in a non-native secondary, tertiary, or quaternary structure.

[0204] The particular variant region of the variant AKAP protein is injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies can be generated for subsequent use in immunoassays to measure the presence and quantity of the protein. Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen (antigen), preferably a purified protein, a protein coupled to an appropriate carrier (e.g., GST, keyhole limpet hemocyanin, etc.), or a protein incorporated into an immunization vector such as a recombinant vaccinia virus (see, U.S. Pat. No. 4,722,848) is mixed with an adjuvant and animals are immunized with the mixture. The animal’s immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the protein of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein is
performed where desired (See, e.g., Coligan, Current Protocols in Immunology, Wiley/Greene, NY (1991); and Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Press, NY (1989)).

2. Western Blotting of Tissue Samples for the Variant D-AKAP2 Protein

Biological samples are homogenized in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), heated at 100°C for 10 min and run on a 14% SDS-PAGE with a 25 mM Tris-HCl, pH 8.3, 250 mM Glycine, 0.1% SDS running buffer. The proteins are electrophoretically transferred to nitrocellulose in a transfer buffer containing 39 mM glycine, 48 mM Tris-HCl, pH 8.3, 0.037% SDS, 20% methanol. The nitrocellulose is dried at room temperature for 60 min and then blocked with a phosphate-buffered saline (PBS) solution containing either bovine serum albumin or 5% nonfat dried milk for 2 hours at 4°C.

The filter is placed in a heat-sealable plastic bag containing a solution of 5% nonfat dried milk in PBS with a 1:100 to 1:2000 dilution of affinity purified anti-AKAP peptide antibodies, incubated at 4°C. For 2 hours, followed by three 10 min washes in PBS. An alkaline phosphatase conjugated secondary antibody (i.e., anti-mouse/rabbit IgG), is added at a 1:200 to 1:2000 dilution to the filter containing 39 mM NaCl, 50 mM Tris-HCl, pH 7.5 buffer and incubated for 1 h at room temperature.

The bands are visualized upon the addition of a chromogenic substrate such as 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). The filter is incubated in the solution at room temperature until the bands develop to the desired intensity. Molecular mass determination is made based upon the mobility of pre-stained molecular weight standards (Rainbow Markers, Amersham, Arlington Heights, III.).

3. Microparticle Enzyme Immunoassay (MEIA)

Variant D-AKAP2 protein species and peptides are detected using a standard commercialized antigen competition ELA assay or polyclonal antibody sandwich ELA assay on the IMx® Analyzer (Abbott Laboratories, Abbott Park, III.). Samples containing the D-AKAP2 protein are incubated in the presence of anti-D-AKAP2 coated microparticles. The microparticles are washed and secondary polyclonal anti-D-AKAP2 antibodies conjugated with detectable entities (i.e., alkaline phosphatase) are added and incubated with the microparticles. The microparticles are washed and the bound antibody/antigen/antibody complexes are detected by adding a substrate (i.e., 4-methyl umbelliferyl phosphate) (MUP) that will react with the secondary conjugated antibody to generate a detectable signal.

Intracellular localization of the variant D-AKAP2 protein species can be determined by a variety of in situ hybridization techniques. In one method, cells are fixed with 4% paraformaldehyde in 0.1 M PBS; pH 7.4 for 5 min., rinsed in PBS for 2 min., dilapidated and dehydrated in an ethanol series (50, 70 and 95%) (5 min. each and stored in 95% ethanol at 4°C).

The cells are stained with the primary anti-D-AKAP2 antibody and a mixture of secondary antibodies used for detection. Laser-scanning confocal microscopy is performed to localize the D-AKAP2 protein.

J. Biological Assays


In Vitro Binding Assay

Huang et al. (1997) Proc. Natl. Acad. Sci. USA, 272:8057-8064; Protein preparations containing D-AKAP2 fused to GST are incubated with glutathione resin in PBS for 2 hours at 4°C. With 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5 mM benzamidine, and 5 mM-mercaptoethanol and washed extensively with the same buffer. 200 micrograms of PKA regulatory subunit RII and/or RII were added to the resin and incubated at 4°C. Proteins associated with the D-AKAP2 are eluted and analyzed by Laemmli electrophoresis. The proteins were visualized by Coomassie Staining. PKA proteins can be radiolabeled or labeled with a fluorophore to allow detection; or can be assayed for phosphorylation activity as set forth in the next section.

2.PKA Phosphorylation of Protein Substrate

Cyclic AMP-dependent protein kinase (PKA) catalyzes the transfer of gamma phosphate from adenosine triphosphate (ATP) to a serine or threonine residue in a protein substrate. A short synthetic peptide (Leucine-Arg-Arg-Alanine-Serine-Leucine-Glycine or LRRASLG) is used as a substrate to assay the specific type of PKA activity as described in Pearson et al. (1991) Meth. Enzymol., 200:62-81.

The PKA assay is typically carried out in a reaction of the enzyme with a peptide substrate and gamma 32P-ATP followed by separation of the 32P-peptide product from the unreacted gamma 32P-ATP on a phosphocellulose membrane. This method requires at least one basic amino acid residue in the peptide substrate. The peptide substrate can be tagged with a biotin group so that the biotinylated 32P-peptide product consistently binds to a streptavidin membrane in a manner independent of the peptide sequence as described in

K. Screening Assays for Modulators

[0220] Modulators of D-AKAP2 biological activities may be identified by using any of the disclosed methods related to D-AKAP2 binding to PKA, D-AKAP2 localization in the mitochondria, binding to other signaling enzymes and phosphorylation by PKA. D-AKAP2 proteins are involved in signal transduction as they bind to protein kinase A (PKA) and are thought to anchor the kinase at a location, e.g., the mitochondria, where PKA acts to phosphorylate a specific substrate, either at the mitochondria or at an ion channel. Thus, an alteration in D-AKAP2 binding to PKA, localization to the mitochondria, or phosphorylation by PKA, among other steps, will result in an alteration in signal transduction.

[0221] Accordingly, provided herein are methods of screening for agents that decrease or disrupt the binding of a Val646 variant of D-AKAP2 with RlTar PKA, comprising combining a candidate agent with a cell comprising a nucleotide sequence which encodes a Val646 variant D-AKAP2 protein corresponding to SEQ ID NO:65, operably linked to a promoter such that the nucleotide sequence is expressed as a D-AKAP2 peptide in the cell; and determining the effect of the agent upon the localization of PKA to the mitochondria, wherein a decrease in localization to the mitochondria identifies an agent that decreases the binding of a Val646 variant of D-AKAP2 with RlTar PKA. Also provided are high-throughput methods of screening for agents that decrease or disrupt the binding of a Val646 variant of D-AKAP2 to an RlTar subunit of PKA, comprising combining a candidate agent with an admixture comprising RlTar and a D-AKAP2 peptide sequence that binds to RlTar; and determining the effect of the agent upon the localization of PKA to a mitochondria. The candidate agent can be combined with the admixture in a cell-free system. The candidate agent is combined with the admixture intracellularly. The peptide sequence can be any of the D-AKAP2 peptides described herein.

[0222] In particular, once a variant D-AKAP2 proteins species or variant D-AKAP2 peptide provided herein is contacted with a potential modulating molecule, the effect of the molecule on the binding between AKAP protein or peptide and PKA can be determined using the assays disclosed herein. For example, mitochondria can be isolated from cells exposed to the potential modulating molecule. PKA protein can then be isolated and quantitated or phosphorylation can be determined using the disclosed PKA assay. An increase in the amount of PKA protein in the mitochondria or the quantity of test peptide phosphorylated by mitochondrial isolated PKA would indicate a positive effect of the test molecule. Binding of the particular variant D-AKAP2 protein species, or peptide fragment thereof, and PKA could be directly assessed using an in vitro binding assay, or other disclosed binding assays such as set forth in Example 3 herein, or by immunoassays such as immunoprecipitation.

L. Assay Formats and Selection of Test Substances that Modulate at Least One D-AKAP2-Mediated Activity of a PKA Holoenzyme

[0223] Methods for identifying agents that modulate at least one D-AKAP2-mediated activity of a PKA holoenzyme are provided. The methods include phage display and other methods for assessing alterations in the activity of a D-AKAP2 protein and/or a PKA holoenzyme. Such methods or assays can use any means of monitoring or detecting the desired activity. A variety of formats and detection protocols are known for performing screening assays. Any such formats and protocols can be adapted for identifying modulators of D-AKAP2-mediated PKA holoenzyme activities. The following includes a discussion of exemplar protocols.

[0224] 1. High Throughput Screening Assays

[0225] Although the above-described assay can be conducted where a single D-AKAP2 protein or peptide and/or PKA holoenzyme is screened, and/or a single test substance is screened in one assay, the assay typically is conducted in a high-throughput screening mode, i.e., a plurality of the PKA holoenzymes are screened against and/or a plurality of the test substances are screened simultaneously (See, generally, High Throughput Screening: The Discovery of Bioactive Substances (Devlin, Ed.) Marcel Dekker, 1997; Sittampalam et al. (1997) Curr. Opin. Chem. Biol., 1:384-91; and Silverman et al. (1998) Curr. Opin. Chem. Biol., 2:397-403). For example, the assay can be conducted in a multi-well (e.g., 24-, 48-, 96-, 384-, 1536-well or higher density), chip or array format.

[0226] High-throughput screening (HTS) is the process of testing a large number of diverse chemical structures against disease targets to identify “hits” (Sittampalam et al. (1997) Curr. Opin. Chem. Biol., 1:384-391). Current state-of-the-art HTS operations are highly automated and computerized to handle sample preparation, assay procedures and the subsequent processing of large volumes of data.


[0228] 2. Test Substances

[0229] Test compounds, including small molecules, antibodies, proteins, nucleic acids, peptides, and libraries and collections thereof, can be screened in the above-described assays and assays described below to identify compounds that modulate the D-AKAP2-mediated activity of a PKA holoenzyme. Rational drug design methodologies that rely on computational chemistry can be used to screen and identify candidate compounds.
The compounds identified by the screening methods include inhibitors, such as antagonists, and can be agonists. Compounds for screening include any compounds and collections of compounds available, known or that can be prepared.

**Selection of Compounds**

Comounds can be selected for their potency and selectivity of modulating either the phosphorylation activity of a PKA holoenzyme or the translocation (e.g., localization to mitochondria) of the PKA holoenzyme. As described herein, and as generally known, a variant D-AKAP2 protein species, or peptide fragment thereof (e.g., SEQ ID NOs:17-54, and the like), a target PKA holoenzyme and its substrate are combined under assay conditions permitting reaction of the compound with its substrate. The assay is performed in the absence of test compound, and in the presence of increasing concentrations of the test compound. In addition, to identify test compounds that modulate D-AKAP2 mediated PKA activity, these assays can be performed in the absence of D-AKAP2, or fragments thereof. Those of skill in the art will understand that if a test compound demonstrates modulating activity of PKA in the presence of D-AKAP2, or fragments thereof, and not in its absence, then that compound is identified and selected as a D-AKAP2 mediated modulator of PKA activity. The concentration of test compound at which 50% of the enzymatic activity (e.g., phosphorylation activity) is inhibited by the test compound is the IC_{50} value (Inhibitory Concentration) or EC_{50} (Effective Concentration) value for that compound. Within a series or group of test compounds, those having lower IC_{50} or EC_{50} values are considered more potent inhibitors of the PKA enzymatic activity than those compounds having higher IC_{50} or EC_{50} values. The IC_{50} measurement is often used for more simplistic assays, whereas the EC_{50} is often used for more complicated assays, such as those employing cells.

**Typically candidate compounds have an IC_{50} value of 100 nM or less as measured in an in vitro assay for inhibition of PKA holoenzyme activity. The test compounds are also evaluated for selectivity toward a particular isoform of PKA, such as an RIIα or RIIβ containing PKA. As described herein, and as generally known, a test compound is assayed for its potency toward a panel of variant D-AKAP2 protein species, or peptide fragments thereof (e.g., SEQ ID NOs:17-54, and the like) and/or a target PKA holoenzyme, and other enzymes and an IC_{50} value or EC_{50} value is determined for each test compound in each assay system. A compound that demonstrates a low IC_{50} value or EC_{50} value for the target enzyme, e.g., PKA holoenzyme, and a higher IC_{50} value or EC_{50} value for other enzymes within the test panel (e.g., urokinase-type plasminogen activator, thrombin, Factor Xa), is considered to be selective toward the target enzyme. Generally, a compound is deemed selective if its IC_{50} value or EC_{50} value in the target enzyme assay is at least one order of magnitude less than the next smallest IC_{50} value or EC_{50} value measured in the selectivity panel of enzymes.

**Comounds are also evaluated for their activity in vivo. The type of assay chosen for evaluation of test compounds depends on the pathological condition to be treated or prevented by use of the compound, as well as the route of administration to be evaluated for the test compound.

**The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention. The practice of methods and development of the products provided herein employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Molecular Cloning A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glover, ed., 1985); Oligonucleotide Synthesis (M. J. Gait, ed., 1984); Mullis et al., U.S. Pat. No. 4,683,195; Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins, eds., 1984); Transcription and Translation (B. D. Hames & S. J. Higgins, eds., 1984); Culture of Animal Cells (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells and Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., New York); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos, eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al., eds.); Immunochemical Methods In Cell and Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

**EXAMPLE 1**

Assay of the Binding of D-AKAP2, A-Kinase Binding (AKB) Domain Ile/Val Variants to PKA

**Details**

The variable amino acid (Ile/Val) is located at amino acid 646 of SEQ ID NOs:64 and 65 in the AKB domain of D-AKAP2. This domain is the docking site for PKA and is highly conserved among species. Similar to other AKAPs, the AKB domain forms an amphipathic helix with hydrophobic amino acids on one face of the helix. To determine whether the D-AKAP2 Ile/Val variant resulted in an alteration in its binding properties to PKA, the binding of each variant to the regulatory subunit of PKA was investigated.

**Details**

Binding of each AKB domain variant to PKA was first examined using an in vitro pull-down assay, in which the 40 C-terminal residues of D-AKAP2 (amino acids 623-662 of SEQ ID NO:64) containing the AKB were fused to glutathione-S-transferase (GST). The GST fusion constructs were made by fusing the 40 C-terminal amino acids of D-AKAP2 to the C-terminus of GST and subcloning between the Ndel and BamHI sites of pRSET (Invitrogen, Carlsbad, Calif.). The constructs were transfected into BL21 cells and expressed for 6 hours at 20°C. The cells were lysed in PBS with 5 mM BME and 0.1% Triton X-100. 3 μl of supernatant were added to 200 μl of this buffer and 1 Op glutathione beads. After three washes, RIIα (53 μg, 20 μM) and RIIβ (2.4 μg, 2 μM) were added to the beads, respectively, and the total volume was adjusted to 40 μl. After incubating for 30 min at 4°C, the beads were washed three times, and separated in a 10% acrylamide gel.

**Details**

The Ile/Val substitution resulted in an isofrom specific difference in PKA binding. The RII isoform of PKA bound with a significantly higher affinity to the Val variant. The higher affinity was seen for both the mouse and the human ARB domains. The residue differences between mouse and human downstream of the Ile/Val position had no
Assay of the Binding Affinity of each Ile/Val AKB Domain Variant to PKA R-Subunit Isoforms

To assess the magnitude of the affinity difference, binding of both Ile/Val variants to the R-subunit isoforms of PKA was analyzed in a quantitative assay. Twenty-seven residue peptides containing the two AKB domain Ile/Val variants (SEQ ID Nos: 1 and 2, respectively) of D-ÅKAP2 with a C-terminal cysteine were synthesized by Synpep Corporation (Dublin, Calif.). The peptides were HPLC (high performance liquid chromatography) purified and mass spectrometry checked. Both peptides were labeled with tetramethyl rhodamine-5-maleimide (Molecular Probes, Eugene, Oreg.) at the cysteine residue and HPLC purified. Increasing concentrations of RÎ± and RÎ±β, respectively, were equilibrated with 10 nM of peptide for at least one hour at room temperature in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20. For RÎ±K tests, 1 nM of labeled peptide was used since the binding affinity to RÎ±K was higher than to RÎ± and RÎ±β. Fluorescence anisotropy was monitored using a Fluoromax-2 (Spex, Jobin Yvon Horiba, Edison, N.J.) equipped with photometers. The fluorophore was excited at 541 nm (5-10 nm bandwidth) and emission was monitored at 575 nm (5-10 nm bandwidth). For each protein three separate binding experiments were averaged and fit to a 1:1 binding model using the non-linear regression application in GraphPad Prism version 3.00 (GraphPad Software, San Diego, Calif.).

Peptides of each AKB Ile/Val variant (SEQ ID Nos: 1 and 2) were synthesized and fluorescence-labeled. Binding of the labeled peptides to the regulatory subunit isoforms was monitored using a fluorescence assay in which binding of the peptide was proportional to an increase in steady state anisotropy. There was no difference in binding of the AKB variants to either the RÎ± or RÎ±β isoforms. In addition, the RÎ± isoforms bound tighter to the variants than the RÎ±β isoform. However, as indicated by the pull-down experiments, RÎ± displayed differential binding to the AKB variants. The Val variant (SEQ ID NO: 2) had a nearly three-fold increase in binding affinity when compared to the Ile variant (SEQ ID NO: 1). The interaction was specific since no binding was observed to a deletion construct of RÎ± that lacked the AKAP binding domain (delta 1-91 RÎ±).

In Vivo Assays of the Association of the AKB Ile646Val Variants and the PKA Regulatory Subunits and Targeting to Mitochondria

To determine whether the observed in vitro affinity differences resulted in a difference in cellular compartmentalization, the association of the AKB Ile/Val variants and the PKA regulatory subunits in vivo was examined. The 30 amino acid mitochondrial anchoring domain of D-ÅKAP1 (Chen et al. (1997) J. Biol. Chem., 272:15247-57; and Huang et al. (1997) J. Biol. Chem., 272:8057-8064) was fused to the C-terminal 156 residues of mouse (amino acids 217-372 of SEQ ID NO: 55) and human (amino acids 507-662 of SEQ ID NO: 64) D-ÅKAP2, respectively, followed by a Flag-tag and sub-cloned into pcDNA4 (Invitrogen, Carlsbad, Calif.). The dimerization/docking (D/D) domain of RÎ± was each fused with green fluorescent protein (GFP) and cloned into pEGFPN1 (Clontech, Palo Alto, Calif.). Equimolar ratios of the Flag-tagged D-ÅKAP2 constructs and either RÎ±-GFP or RÎ±-D-GFP constructs were mixed and transfected into 10T(1/2) cells using cytovectene (BioRad, Hercules, Calif.). The AKB domain was detected by immuno-staining with monoclonal antibodies against the Flag-tag (Kodak, Rochester, N.Y.) followed by rhodamine-conjugated secondary antibody (Jackson Lab, Bar Harbor, Me.). The cells were imaged using a Zeiss microscope equipped with a digital camera. Each channel was exposed for the same amount of time.

In this assay, the AKB domain within the C-terminal 156 residues of D-ÅKAP2 from mouse (Val) and human (Ile) was fused to the mitochondrial anchoring domain from D-ÅKAP1 and tethered to the outer mitochondrial membrane (Chen et al. (1997) J. Biol. Chem., 272:15247-15257, and Huang et al. (1997) J. Biol. Chem., 272:8057-8064). The binding of the AKB domain with the PKA regulatory domains was detected as the co-transfected PKA regulatory domains co-localized to the mitochondria. Both the human and mouse AKB domains can target RÎ± to the mitochondria effectively; in accordance with their similar affinity in vitro. However, for RÎ± there was a difference in co-localization between the variants. The Val variant effectively targeted RÎ± to the mitochondria. The Ile variant, however, was unable to target RÎ±, which was evenly diffused in the cytosol suggesting that the Ile variant of D-ÅKAP2 may have impaired ability to sequester RÎ±.

The D-ÅKAP2 variants at amino acid 646 of SEQ ID NO: 64 and 65 described herein map to the conserved AKB domain of D-ÅKAP2, which was previously shown to interact with the regulatory subunit of PKA. In accordance with the methods provided herein, it has been demonstrated that this variation impacts the binding to PKA in an isoform specific manner both in vitro and in vivo. The Val(646) variant at amino acid 646 of SEQ ID NO: 65, which has previously been identified as a deleterious allele associated with morbidity in the age-stratified approach (see, e.g., US Patent Application US20020040130A1 and PCT WO 02/04489), binds three-fold tighter to the RÎ± isoform when compared to the Ile(646) variant. At the cellular level, this affinity difference resulted in a decrease in mitochondrial localization of the Ile (646) variant.

Development of a Detection System to Verify Binding of 27-mer Peptides to PKA Homodimers

A peptide SPOT-synthesis technique was applied to study the interaction of a 27-mer D-ÅKAP2-derived peptide VQGNTEAQEEELAWKIAMIVSDIMQQ (SEQ ID NO: 11) with the regulatory subunit PKA-RÎ±. However, prior to binding studies on cellulose membranes, the interaction of the 27-mer with PKA was confirmed by ELISA (enzyme-linked immunosorbent assay).

The peptide biotin-ba-ba-VQGNTEAQEEELAWKIAMIVSDIMQQ (SEQ ID NO: 14) was synthesized as a C-terminal amide in milligram quantity and purified to a 99% purity by HPLC. Its identity was confirmed by MALDI TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry. The peptide was immobi-
ized in neutravidin (NA)-coated 96-well microtiter plates and incubated with various concentrations of PKA-RIIα-GFP. Binding was detected using an anti-GFP antibody (3E6, Quantum Biotechnologies, QBiogene, Carlsbad, Calif.) in combination with a secondary horseradish peroxidase labeled antibody. Microtiterplates were coated with 40 μg/ml Neutravidin (NA) and subsequently incubated with 25 μM peptide. Various concentrations (0-5 μg/ml) of PKA-RIIα-GFP were added. Bound subunit was detected using a mouse anti-GFP antibody (1 μg/ml) and an anti-mouse-IgG antibody labeled with peroxidase (1 μg/ml). Negative controls were utilized that contained either a microtiter plate with only neutravidin or only peptide, or neither one. The only signal detected was in wells containing both neutravidin and peptide, indicating that the 27-mer peptide corresponding to SEQ ID NO:1, when immobilized on Neutravidin coated wells, is able to bind to PKA-RIIα in the same experiment.

EXAMPLE 5

Assay of Binding Properties of the 27-mer Peptide
Dependent Upon Amino Acid Sequence Composition and Length

[0247] a) Identification of Critical Residues for Binding PKA within the 27-mer Peptide (SEQ ID NO:1) Corresponding to the Binding Domain of D-AKAP2.

[0248] To identify key residues within the 27-mer Ile/Val peptides (SEQ ID NO:1) for interaction with PKA, an alanine, an aspartic acid, and a lysine scan was performed. The filters for the peptide arrays were prepared by SPOT-synthesis and incubated with PKA-RIIα-GFP (see, e.g., Frank R., (1992) Tetrahedron, 48(42):9217-9232; Kramer et al. (1994) Comp. Meth. Enzymol., 6:388-395; Kramer et al. (1997) Meth. Mol. Biol., 87:25-39; and Kramer et al. (1999) J. Peptide Res., 54:319-327). Binding was detected using an anti-GFP antibody (3E6, Quantum Biotechnologies, QBiogene, Carlsbad, Calif.) in combination with a secondary antibody and a chemiluminescence-imager. All scans revealed a clear key residue pattern.

[0249] Residues of the 27-mer peptide that cannot be substituted for while retaining the ability to bind RIIC; in the Ala-scan are residues 16, 20 and 21; in the Asp-scan are residues 12, 13, 16, 17, 20, 21, and 24; in the Lys-scan are residues 12, 13, 16, 17, 20, 21, and 24 of the 27-mer peptide (SEQ ID NO:1). Residues that result in reduced binding upon substitution in the Ala-scan are residues 10, 12, and 23; in the Asp-scan are residues 14 and 15; and in the Lys-scan is residue 14 of the 27-mer peptide (SEQ ID NO:1). These results indicate a helical structure of the binding domain. Although the variable position (Ile/Val) occurs at amino acid residue 24, in this experiment only isoleucine containing peptides were synthesized.

[0250] b) Amino- and Carboxy-Terminal Truncation Experiments to Identify Minimal Peptide Length Capable of Binding to PKA-RIIα and PKA-RIIC.

[0251] Several truncation analogs of the 27-mer peptide corresponding to SEQ ID NO:1 have been synthesized and tested for binding. N-terminal truncations, C-terminal truncations, and N/C-terminal truncations were performed. For example, Table 3 sets forth N/C-terminal truncations where, sequentially, both an N- and a C-terminal amino acid were omitted in each SPOT-synthesis.

TABLE 3

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<th>Peptides</th>
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<td>+++</td>
<td>+++</td>
<td>SEQ ID NO:1</td>
</tr>
<tr>
<td>QCQNDRAEQBELWIKEMIVSDIM</td>
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<td>+++</td>
<td>SEQ ID NO:6</td>
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<td>+++</td>
<td>+++</td>
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<td>+++</td>
<td>+++</td>
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<td>++</td>
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<td>++</td>
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<td>--</td>
<td>+</td>
<td>SEQ ID NO:4</td>
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<td>--</td>
<td>--</td>
<td>SEQ ID NO:10</td>
</tr>
<tr>
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</table>
In this experiment, only the isoleucine variant 27-mer peptide (SEQ ID NO:1) was used (amino acid position of variation corresponds to position 24 of SEQ ID NO:1). Incubation with PKA-Rlα-GFP and PKA-Rlα-GFP and detection with antibodies was performed as described above. It has been found that several residues from the N- and C-terminus can be omitted without significant loss of binding. The shortest peptide with no reduced signal intensity identified for both Rlα and Rlα is a 21-mer with the following sequence: NH2-NTDEAQELAWKIAKMIVSDI-COOH (SEQ ID NO:3 and 7).

It is important to note that the Ile/Val polymorphic amino acid position corresponding to the last residue in the above peptide (SEQ ID NO:3 and 7) (or residue 24 in SEQ ID NO:1) is essential for binding to the Rlα subunit. If the peptide is truncated to exclude residue 24 in SEQ ID NO:1, the remaining peptide does not bind to Rlα at all. In addition, if the peptide is truncated to exclude residue 24 in SEQ ID NO:1, binding to Rlα is reduced. In this particular N/C-terminal dual truncation assay, it has been found that the shortest peptide that still binds to Rlα is a 15-mer: NH2-NTDEAQELAWKIAKMIV-COOH (SEQ ID NO:4).

In addition, the minimal sequence required for regulatory subunit binding was also assessed using N- and C-terminal truncations of the 27-residue human D-AKAP2 sequence. N-terminal or C-terminal truncated peptides were synthesized using POP-3 synthesis on cellulose membrane as described herein. Binding was evaluated by incubating each membrane with GFP-Rlα D/D and GFP-Rlα D/D as indicated below. The dimerization/docking (D/D) domain of bovine Rlα (residues 1-109) and mouse Rlα (residues 1-146), fused to green fluorescence protein (GFP) were subcloned into a pRSET expression vector (Invitrogen, Carlsbad, Calif.) downstream of a histidine tag. The proteins, GFP-Rlα D/D and GFP-Rlα D/D were expressed in E. coli BL21 (DE3) and purified using Talon (Clontech, Palo Alto, Calif.) resin. The His tag was cleaved using thrombin and the protein further purified using an S75-Sephadex (16/60) gel filtration column (Pharmacia, Peapack, N.J.) in 50 mM MES pH 5.8, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT. The protein was stored at 4°C.

Bound protein was detected using a primary antibody against GFP and enzyme conjugated secondary antibody for amplification of signal. The membrane was then analyzed by chemiluminescence. The results are set forth in Tables 4 and 5.

### TABLE 4-continued

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### TABLE 5

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<td>VQGNTDEAEQLR</td>
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[0255] The C-terminal truncations defined clearly the C-terminal boundary for binding to the isomers. There was an absence of binding to both regulatory subunits at a defined residue from the C-terminus. For R1o, binding abruptly stopped after the C-terminal isoleucine (VQGNTDEAQELAWKIAKMKIVSDI; SEQ ID NO:85), suggesting that the C-terminal (...MQQ) residues are not required for binding (Table 5). This C-terminal isoleucine residue is the location of a single nucleotide polymorphism of D-AKAP2, which codes for either a valine or isoleucine at this position (1646V; SEQ ID NOs:64 and 65). For R1o, binding abruptly stopped at the upstream valine position (VQGNTDEAQELAWKIAKMKIV; SEQ ID NO:88), suggesting that more C-terminal residues (...SDIMQQ; SEQ ID NO:1, amino acids 22-27), which contain the polymorphism, are dispensable for the R1o binding site (Table 5). This is consistent with the 1646V polymorphic site only having an effect on binding to the R1o isoform.

[0256] The N-terminal truncations did not result in a clear cut boundary, but rather there was a titratable decrease in signal for both R1o and R1o isoforms starting at the glutamine (QUELAWKIAKMKIVSDIMQQ; SEQ ID NO:73) (Table 4). This suggests that the N-terminal negative charges play a role in enhancing the affinity to both isoforms.

EXAMPLE 6
Assay of the Binding Properties of the Two Naturally Occurring Allelic Variants of the D-AKAP2 Protein

[0257] a) Optimization of Peptide Density and Regeneration Protocols
[0258] First, the optimal peptide density for further synthesis was determined to improve quantification of binding differences between both PKA isoforms. This was achieved by synthesizing two sets of membranes containing two peptide sequences representing the Ile and Val alleles, respectively:

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<thead>
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<th>PEPTIDES</th>
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</tr>
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<td>VQGNTDEAQELAWKIAKMKIVSDIMQQ</td>
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</tr>
<tr>
<td>VQGNTDEAQELAWKIAKMKIVSDIMQQ</td>
<td>2</td>
</tr>
</tbody>
</table>

[0259] Both peptides were synthesized as 5 spots varying in peptide density from 50% to 0.1% membrane saturation. Then, set 1 was incubated with PKA-R1o-GFP, set 2 with PKA-R1o-GFP to determine the optimal peptide density for the binding assay. Subsequently, the membranes were regenerated and incubated with the other regulatory subunit, respectively (set 1 with PKA-R1o-GFP, set 2 with PKA-R1o-GFP). This was to verify the feasibility of regenerating the membranes, which was beneficial for the following experiments as well as to identify the preferred order of incubation.

[0260] Membranes after incubation and GFP-signal development, as well as quantification of the signal intensities revealed that the signal intensity for PKA-R1o-GFP decreases with reduction of the peptide density, whereas the highest signals for PKA-R1o-GFP are observed for reduced peptide densities between 5 and 10%. These findings correlate with a higher affinity of the peptides for PKA-R1o-GFP. A reduced peptide density of 10% suitable for both regulatory subunits was suggested for further experiments.

[0261] For PKA-R1o-GFP, an approximate 10-fold higher signal intensity compared to PKA-R1o-GFP was measured. Since both membranes were incubated simultaneously under the same conditions this finding must be due to the higher affinity of PKA-R1o-GFP for the D-AKAP2-derived peptide. No significant PKA-R1o-GFP binding differences between the Ile and Val variants of the D-AKAP2-derived peptide were observed. However, allele-specific differences were obtained for PKA-R1o-GFP.

[0262] After stripping and regeneration of membranes similar results were obtained compared to a fresh filter set. The signal intensity was about 10% lower. However, the regeneration protocol is suitable since no signals were observed in a control experiment with the detection antibodies alone. The preferred order for further experiments was determined to be incubation of peptide membranes with PKA-R1o-GFP followed by PKA-R1o-GFP due to the differences in signal intensity.

[0263] b) Quantification of Allele-Specific Binding Differences.

[0264] Two 27-mer peptides representing the Ile and Val variants (SEQ ID NOs:1 and 2, respectively) were synthesized and purified in milligram quantities and the affinity to PKA-R1o-GFP was determined using well-known surface plasmon resonance in combination with BLAcore chips (see, e.g., Current Opinion in Biotechnology (1997) 8:50-57; Current Opinion in Biotechnology (1994) 5:389-395; Current Opinion in Biotechnology (1994) 5:65-71; Structure (1995) 3:969; Current Biology (1995) pp. 699-705; Analytical Biochemistry (1991) 201:197-210; and macinfac.bio.unc.edu/bla.core.html). PKA-R1o-GFP was coupled to BLAcore CM5 chips. BSA was immobilized on control chips.

Chip 1: PKA-R1o-GFP (1681 RU) Chip 2: BSA (1424 RU)

[0265] TABLE 6

<table>
<thead>
<tr>
<th>PKA-R1o-GFP binding affinity</th>
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<td>Ac-VQGNTDEAQELAWKIAKMKIVSDIMQQ</td>
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<td>Ac-VQGNTDEAQELAWKIAKMKIVSDIMQQ</td>
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</table>

[0266] The affinities in this assay are lower compared to those determined by fluorescence anisotropy. The most likely reason is that in this study the signal difference between the flow cell with the regulatory subunit and the control protein BSA was measured, such that slight unspecific binding to BSA directly reduces the apparent affinity. However, similar
to previous results, an affinity difference of PKA-R1α-GFP to both allelic 27-mer peptides was observed (Table 6).

EXAMPLE 7

Identification of Substituted Peptide Sequences that are able to Disrupt the Interaction Between D-AKAP2 and PKA Completely by Either Binding Specifically PKA-R1α- or R1α or by Binding both PKA Isoforms

[0267] Substitution analysis of both allelic peptides (VQGNTDEAQLAEWKLAKMIVSDVMQEQ; SEQ ID NO:2 and VQGNTDEAQELAEWKLAKMIVSDVMQEQ; SEQ ID NO:1) was conducted. Each amino acid of the 27-mer peptide was substituted, one amino acid at a time per peptide, by any naturally occurring L- and D-amino acid. For both peptides complete L- and D-substitution analysis membranes have been screened for binding of PKA-R1α-GFP. After regeneration, the peptide arrays were incubated with PKA-R1α-GFP. After binding, detection was carried out with an anti-GFP antibody in combination with a peroxidase-labeled secondary antibody.

[0268] a) Peptide Array Synthesis

[0269] The cellulose-bound peptide libraries were automatically prepared according to standard SPOT synthesis protocols (Frank, R., (1992) Tetrahedron, 48:9217-9232) using a SPOT synthesizer (Abimed GmbH, Langenfeld, Germany) as described in Kramer et al. (1998) Methods Mol. Biol., 87:25-30 and Wenschuh et al. (2000) Biopolymers, 55:188-206. The peptides were synthesized on an amino functionalized cellulose membrane as distinct spots. A β-alanine dipeptide spacer was inserted between the C-terminus of the peptide and the membrane support. The peptide loading of the membranes was reduced by mixing 10% Fmoc-β-alanine-OOPp and 90% acetylated β-alanine-OOPp active esters for the first coupling step. The peptide loading was optimized in advance by varying the Fmoc-β-alanine-OOPp percentage from 0.1% to 50%. The peptide was extended stepwise using standard Fmoc solid-phase peptide synthesis followed by cleavage of the side chain protecting groups under trifluoroacetic acid (TFA) conditions. Sequence files were generated with the software DIGEN (Jerini A G, Berlin, Germany). All peptides were N-terminally acetylated. For synthesis quality control, a selection of peptides that was synthesized in duplicate was cleaved from the solid support by ammonia vapor in the dry state. Subsequently, identity was verified by MALDI-MS (Voyager-DE, Applied Biosystems, Foster City, Calif., USA).

[0270] b) Peptide Array Screening

[0271] The peptide arrays were pre-incubated with T-TBS blocking buffer (TBS pH 8.0, 0.05% Tween 20 in the presence of blocking reagent; Roche Diagnostics Chemiluminescence detection kit 1506964, Mannheim, Germany). Subsequently, the peptide arrays were incubated with solutions of GFP-R1α D/D or GFP-R1α D/D at a final concentration of 1.0 μg/ml for 2 h in T-TBS blocking buffer. After washing three times for 10 min with T-TBS, the anti-GFP antibody (1/1000, Alexa Fluor 555, Invitrogen, Carlsbad, Calif.) was added to a final concentration of 1 μg/ml in T-TBS blocking buffer for 1 h followed by washing three times for 10 min with T-TBS. Finally, the arrays were incubated with a second anti-mouse IgG peroxidase-labeled antibody (Catalog #: A5906, Sigma, Deisenhofen, Germany) which was applied at a concentration of 1 μg/ml in T-TBS blocking buffer for 1 h, followed by washing three times for 10 min with T-TBS. Analysis and quantification of peptide-bound GFP-R1α D/D or GFP-R1α D/D-antibody complexes was done using a chemiluminescence substrate and the Lumi-Imager® (Roche Diagnostics, Mannheim, Germany). All steps were carried out at room temperature. Binding of the detection antibodies to the peptides was excluded by control incubations with antibody 3E6 and the anti-mouse IgG peroxidase-labeled antibody alone. For a given membrane, binding of GFP-R1α D/D was performed first. Subsequently, the membrane was regenerated using detergent (Kramer et al. (1998) Methods Mol. Biol., 87:25-30) and complete removal of the GFP-R1α D/D was proven by a control incubation with antibody 3E6 and the anti-mouse IgG peroxidase-labeled antibody alone. Afterwards, binding of GFP-R1α D/D to the peptide array was assessed.

[0272] The results of the incubated membranes containing the single amino acid substitutions using L-amino acids are shown in FIG. 1. FIG. 1 shows the amino acid substitution analysis of both isoforms (V1/24; SEQ ID NO:1 and 2; of a 27-mer D-AKAP2 peptide that is the binding domain for the R1α and R1α subunits of protein kinase A (PKA). Each amino acid of the respective D-AKAP2 peptide (vertical lane) was substituted, one amino acid at a time, by a single L-amino acid (horizontal line). The top filters show the results of substituting amino acid residues in the Valine variant (SEQ ID NO:2; and the bottom filters represent the substitutions in the Isoleucine variant (SEQ ID NO:1). The left hand filters were analyzed with PKA-R1α-GFP and right hand filters with PKA-R1α-GFP, respectively. Key amino acid residues for the binding to both R1α and R1α are indicated to be residues 12, 13, 16, 17, 20 and 21. The vertical dark boxes occurring at residues 10-21 of the V1/24 peptides in the prolinc column indicate loss of binding to both isoforms after prolinc substitution in the middle part of the peptide. The light colored boxes mark substitutions specifically disrupting or significantly decreasing the binding to PKA-R1α-GFP. Substitutions that significantly enhance the binding of both V1/24 peptides to PKA-R1α-GFP are dark-circled and correspond to the substitution in SEQ ID NO:1 and 2 of one, two or more: of Q at residue 9 with F, I, L, V, H, M, R, T, W or Y; of L at residue 12 with F, W or Y; of V at residue 21 with F, W or Y; and of M at residue 25 with F, I, L, T, V, W or Y. For light-colored boxes and circled markings, the binding to PKA-R1α-GFP can be either increased, normal, decreased or even completely disrupted.

[0273] Key residues for binding of D-AKAP2 peptide variants to PKA-R1α-GFP and PKA-R1α-GFP are 116, A17, 120, and V21. For binding to both PKA-GFP subunits (R1α and R1α) these critical residues cannot be substituted on the peptide without loss of binding except by physicochemically very similar ones, which will lead to reduced binding (e.g., A17 by I or V). The substitution pattern reflects the postulated helical binding structure of the peptide. Key residues are found on one side of the helical wheel and prolinc substitutions in the central part of the peptide corresponding to residues 10-21 are disadvantageous for the binding to both isoforms of PKA.

[0274] In this substitution analysis only slight differences between the valine and isoleucine variants were observed. However, it was not the objective to compare both allelic peptides quantitatively.

[0275] D-amino acid substitutions in the central part of the peptide reduce the binding ability to both PKA subunits. The
binding to PKA-RIIα-GFP was regularly decreased throughout the entire peptide for all substitutions. There were a few substitutions, which enhanced the binding to PKA-RIIα-GFP in the context of all other substitutions. The following list indicates those peptides and substitutions with D-amino acid analogs in underlined and bold one-letter code.

VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:17)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:18)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:19)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:20)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:21)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:22)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:23)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:24)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:25)

and VQGNTD/EASLKWIAKIVMDI/V/MQQ. (SEQ ID NO:26)

EXAMPLE 8
Identification of Peptide Sequences that Show Preferential and/or Enhanced Binding to Either PKA-Rlc or -RIIα

[0276] As a result of the substitution analysis conducted as described in Example 7 and set forth in FIG. 1, it has been found that amino acid residues L12 and A13 are key residues for PKA-Rlc binding but not for PKA-RIIα binding, which indicates that binding of the 27-mer peptides to PKA-Rlc-GFP is more entailed compared to PKA-RIIα-GFP. Also residues D23 and V724 (corresponding to the polymorphic position within the 27-mer peptides), are residues with a limited preference for certain amino acids regarding the binding to PKA-Rlc-GFP.

[0277] Exemplary substitution analogs leading to preferred or exclusive binding of the 27-mer peptides to PKA-Rlc-GFP relative to PKA-RIIα-GFP, or enhanced binding to both Rlc and RIIα isofroms correspond to the substitution in SEQ ID NOs:1 and/or 2 of one, two or more: of Q at residue 9 with F, I, L, V, H, M, K, T, W or Y; of L at residue 12 with F, W or Y; of V at residue 21 with I, L or W; and of M at residue 25 with F, I, L, T, V, W or Y.

[0278] For the 27-mer Val-isofrom (SEQ ID NO:2), exemplatory substitution analogs leading to preferred or exclusive binding to PKA-Rlcs-GFP relative to PKA-Rlc-GFP correspond to the substitution in SEQ ID NO:2: of L at residue 12 with A, C, or K; of A at residue 13 with F, H, I, K, L, M or N; of W at residue 14 with C; of K at residue 15 with I; of K at residue 16 with C; of M at residue 19 with C; of S at residue 22 with C; and of D at residue 23 with C.

[0279] For the 27-mer Ile-isofrom (SEQ ID NO:1), exemplatory substitution analogs leading to a preferred or exclusive binding to PKA-Rlcs-GFP relative to PKA-Rlc-GFP correspond to the substitution in SEQ ID NO:1: of A at residue 13 with F, H, I, L, M and S; of W at residue 14 with C; of K at residue 15 with C; of K at residue 16 with C; of M at residue 19 with C; of S at residue 22 with C; and of D at residue 23 with C.

[0280] The following is an exemplary list of peptides substituted with L- and D-amino acid residues bearing specific novel binding properties. Since the design of these experiments was not done to disclose binding differences between the two allelic peptides (SEQ ID NO:1 and 2), both peptide syntheses can be used as mutual replications. Peptides that show similar binding properties in two independent experiments are indicated below.

[0281] A. Peptides with Substantially No Binding to PKA-Rlc-GFP but Normal Binding to PKA-RIIα-GFP. Substituted L-Amino Acids are Indicated in Bold, and the Polymorphic Site in Brackets:

VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:27)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:28)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:29)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:30)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:31)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:32)
VQGNTD/EASLKWIAKIVMDI/V/MQQ. (SEQ ID NO:33)

[0282] B. Peptides with Substantially No Binding to PKA-Rlc-GFP and Reduced Binding to PKA-RIIα-GFP. Substituted L-Amino Acids are Indicated in Bold, and the Polymorphic Site in Brackets:

VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:34)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:35)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:36)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:37)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:38)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:39)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:40)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:41)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:42)
VQGNTD/EASLKWIAKIVMDI/V/MQQ. (SEQ ID NO:43)

[0283] C. Peptides with Enhanced Binding to Both, PKA-Rlc-GFP and PKA RIIs-GFP. Substituted L-Amino Acids are Indicated in Bold, and the Polymorphic Site in Brackets:

VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:44)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:45)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:46)
VQGNTD/EASLKWIAKIVMDI/V/MQQ; (SEQ ID NO:47)
VQGNTD/EASLKWIAKIVMDI/V/MQQ. (SEQ ID NO:48)
Additional multiple substitution peptides (PV-37, 38, 47, 48, 49) were synthesized by Peptron (Korea). All peptides contained a C-terminal Cys for conjugation of the fluorescence probe and contained an amide protected C-terminus. The peptides were HPLC purified and the molecular mass verified by mass spectrometry. Peptide purities were greater than 95%

[0291] Each peptide was fluorescently labeled using a 25 mM solution of tetramethylrhodamine-5-maleimide (catalog number T-6027, Molecular Probes, Eugene, Ore.) dissolved in DMSO. The peptides were labeled by incubating with a three-fold molar excess of the label for 16 hr at 4°C in 20 mM Tris, pH 7.0 and 1 mM tris-(2-carboxyethyl) phosphine, hydrochloride (TCEP) (non-thiol reducing agent, Molecular Probes). The sample was quenched with 1 mM β-mercaptoethanol to bind to any unreacted maleimide and diluted with 0.1% TFA for purification by HPLC. The labeled peptides were resolved using a C18 column with a water/acetonitrile gradient containing 0.1% TFA. The concentration of each peptide was determined by absorbance at 541 nm after diluting into 100% methanol and using an extinction coefficient of 91,000 M⁻¹ cm⁻¹ for absorbance of the rhodamine label at 541 nm (Molecular Probes Catalog). The peptides were stored at 4°C in 50% acetonitrile.

[0292] b) Rlx and Rlx Production

[0293] Full-length murine Rlx was expressed in E. coli BL21 (DE3). Full-length bovine Rlx was expressed in E. coli 222. The proteins were purified as previously described using a cAMP affinity resin (Hamuro et al. (2002) J. Mol. Biol., 321:703). The protein concentrations were determined using the following extinction coefficients at 280 nm, which were calculated using a standard concentration of protein calibrated using quantitative amino acid analysis: Rlx 52,603 M⁻¹ cm⁻¹ and Rlx 62,456 M⁻¹ cm⁻¹. The proteins were stored at 4°C in 50 mM MES pH 5.8, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM DTT.

[0294] c) Fluorescence Anisotropy

[0295] Binding of each fluorescently labeled peptide to the regulatory subunits was monitored using fluorescence anisot-
therapy. Rlxα and RIIα were serially diluted beginning at 1 μM and 0.1 μM, respectively, into 10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (Biocore) containing either 10 nM or 1 nM of fluorescently labeled peptide for Rlxα and RIIα, respectively. The samples were equilibrated for at least 1 hour at room temperature and fluorescence anisotropy monitored using a Fluoromax-2 (Jobin Yvon Horiba, SPEX Division, Edison, N.J.) equipped with Glan-Thompson polarizers. The rhodamine-labeled peptide was excited at 541 nm (5-10 nm bandpass) and emission monitored at 575 nm (5-10 nm bandpass). The anisotropy was calculated directly with the Fluoromax software using the following equation:

$$r = \frac{I_{xy} - G_{up} + 2G_{down}}{I_{xx} - 2G_{up} - G_{down}}$$

where $r$ is the steady state anisotropy, $I_{xy}$ is the fluorescence intensity with the excitation and emission polarizers oriented in the vertical position (0° from normal), $I_{xx}$ is the fluorescence intensity with the excitation polarizer in the vertical position and the emission polarizer oriented in the horizontal position (90° relative to the excitation polarizer, G is the monochromator grating factor which is equal to ($I_{xx}$/$I_{xy}$), with the first subscript indicating the position of the excitation polarizer and the second subscript indicating position of the emission polarizer. Three separate binding experiments were averaged and fit to a 1:1 binding model using the non-linear regression application in GraphPad Prism version 3.00 (GraphPad Software, San Diego, Calif.).

TABLE 7

Dissociation constants ($K_d$) with standard error (n = 3) for peptides binding to Rlx and RIIα determined using a fluorescence anisotropy binding assay.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rlxα ($K_d$) (nM)</th>
<th>RIIα ($K_d$) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKB (null) (SEQ ID NO:108)</td>
<td>48 ± 4</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>VQGNTDEAQELANKIQAKVIMSDVMQQC</td>
<td>2493 ± 409</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>AKB (RI), RII specific (SEQ ID NO:109)</td>
<td>5.2 ± 0.5</td>
<td>456 ± 33</td>
</tr>
<tr>
<td>VQGNTDEAQELANKIQAKVIMSDVMQQC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKB (RI), RII specific (SEQ ID NO:103)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VQGNTDEAQELANKIQAKVIMSDVMQQC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Substituted residues are underlined and in bold.

TABLE 8

E.D. (pM)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Rlxα ($K_d$) (nM)</th>
<th>RIIα ($K_d$) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQ ID NO:108 (residues 9-28)</td>
<td>48 ± 4</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>. . . QERLANQIAKAMVIDVMQQC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEQ ID NO:111 (residues 9-28)</td>
<td>120 ± 13</td>
<td>83 ± 7</td>
</tr>
<tr>
<td>. . . QERLANQIAKAMVIDVMQQC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEQ ID NO:112 (residues 9-28)</td>
<td>773 ± 49</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>Ac-DLANKIQAKMVIDVMQQC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(e.g., very weak binding) to RIIα compared to unmodified SEQ ID NO:108, AKB (RI), and is indicated with an asterisk.
TABLE 8-continued

<table>
<thead>
<tr>
<th></th>
<th>RIA</th>
<th>RIIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV-37</td>
<td>FEILAWKIAMNISDVPQQC</td>
<td>19 ± 0.7</td>
</tr>
<tr>
<td>PV-38</td>
<td>FEILAWKIAMNISDVPQQC</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>PV-47</td>
<td>QUEFPWAKIAMNISDVPQQC</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>PV-48</td>
<td>QUEFPWAKIAMNISDVPQQC</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>PV-49</td>
<td>FEILAWKIAMNISDVPQQC</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

PV-37 (SEQ ID NO:104); PV-38 (SEQ ID NO:103); PV-47 (SEQ ID NO:105); PV-48 (SEQ ID NO:106); PV-49 (SEQ ID NO:107)

[0299] To determine whether the N-terminal negative charges were important for high affinity binding as suggested by the truncation data, a truncation peptide corresponding to SEQ ID NO:102 was synthesized that did not contain the two N-terminal Glu residues, but instead contained an N-terminal α-acetylated Asp. This peptide also showed reduced binding to both regulatory subunits confirming the requirement of at least one of the N-terminal negative charges. Also evident from these mutations is the importance of the bulky hydrophobic tryptophan at position 21 in selectively reducing the affinity to the type II isomter. The single mutant V21W (SEQ ID NO:111) showed dramatically reduced binding to RI, while only modestly decreased affinity toward RIa (Table 8). This position is therefore a critical position along the helix for establishing RI/RIa selectivity. In the background of V21W, further substitutions at position 9 and 25 dramatically enhanced binding to RIa while further disrupting RIa binding (see PV-37 and PV-38, Table 8). RIIa also seemed to be less tolerant of the Leu to Phe substitution at position 12 (PV-47 and PV-48, Table 8). PV-49, which is identical to PV-38 except that it has an Ile instead of a Trp at position 21, bound with the greatest affinity to RIa (Table 8). However, this peptide also bound very tightly to RIa. This again reinforced that a bulky aromatic residue at position 21 was important to select against RIa binding. Interestingly, the Phe at positions 9 and 25 are only disruptive to RIa binding when Trp is present at position 21 (PV-38). When Trp at position 21 is replaced with Ile, the affinity for RIa is restored and the affinity for RIa further enhanced (PV-49). Thus, it is contemplated herein that Trp at position 21 (I) and the Phe at position 25 (I+4) may interact to form additional unfavorable interactions for RIa binding.

EXAMPLE 10

In Vivo Assays of the Association of AKB Variants and the PKA Regulatory Subunits and Targeting to the Mitochondria

[0300] To test the ability of the mutations set forth in Table 7 [AKB(dual), AKB(RI), AKB(RII) and AKB(null)] to co-localize with selected PKA isoforms in cells, a flag-tagged AKB domain construct targeted to the mitochondria was prepared using the AKB domain and a mitochondria targeting sequence from D-AKAP1 as set forth in Example 3. The respective mutants were then incorporated into the AKB domain of D-AKAP2 to test for selectivity in the cell. The targeting constructs of the AKB domain were made by fusing the C-terminal 156 residues of mouse D-AKAP2 with the N-terminal mitochondrial-targeting domain of D-AKAP1 (residues 1-30 of D-AKAP1). After adding a flag tag to the C-termims, the constructs were sub-cloned into the pC1 vector (Promega, Madison, Wis.). The mutations of the AKB domain corresponding to those set forth in Table 7 herein were made with the QuickChange mutagenesis method (Makarova et al. (2000) Biotecniques, 29:970-972). Full-length bovine RIa and mouse RIa were fused with green fluorescent protein (GFP) by subcloning into pEGFPN1 (Clontech, Palo Alto, Calif.). Equal amounts of the targeting constructs and either GFP-Rlax or GFP-RIa constructs were co-transfected into 10H(1/2) cells using Lipofectamine (Invitrogen, Carlsbad, Calif.). The AKB domain was detected by immunostaining with monoclonal antibodies against the Flag-tag (Kodak, Rochester, N.Y.) followed by a rhodamine-conjugated secondary antibody (Jackson Lab, Bar Harbor, Me.). The cells were imaged using a radiance confocal microscope (BioRad, Hercules, Calif.).

[0301] By concentrating D-AKAP2 at the mitochondria, the co-localization of the AKB domain and the R isoforms was easily detected. GFP constructs of RIa and RIa were co-transfected into 10H(1/2) cells with Flag-tagged wild type AKB(dual), AKB(RI), AKB(II) and AKB(null). The results indicated that all of the AKB domains localized well to the mitochondria. The AKB(dual) was able to recruit both GFP-RIa and GFP-RIa to the mitochondria. The RI-specific peptide, AKB(RI), recruited RI to the mitochondria but was incapable of recruiting GFP-RI. In contrast, the RI-specific peptide, AKB(RI), only localized GFP-RI to the mitochondria; GFP-RI was not localized by the targeted AKB(RI) peptide. The null peptide, AKB(null), could not co-localize either GFP-Rlax or RIa.

EXAMPLE 11

Construction of Transgenic Knock-In Mice Containing Single Specific Amino Acid Substitutions in D-AKAP2

[0302] Constructs to knock-in specific amino acid substitutions in mice are designed as follows, which results in a change in the binding of D-AKAP2 to both PKA isoforms (containing RI or RII subunits) and subsequently the D-AKAP2 mediated function. The mouse D-AKAP2 cDNA sequence has been cloned (see, Huang et al. (1997) PNAS, 94:11184-11189) and is available at accession # NM_019921. Although Huang et al., believed the 372 amino
acid protein was full-length, it may represent a truncated version of the mouse D-AKAP2 protein. The genomic organization and structure of the mouse D-AKAP2 gene is available in draft form from Genbank (accession #AL646042 and #AC084019). The mouse protein and nucleic acid sequences are set forth in SEQ ID NOs: 55 and 56. In SEQ ID NOs: 55 and 56, the first amino acid (met) corresponds to amino acid position 291 in the human D-AKAP2 protein set forth as SEQ ID NOs: 64 and 65. The PKA binding sequence (27-amino acid peptide) corresponds to amino acids 333-359 of the mouse D-AKAP2 protein SEQ ID NO: 56 and to amino acids 623-649 of the human D-AKAP2 protein SEQ ID NOs: 64 and 65.

[0303] A) Based on the mouse cDNA sequence set forth in SEQ ID NO: 55, a modified D-AKAP2 knock-in mouse is created, using well-known methods, by introducing a Q91 substitution in the region of mouse D-AKAP2 corresponding to the 27-mer peptide region set forth in SEQ ID NOs: 1 and 2 (which corresponds to a Q3411 substitution in the mouse D-AKAP2 protein set forth in SEQ ID NO: 56). The knock-in is created by replacing the codon corresponding to nucleotides 1021-1023 of SEQ ID NO: 55 with either one of the codons “ATT” or “ATC”. The phenotype of this particular Q3411 knock-in mouse corresponds to enhanced binding of the modified D-AKAP2 protein to PKA-RIα-GFP, but normal binding to PKA-RIα-GFP.

[0304] B) Based on the mouse cDNA sequence set forth in SEQ ID NO: 55, a modified D-AKAP2 knock-out mouse is created, using well-known methods, by introducing an A13L substitution in the region of mouse D-AKAP2 corresponding to the 27-mer peptide region set forth in SEQ ID NOs: 1 and 2 (which corresponds to a A3451 substitution in the mouse D-AKAP2 protein set forth in SEQ ID NO: 56). The knock-in is created by replacing the codon corresponding to nucleotides 1033-1035 of SEQ ID NO: 55 with either one of the codons “TTA”, “TTG”, “CTT”, “CTC”, “CTA” or “CTG”. The phenotype of this particular A3451 knock-out mouse corresponds to disrupted binding of the modified D-AKAP2 protein to PKA-RIα-GFP, but normal binding to PKA-RIα-GFP.

[0305] C) Based on the mouse cDNA sequence set forth in SEQ ID NO: 55, a modified D-AKAP2 knock-in mouse is created, using well-known methods, by introducing a V21W substitution in the region of mouse D-AKAP2 corresponding to the 27-mer peptide region set forth in SEQ ID NOs: 1 and 2 (which corresponds to a V353W substitution in the mouse D-AKAP2 protein set forth in SEQ ID NO: 56). The knock-in is created by replacing the codon corresponding to nucleotides 1057-1059 of SEQ ID NO: 55 with either one of the codons “TGA” or “TGG”. The phenotype of this particular V353W knock-in mouse corresponds to normal binding of the modified D-AKAP2 protein to PKA-RIα-GFP, but disrupted binding to PKA-RIα-GFP.

EXAMPLE 12
Construction of Transgenic Triple-Mutant Knock-In Mice Containing Three Specific Amino Acid Substitutions in D-AKAP2

[0306] Based on the mouse cDNA sequence set forth in SEQ ID NO: 55, a modified D-AKAP2 knock-in mouse is created, using well-known methods, by introducing a Q9F, V21W, M25F triple-mutant substitution in the region of mouse D-AKAP2 corresponding to the 27-mer peptide region set forth in SEQ ID NOs: 1 and 2 (which corresponds to a Q3411F, V353W, M357F substitution in the mouse D-AKAP2 protein set forth in SEQ ID NO: 56). The knock-in is created by replacing the codon corresponding to nucleotides 1021-1023 (9QF) of SEQ ID NO: 55 with either one of the codons “TTT” or “ TTC”; by replacing the codon corresponding to nucleotides 1057-1059 of SEQ ID NO: 55 with either one of the codons “TGA” or “TGG”; and replacing the codon corresponding to nucleotides 1069-1071 of SEQ ID NO: 55 with either one of the codons “TTT” or “TTT”. The phenotype of this particular Q3411F, V353W, M357F triple-mutant knock-in mouse corresponds to RIα-specific binding (e.g., corresponding to approximately 10-fold increased binding to RIα and approximately 220-fold decreased binding affinity for RIαc; see Table 7).

[0307] Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.
<490> SEQUENCE: 2
Val Glu Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile
      1     5      10      15
Ala Lys Met Ile Val Ser Asp Val Met Gin Gin
      20      25

<210> SEQ ID NO 3
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3
Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met
      1     5      10      15
Ile Val Ser Asp Ile
      20

<210> SEQ ID NO 4
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4
Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val
      1     5      10      15

<210> SEQ ID NO 5
<211> LENGTH: 25
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5
Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala
      1     5      10      15
Lys Met Ile Val Ser Asp Ile Met Gin
      20      25

<210> SEQ ID NO 6
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6
Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys
      1     5      10      15
Met Ile Val Ser Asp Ile Met
      20

<210> SEQ ID NO 7
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7
Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met
      1     5      10      15
Ile Val Ser Asp Ile
      20
Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp

Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser

 Ala Gln Glu Leu Ala Trp Lys Ile Ala Lys Met Ile

 Glu Leu Ala Trp Lys Ile Ala Lys

 Glu Leu Ala Trp Lys Ile Ala Lys

 Glu Leu Ala Trp Lys Ile Ala Lys

 Glu Leu Ala Trp Lys Ile Ala Lys
<222> LOCATION: (1)...(2)
<223> OTHER INFORMATION: bAla

<400> SEQUENCE: 14

Xaa Xaa Val Gln Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Ala Trp
  1  5 10 15
Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gin Gln
  20 25

<210> SEQ ID NO 15
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: ac-Val

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Val Gln Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Ala Trp Lys Ile
  1  5 10 15
Ala Lys Met Ile Val Ser Asp Val Met Gin Gln
  20 25

<210> SEQ ID NO 16
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<212> TYPE: PRT
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<220> FEATURE:
<221> NAME/KEY: MOD RES
<222> LOCATION: (1)
<223> OTHER INFORMATION: ac-Val

<400> SEQUENCE: 16

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  1  5 10 15
Ala Lys Met Ile Val Ser Asp Ile Met Gin Gln
  20 25

<210> SEQ ID NO 17
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
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<222> LOCATION: (6)
<223> OTHER INFORMATION: D-Thr

<400> SEQUENCE: 17

Val Gln Gly Asn Thr Thr Glu Ala Gin Glu Glu Leu Ala Trp Lys Ile
  1  5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gln
  20 25
Val Gln Gly Asn Thr Asp Glu Ala Phe Glu Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

Val Gln Gly Asn Thr Asp Glu Ala Ile Glu Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

Val Gln Gly Asn Thr Asp Glu Ala Leu Glu Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

Val Gln Gly Asn Thr Asp Glu Ala Phe Glu Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25
<210> SEQ ID NO 22
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (9)
<223> OTHER INFORMATION: D-Tyr

Val Gln Gly Asn Thr Asp Glu Ala Tyr Glu Leu Ala Trp Lys Ile
1 5 10 15

Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20 25

<210> SEQ ID NO 23
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (21)
<223> OTHER INFORMATION: D-Leu

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
1 5 10 15

Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20 25

<210> SEQ ID NO 24
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
1 5 10 15

Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20 25
Val Glu Asp Ala Gin Lys Leu Phe Thr Arg Ile
Val Gin Asp Ala Gin Lys Leu Phe Thr Arg Val

SEQ ID NO: 25
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
FEATURE: MOD RES
LOCATION: (25)
OTHER INFORMATION: Ile or Val
Val Gin Asp Ala Gin Lys Leu Phe Thr Arg Ile

SEQ ID NO: 26
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
FEATURE: MOD RES
LOCATION: (26)
OTHER INFORMATION: D-Phe
Val Gin Asp Ala Gin Lys Leu Phe Thr Arg Ile

SEQ ID NO: 27
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
FEATURE: MOD RES
LOCATION: (27)
OTHER INFORMATION: Gln
Val Gin Asp Ala Gin Lys Leu Phe Thr Arg Ile

SEQ ID NO: 28
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
FEATURE: MOD RES
LOCATION: (28)
OTHER INFORMATION: Other
Val Gin Asp Ala Gin Lys Leu Phe Thr Arg Ile

-Continued-
Val Gln Gly Arg Thr Asp Glu Ala Gln Gln Glu Leu Ile Trp Lys Ile
  1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
  20  25

<210> SEQ ID NO 29
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<212> TYPE: PRT
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  <222> LOCATION: (24)
  <223> OTHER INFORMATION: Ile or Val
<400> SEQUENCE: 29
Val Gln Gly Arg Thr Asp Glu Ala Gln Gln Glu Leu Leu Trp Lys Ile
  1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
  20  25

<210> SEQ ID NO 30
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  <222> LOCATION: (24)
  <223> OTHER INFORMATION: Ile or Val
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Val Gln Gly Arg Thr Asp Glu Ala Gln Gln Glu Leu Ala Trp Cys Ile
  1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
  20  25

<210> SEQ ID NO 31
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
  <221> NAME/KEY: MOD_RES
  <222> LOCATION: (24)
  <223> OTHER INFORMATION: Ile or Val
<400> SEQUENCE: 31
Val Gln Gly Arg Thr Asp Glu Ala Gln Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15
Ala Cys Met Ile Val Ser Asp Xaa Met Gln Gln
  20  25

<210> SEQ ID NO 32
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<212> TYPE: PRT
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<220> FEATURE:
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  <223> OTHER INFORMATION: Ile or Val
<400> SEQUENCE: 32
Val Gln Gly Arg Thr Asp Glu Ala Gln Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15

Ala Lys Cys Ile Val Ser Asp Xaa Met Gln Gln

20  25

<210> SEQ ID NO 33
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<220> FEATURE:
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<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 33

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile
1   5   10
Ala Lys Met Ile Val Cys Asp Xaa Met Gln Gln
20  25

<210> SEQ ID NO 34
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<220> FEATURE:
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<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 34

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Cys Ala Trp Lys Ile
1   5   10
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20  25

<210> SEQ ID NO 35
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
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<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 35

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1   5   10
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20  25

<210> SEQ ID NO 36
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<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 36

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1   5   10
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20  25
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<212> TYPE: PRT
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<220> FEATURE:
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<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 37

Val Gin Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Trp Lys Ile
1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

<210> SEQ ID NO 38
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 38

Val Gin Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Met Trp Lys Ile
1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

<210> SEQ ID NO 39
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 39

Val Gin Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Ile Gin Ile
1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

<210> SEQ ID NO 40
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 40

Val Gin Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Val Trp Lys Ile
1  5  10  15
Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20 25

<210> SEQ ID NO 41
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<210> SEQ ID NO 42
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

Val Gln Gly Asn Thr Asp Glu Ala Gln Gln Glu Leu Trp Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20 25

<210> SEQ ID NO 43
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

Val Gln Gly Asn Thr Asp Glu Ala Gln Gln Glu Leu Tyr Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20 25

<210> SEQ ID NO 44
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

Val Gln Gly Asn Thr Asp Glu Ala Glu Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Cys Xaa Met Gln Gln
20 25

<210> SEQ ID NO 45
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

Val Gln Gly Asn Thr Asp Glu Ala Phe Glu Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20 25
Val Gly Gly Asn Thr Asp Glu Ala Ile Glu Glu Leu Ala Trp Lys Ile
1      5     10    15

Ala Lys Met Ile Val Ser Asp Xaa Met Gln Gln
20    25

-continued

SEQ ID NO 47
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
NAME/KEY: MOD_RES
LOCATION: (24)
OTHER INFORMATION: Ile or Val

SEQ ID NO 48
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
NAME/KEY: MOD_RES
LOCATION: (24)
OTHER INFORMATION: Ile or Val

SEQ ID NO 49
LENGTH: 27
TYPE: PRT
ORGANISM: Homo sapiens
NAME/KEY: MOD_RES
LOCATION: (24)
OTHER INFORMATION: Ile or Val
Ala Lys Met Ile Val Ser Asp Xaa Ile Gin Gin
20     25

<210> SEQ ID NO  50
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 50

Val Gin Gly Asn Thr Asp Glu Ala Gin Gin Gin Leu Ala Trp Lys Ile
1     5     10     15

Ala Lys Met Ile Val Ser Asp Xaa Leu Gin Gin
20     25

<210> SEQ ID NO  51
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 51

Val Gin Gly Asn Thr Asp Glu Ala Gin Gin Gin Leu Ala Trp Lys Ile
1     5     10     15

Ala Lys Met Ile Val Ser Asp Xaa Met Gin Gin
20     25

<210> SEQ ID NO  52
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 52

Val Gin Gly Asn Thr Asp Glu Ala Gin Gin Gin Leu Ala Trp Lys Ile
1     5     10     15

Ala Lys Met Ile Ile Ser Asp Xaa Met Gin Gin
20     25

<210> SEQ ID NO  53
<211> LENGTH: 27
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24)
<223> OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 53

Val Gin Gly Asn Thr Asp Glu Ala Gin Gin Gin Leu Ala Trp Lys Ile
1     5     10     15

Ala Lys Met Ile Leu Ser Asp Xaa Met Gin Gin
20     25
<210> SEQ ID NO 54
<211> LENGTH: 27
<212> TYPE: PRO
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: MOD_RES
<222> LOCATION: (24) OTHER INFORMATION: Ile or Val

<400> SEQUENCE: 54

Val Gln Gly Arg Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
1   5   10   15

Ala Lys Met Ile Trp Ser Asp Xaa Met Gln Gln
20  25

<210> SEQ ID NO 55
<211> LENGTH: 1119
<212> TYPE: DNA
<213> ORGANISM: Mus musculus
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1)...

<400> SEQUENCE: 55

atg aac gaa cag gca gtc ctc act ttt acc aac tat ata
Met Lys Ser Ile Glu Asp Ala Val Arg Thr Phe Thr Lys Tyr Ile
1   5   10   15

ttc cca gat gct gct aag cca ata cca att aca gas gcc atg aag gcc
Ser Pro Asp Ala Ala Lys Pro Ile Pro Ala Thr Glu Ala Met Arg Aam
20  25  30

gac atc atc gca aag att tgt gga gaa gat gga cag gtc gat gcc aac
Asp Ile Ile Ala Lys Ile Cys Gly Glu Asp Gly Gln Val Asp Pro Aam
40  45  50  55

tgt ttc gtt ctc gac acg gct gtc ttt aat gca atg gag cca gag
cys Phe Val Leu Asp Thr Ala Val Tyr Val Phe Ser Met Glu Glu Glu
60  65  70  75  80

cac ttt aag gga tgt ctc gca aag cgt ttc tat aac gag cag gat
his Phe Ser Glu Phe Leu Arg Ser His His Phe Cys Tyr Tyr Ile
90  95 100 105 110

gaa gtt gtc acc aat tgt cac aat gct gat gat atc ttc ttt tgt
glu Val Leu Thr Ser Gly Thr Val Tyr Leu Ala Asp Ile Leu Phe Cys
136 141 146 151 156

ggt ttc ggc ctc ttt tat ttt cgg tac atg gas aac gaa gat gca
ser Asp Alal Phe Tyr Phe Ser Gly Tyr Met Glu Gly Glu Asp Ala
182 187 192 197 202

gcc ctc ttt ttt tgg ttc gca gcc ggt tat gat ttc carr ctc tcc
gal Val Asp Ile Leu Glu Gln Phe Thr Asp Ala Asp Phe Glu Ser Glu
238 243 248 253 258

cgg atc aat tgt ctc tgg gaa ggc cag gat aag gta gaa cag ggc cag
arg Ala Ala Lys Lys Gln Tyr Asp Gly Gln Glu Ala Asp Aam
294 300 306 312 318

gcc gga ttt gat gtt tct cga tta gas att gas cct aat ttc tlg
leu Gly Phe Asp Asp Val Val Arg Leu Glu Ile Glu Ser Asn Ile Cys
354 360 366 372 378

agg gaa ggt gga cca ctt cct aat tgt ttc aca act cca tta cgt cag
arg Glu Gly Gly Pro Leu Pro Asn Cys Phe Thr Thr Pro Leu Arg Glu
414 420 426 432 438
GCC TGG ACA ACC ATG GAG AAG GTG TTT TTG CCT GGT TTT CTC TCC AGC
Ala Trp Thr Thr Met Glu Lys Val Phe Leu Pro Gly Phe Leu Ser Ser
190 

GTC TAT TAC AAA TAT TTG AAT GTC TCC TGT GCT CAT TCA GCT GTA GGA
Gln Leu Tyr Tyr Leu Leu Asp Leu Ala Ala His Ser Val Arg Gly
210 

GAT CTT TTA ACG GCA CCG GCT GCT TCC AGC
Trp Glu Phe Leu Gly Gly Asp Val Ser Leu Ala His Ser Gly Ser Val
220 

GTT CTT CCT GAG GAG CCT GCC CCT CAC GGC CTT TGG
Val Leu Pro Glu Glu Pro Leu His Ser Val His Ser Asp Pro Leu Val
240 

CAG TCT GTC AAA AAA GCC AGT ATT AAA ATT CTG AAA AAT TTG GAT
Gln Ser Ser Val Lys Ala Ser Ille Lys Leu Lys Arg Asp Ser Asp
260 

GAA GCA ATA ATT GTG GAT GCT GCA AGT CTG GAC CCA GAA TCT TTA TAT
Glu Ala Ile Ille Val Asp Ala Ala Ser Leu Asp Pro Glu Ser Leu Tyr
280 

CAG ACA TAT GCA GGG AAG ATG TCC TTT GGG AGA GTT AGT GAT TGG
Gin Thr Tyr Thr Gln Gln Arg Val Ala AAG ATG
300 

GGG CAG TTC ATC CGA GAG TCT GAG CCT GAA CCT GAT GTG AAG AAA TCA
Gly Gin Phe Ile Arg Glu Ser Glu Pro Glu Pro Asp Val Lys Ser
320 

AAA GGA TTC ATG TTC TCA CAA GCT ATG AAG AAG TGG GTG CAA GGA SAT
Lys Gin Ser Gln Ser Val Asp Gln Leu Leu Ala Thr Thr Lys Leu
340 

ACT GAC GAG GCC CAA GAG CTA GCT TGG AAG ATT GCA AAA ATG ATA
Thr Asp Glu Gin Ala Gln Gin Ala Pro Leu Thr Ser Asp Thr Lys Lys
360 

GTG ATG CTA GAG CAG CAC CAT GAT CAA CGA CTG AAG
Val Val Pro Glu Thr Gln His His His Phe Lys Leu Gln
380 

TCT ACA AAG CTA TGA
Ser Thr Lys Leu
400 

SEQ ID NO: 56
LENGTH: 372
TYPE: PRT
ORGANISM: Mus musculus
SEQUENCE: 56
Met Lys Ser Ile Glu Gin Asp Ala Val Asn Thr Phe Thr Lys Tyr Ile
1 5 10 15
Ser Pro Asp Ala Ala Lys Pro Ile Pro Ile Thr Glu Ala Met Arg Asn
20 25 30
Amp Ile Ile Ala Lys Ille Cys Gin Glu Asp Gly Gin Val Asp Pro Asn
35 40 45
Cys Phe Val Leu Asp Thr Ala Val Phe Ser Ala Met Glu Gin Glu
50 55 60
His Phe Ser Gin Phe Leu Arg Ser His His Phe Cys Thr Tyr Gin Ile
65 70 75 80
Glu Val Leu Thr Ser Gly Thr Val Tyr Leu Ala Asp Ile Leu Phe Cys
85 90 95
<table>
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<th>Glu Ser Ala Leu Phe Tyr Phe Ser Glu Tyr Met Glu Lys Glu Asp Ala</th>
<th>100 105 110</th>
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<td>Val Asn Ile Leu Gln Phe Trp Leu Ala Ala Asp Asn Phe Gln Ser Gln</td>
<td>115 120 125</td>
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<td>Leu Ala Ala Lys Lys Gly Gln Tyr Asp Gly Gln Ala Gln Asn Asp</td>
<td>130 135 140</td>
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<tr>
<td>Ala Met Ile Leu Tyr Asp Lys Tyr Phe Ser Leu Gln Ala Thr His Pro</td>
<td>145 150 155 160</td>
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<tr>
<td>Leu Gly Phe Asp Asp Val Val Arg Leu Glu Ile Glu Ser Asn Ile Cys</td>
<td>165 170 175</td>
</tr>
<tr>
<td>Arg Glu Gly Gly Pro Leu Pro Asn Cys Phe Thr Thr Pro Leu Arg Glu</td>
<td>180 185 190</td>
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<tr>
<td>Ala Trp Thr Thr Met Glu Lys Val Phe Leu Pro Gly Phe Leu Ser Ser</td>
<td>195 200 205</td>
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<tr>
<td>Asn Leu Tyr Tyr Lys Tyr Leu Asn Asp Leu Ile His Ser Val Arg Gly</td>
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<tr>
<td>Asp Glu Phe Leu Gly Gly Asn Val Ser Ala Ala Asp His Gly Ser Val</td>
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<tr>
<td>Cys Leu Pro Glu Glu Ser His Ser Gly Gly Ser Asp Gly Ser Thr Ala</td>
<td>245 250 255 260</td>
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<td>Gln Ser Ser Val Lys Lys Ala Ser Ile Lys Ile Leu Lys Asn Phe Asp</td>
<td>260 265 270</td>
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<td>Glu Ala Ile Ile Val Asp Ala Ala Ser Leu Asp Pro Glu Ser Leu Tyr</td>
<td>275 280 285</td>
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<tr>
<td>Gln Arg Thr Tyr Ala Gly Lys Met Ser Phe Gly Arg Val Ser Asp Leu</td>
<td>290 295 300</td>
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<tr>
<td>Gly Gln Phe Ile Arg Glu Ser Glu Pro Glu Pro Asp Val Lys Lys Ser</td>
<td>300 310 315 320</td>
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<tr>
<td>Lys Gly Phe Met Phe Ser Glu Met Lys Lys Trp Val Glu Gly Asn</td>
<td>325 330 335</td>
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<tr>
<td>Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile</td>
<td>340 345 350</td>
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<tr>
<td>Val Ser Asp Val Met Gln Gln Ala His His Asp Glu Pro Leu Glu Lys</td>
<td>355 360 365</td>
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| Ser Thr Lys Leu                                               | 370  

<210> SEQ ID NO 57
<211> LENGTH: 159
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE: OTHER INFORMATION: Description of Artificial Sequence: Synthetic nucleotide construct
<220> FEATURE: CDS
<222> LOCATION: (37) ...(159)
<400> SEQUENCE: 57

aaaggttca tggctcaca agctatgaag aagtgg gtaa gga aat act gac
Val Gln Gly Asn Thr Asp  
1 5

Glu Ala Ile Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser
10 15 20

 gag gcc aty gaa gag cta got tgg aag att gca aaa atg ata gtt aat
---continued---

**SEQ ID NO 58**
**LENGTH:** 40
**TYPE:** PRT
**ORGANISM:** Artificial Sequence

**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic peptide

**SEQUENCE:**
Val Gln Gly Amn Thr Aep Glu Ala Ile Glu Leu Ala Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Aep Val Met Gln Gln Ala His His Aep Gln
20 25 30 35
Pro Leu Glu Lys Ser Thr Lys Leu
35 40

---

**SEQ ID NO 59**
**LENGTH:** 159
**TYPE:** DNA
**ORGANISM:** Artificial Sequence

**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic nucleotide construct

**SEQUENCE:**
aaaggtcaagttctcacaagctatgaagagaattgtgcttggccaag aat act gag
Val Gln Gly Amn Thr Aep
1 5

---

**SEQ ID NO 60**
**LENGTH:** 40
**TYPE:** PRT
**ORGANISM:** Artificial Sequence

**FEATURE:**
**OTHER INFORMATION:** Description of Artificial Sequence: Synthetic peptide

**SEQUENCE:**
Val Gln Gly Amn Thr Aep Glu Ala Gln Glu Leu Leu Trp Lys Ile
1 5 10 15
Ala Lys Met Ile Val Ser Aep Val Met Gln Gln Ala His His Aep Gln
20 25 30 35
Pro Leu Glu Lys Ser Thr Lys Leu
35 40
```
Ala Lys Met Ile Val Ser Asp Val Met Gln Gln Ala His His Asp Gln
20  25  30
Pro Leu Glu Lys Ser Thr Lys Leu
35  40

<210> SEQ ID NO 61
<211> LENGTH: 159
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic nucleotide construct
<221> NAME/KEY: CDS
<222> LOCATION: (37)...(159)

<400> SEQUENCE: 61
aaaggaattca tgttctcaaca atcatagaa agaagtgg gttc gag aat act gac
Val Gln Gly Asn Thr Asp 54
1  5
gag gcc caa gaa gga gct gta gtt aag att gca aaa atg ata tgr agt
Glu Ala Gln Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Thr Ser
10  15  20
102
150
159
aag cta tga
Lys Leu
40

<210> SEQ ID NO 62
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic peptide
<400> SEQUENCE: 62
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
1  5  10
15
Ala Lys Met Ile Trp Ser Asp Val Met Gln Gln Ala His His Asp Gln
20  25  30
35  40

<210> SEQ ID NO 63
<211> LENGTH: 2363
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (138)...(2126)

<400> SEQUENCE: 63
gcgcgcgctt gataataggg cgctggtcgc gcgcgtgg gca tcccagggag gcgcgcgcgc
60
tcctcaggc agaaggtggt cctttcgcg ctacgtgca gcgcgcggag gcgcgcgcgc
tcgcgcgcgc gcgcgcgcgc gcgcgcgcgc gcgcgcgcgc gcgcgcgcgc gcgcgcgcgc
120
170
218
```
Ile Thr Glu Ala Met Arg Asn Asp Ile Ile Ala Arg Ile Cys Gly Glu 320

gac gat cag gtc gat ccc aac tgt ttc gtt tgt gca cag tcc ata gtc 335
Amp Gly Glu Val Asp Pro Asn Cys Phe Val Leu Ala Gln Ser Ile Val 340

1178

ttt agt gca atg gag caa gac ccc ttt agt gag ttt tgt gca gat ccc 350
Phe Ser Ala Met Glu Glu His Phe Ser Glu Phe Leu Arg Ser His 355

1226

cat ttc tgt aca tac cag att gaa ggt ctt acc aat gga act gtt tac 365
His Phe Cys Lys Tyr Gln Ile Ile Glu Thr Ser Gly Thr Val Tyr 370

1274

c tg gct gac att ctc ttc tgt gac tca gcc ttc ttt tat ttc tgt gag 380
Leu Ala Asp Ile Leu Phe Cys Glu Ser Ala Phe Tyr Phe Ser Glu 385

1322

tac atg gaa aag gat gca gtt atc atg tta caa ttc tgt gca 390
Tyr Met Glu Gln Ala Asp Val Ala Val Asn Ile Leu Glu Phe Thr Leu Ala 395

1370

gca gat aac ttc cag tgt cag ctt gct gcc aaa aag gca aat gaa 405
Ala Asp Phe Asp Val Leu Ala Ala Lys Lys Gly Tyr Asp 410

1418

gga cag gag gca cag aat ggc gaa gct gaa acc aat aca ttc ttt 415
Gly Glu Glu Asn Asp Val Glu Asp Val Asp Arg Ile Leu Met Arg 420

1466

tcc ttc caa gcc aca cat ctc att gga tgt gtt gta cga tta 430
Ser Leu Glu Ala Thr Pro Leu Gly Asp Arg Val Val Arg Leu 435

1514

gaa att gaa tac aat atc tgc agg gaa ggt ggc oca ctc ccc aac tgt 440
Glu Ile Glu Ser Asn Ile Cys Arg Glu Gly Pro Leu Pro Asn Cys 445

1562

tcc aca act oca tca ctt cag gct tgg aca acc atg gag aag gtc ttt 450
Phe Thr Thr Pro Leu Arg Ala Ala Tyr Arg Ile Leu Phe Tyr Phe 455

1610

ttg cct ggc ttt ctc cag tgc ctt cag tgc aat ctt ttt tat tat aat gat 460
Leu Pro Gly Phe Leu Ser Ser Asn Leu Tyr Tyr Lys Tyr Leu Asp 465

1668

c tac ato cat tcc gtt cga gga gat gaa ttt ctc ggc aac gtc tct 470
Leu Ile His Ser Val Arg Gly Asp Phe Leu Gly Gly Asn Val Ser 475

1706

cgg act gct cct ggg tct gtt ggc cct gct gat gac tct cag cca ggg 480
Pro Thr Ala Pro Gly Ser Val Gly Pro Pro Asp Glu Ser His Pro Gly 485

1754

1802

1850

1898

1946

1994

2042
<210> SEQ ID NO 64
<211> LENGTH: 662
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 64

Met Arg Gly Ala Gly Pro Ser Pro Arg Gin Ser Pro Arg Thr Leu Arg  
1      5      10      15

Pro Asp Pro Gly Pro Ala Met Ser Phe Phe Arg Arg Lys Val Lys Gly  
20     25     30

Lys Gin Gin Lys Thr Ser Asp Val Lys Ser Ile Lys Ala Ser Ile  
35     40     45

Ser Val His Ser Pro Gin Lys Thr Ser Thr Lys Arg His Ala Leu Leu Glu  
50     55      60

Ala Ala Gly Pro Ser His Val Ala Ile Asn Ala Ile Ser Ala Asn Met  
65     70     75     80

Asp Ser Phe Ser Ser Ser Arg Thr Ala Thr Leu Lys Gin Pro Ser  
85     90     95

His Met Gin Ala Ala His Phe Gly Gin Leu Gin Ser Cys Leu Leu Asp  
100    105    110

Tyr Gin Thr Gin Glu Thr Lys Ser Ser Leu Ser Lys Thr Leu Glu Gin  
115    120    125

Val Leu His Asp Thr Ile Val Leu Pro Tyr Phe Ile Gin Phe Met Glu  
130    135    140

Leu Arg Arg Met Gin His Leu Val Lys Phe Thr Leu Gin Ala Gin Ser  
145    150    155    160

Phe His Ser Thr Thr Ser Arg Ile Arg Ala His Ser Leu Asn Thr  
165    170    175

Met Lys Gin Ser Ser Leu Ala Gin Pro Val Pro Ser Lys Lys His  
180    185    190

Glu Thr Thr Ala Ser Phe Leu Thr Asp Ser Leu Asp Gin Arg Leu Gin  
195    200    205

Asp Ser Gly Ser Ala Gin Leu Phe Met Thr His Ser Gin Gly Gin Gin  
210    215    220

Leu Asn Gin Gin Thr Gin Ser Thr Gin Gin Gin Ser Leu Gin Gin Gin  
225    230    235    240

Glu Cys Asp Ser Ala His Ser Leu Arg Leu Gin Met Ala Arg Gin Gin  
245    250    255

Thr His Gin Val Ser Met Gin Thr Gin Glu Ser Ser Ser Thr Leu Thr  
260    265    270
Val Ala Ser Arg Asn Ser Pro Ala Ser Pro Leu Lys Glu Leu Ser Gly

Lys Leu Met Lys Ser Ile Glu Gin Asp Ala Val Asn Thr Phe Thr Lys

Tyr Ile Ser Pro Asp Ala Ala Lys Pro Ile Pro Ile Thr Glu Ala Met

Arg Asn Asp Ile Ala Arg Ile Cys Gly Glu Asp Gly Gin Val Asp

Pro Asn Cys Phe Val Leu Ala Gin Ser Ile Val Phe Ser Ala Met Glu

Gln Glu His Phe Ser Glu Phe Leu Arg Ser His His Phe Cys Lys Tyr

Gln Ile Glu Val Leu Thr Ser Gly Thr Val Tyr Leu Ala Asp Ile Leu

Phe Cys Glu Ser Ala Leu Phe Tyr Phe Ser Glu Tyr Met Glu Lys Glu

Asp Ala Val Asn Ile Leu Gin Phe Trp Leu Ala Ala Asp Asn Phe Gin

Ser Gin Leu Ala Ala Lys Gly Gin Tyr Asp Gly Gin Glu Ala Gin

Asn Asp Ala Met Ile Leu Tyr Asp Lys Tyr Phe Ser Leu Gin Ala Thr

His Pro Leu Gly Phe Asp Asp Val Val Arg Leu Glu Ile Glu Ser Asn

Ile Cys Arg Glu Gly Gly Pro Leu Pro Asn Cys Phe Thr Thr Pro Leu

Arg Glu Ala Trp Thr Thr Met Glu Lys Val Phe Leu Pro Gly Phe Leu

Ser Ser Asn Leu Tyr Tyr Lys Tyr Leu Asn Asp Leu Ile His Ser Val

Arg Gly Asp Glu Phe Leu Gly Gin Aan Val Ser Pro Thr Ala Pro Gly

Ser Val Gly Pro Pro Asp Glu Ser His Pro Gly Ser Ser Asp Ser Ser

Ala Ser Gin Ser Ser Val Lys Ala Ser Ile Lys Ile Leu Lys Asn

Phe Asp Glu Ala Ile Val Asp Ala Ala Ser Leu Asp Pro Glu Ser

Leu Tyr Gin Arg Thr Tyr Ala Gly Lys Met Thr Phe Gly Arg Val Ser

Asp Leu Gly Gin Phe Ile Arg Glu Ser Glu Pro Glu Pro Asp Val Arg

Lys Ser Lys Gly Ser Met Phe Ser Gin Ala Met Lys Lys Trp Val Gin

Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Ala Trp Lys Ile Ala Lys

Met Ile Val Ser Asp Ile Met Gin Gin Ala Gin Tyr Asp Gin Pro Leu

Glu Lys Ser Thr Lys Leu
Met Arg Gly Ala Gly Pro Ser Pro Arg Gln Ser Pro Arg Thr Leu Arg
Pro Asp Pro Gly Pro Ala Met Ser Phe Phe Arg Arg Lys Val Lys Gly
Lys Glu Gln Glu Lys Thr Ser Asp Val Lys Ser Ile Lys Ala Ser Ile
Ser Val His Ser Pro Gln Lys Ser Thr Lys Asn His Ala Leu Leu Glu
Ala Ala Gly Pro Ser His Val Ala Ile Asn Ala Ile Ser Ala Asn Met
Asp Ser Phe Ser Ser Ser Arg Thr Ala Thr Leu Lys Gln Pro Ser
His Met Glu Ala Ala His Phe Gly Asp Leu Gly Arg Ser Cys Leu Asp
Tyr Glu Thr Glu Glu Thr Lys Ser Ser Leu Ser Lys Thr Leu Gly Gln
Val Leu His Asp Thr Ile Val Leu Pro Tyr Phe Ile Gln Phe Met Glu
Leu Arg Arg Met Glu His Leu Val Lys Phe Trp Leu Gly Ala Gly Ser
Phe His Ser Thr Thr Thr Ser Arg Ile Arg Ala His Ser Leu Asn Thr
Met Lys Gln Ser Ser Leu Ala Gly Pro Val Ser Pro Ser Lys Lys His
Glu Thr Thr Ala Ser Phe Leu Thr Asp Ser Leu Asp Lys Arg Leu Glu
Asp Ser Gly Ser Ala Gln Leu Phe Met Thr His Ser Glu Gly Ile Asp
Leu Asn Asn Arg Thr Asm Ser Thr Gin Asm His Leu Leu Ser Gin
Glu Cys Asp Ser Ala His Ser Leu Arg Leu Glu Met Ala Arg Ala Gly
Thr His Gin Val Ser Met Gin Thr Gin Ser Ser Ser Thr Leu Thr
Val Ala Ser Arg Asn Ser Pro Ala Ser Pro Leu Lys Glu Leu Ser Gly
Lys Leu Met Lys Ser Ile Glu Gin Asp Ala Val Asn Thr Phe Thr Lys
Tyr Ile Ser Pro Asp Ala Ala Lys Pro Ile Pro Ile Pro Thr Glu Ala Met
Arg Asp Ile Ile Ala Arg Gin Gly Glu Asp Gly Gin Val Asp
Pro Asn Gin Cys Phe Val Leu Ala Gin Ser Ile Val Phe Ser Ala Met Glu
Gln Glu His Phe Ser Glu Phe Leu Arg Ser His His Phe Cys Lys Tyr
-continued

Gln Ile Glu Val Leu Thr Ser Gly Thr Val Tyr Leu Ala Asp Ile Leu 355 360 365
Phe Cys Glu Ser Ala Leu Phe Tyr Phe Ser Glu Tyr Met Glu Lys Glu 370 375 380
Asp Ala Val Asn Ile Leu Gln Phe Trp Leu Ala Ala Asp Asn Phe Gln 385 390 395 400
Ser Gin Leu Ala Ala Lys Lys Gin Tyr Asp Gly Gln Glu Ala Gin 405 410 415
Asn Asp Ala Met Ile Leu Tyr Asp Lys Tyr Phe Ser Leu Gin Ala Thr 420 425 430
His Pro Leu Gly Phe Asp Asp Val Val Arg Leu Glu Ile Glu Ser Asn 435 440 445
Ile Cys Arg Glu Gly Pro Leu Pro Asn Cys Phe Thr Thr Pro Leu 450 455 460
Arg Gin Ala Trp Thr Thr Met Glu Lys Val Phe Leu Pro Gly Phe Leu 465 470 475 480
Ser Ser Asn Leu Tyr Tyr Tyr Leu Asn Asp Leu Ile His Ser Val 485 490 495 500 505
Arg Gin Asp Gly Phe Leu Gly Glu Asn Val Ser Pro Thr Ala Pro Gly 510 515 520 525
Ser Val Gly Pro Pro Asp Glu Ser His Pro Gly Ser Asp Ser Ser 530 535 540
 Ala Ser Gin Ser Ser Val Lys Ala Ser Ile Lys Ile Leu Lys Asn 545 550 555 560
Phe Asp Glu Ala Ile Ile Val Asp Ala Ala Ser Leu Asp Pro Glu Ser 565 570 575
Leu Tyr Gin Arg Thr Tyr Ala Gly Lys Met Thr Phe Gly Arg Val Ser 580 585 590
Asp Leu Gly Gin Phe Ile Arg Glu Ser Glu Pro Glu Pro Asp Val Arg 595 600 605
Lys Ser Lys Gly Ser Met Phe Ser Gin Ala Met Lys Lys Trp Val Gin 610 615 620
Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Ala Trp Lys Ile Ala Lys 625 630 635 640
Met Ile Val Ser Asp Val Met Gin Glu Ala Gin Tyr Asp Gin Pro Leu 645 650 655
Glu Lys Ser Thr Lys Leu

<210> SEQ ID NO 66
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 66
Gln Gly Asn Thr Asp Glu Ala Gin Glu Glu Leu Ala Trp Lys Ile Ala 1 5 10 15
Lys Met Ile Val Ser Asp Ile Met Gin Gin 20 25

<210> SEQ ID NO 67
<211> LENGTH: 25
<210> SEQ ID NO 68
<211> LENGTH: 24
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln

<210> SEQ ID NO 69
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln

<210> SEQ ID NO 70
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln

<210> SEQ ID NO 71
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln

<210> SEQ ID NO 72
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

Ala Gln Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp
Ile Met Gln Gln

<210> SEQ ID NO 73
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 73
Gln Gln Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met
1 5 10 15

Gln Gln

<210> SEQ ID NO 74
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 74
Glu Gln Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met
1 5 10 15

Gln Gln

<210> SEQ ID NO 75
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 75
Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln
1 5 10 15

Gln

<210> SEQ ID NO 76
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 76
Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln
1 5 10 15

Gln

<210> SEQ ID NO 77
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 77
Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln
1 5 10 15

Gln

<210> SEQ ID NO 78
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 78
Trp Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln
1 5 10
Lys Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln
1      5      10

Ile Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln
1      5      10

Ala Lys Met Ile Val Ser Asp Ile Met Gln Gln
1      5      10

Lys Met Ile Val Ser Asp Ile Met Gln Gln
1      5      10

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile
1      5      10      15

Ala Lys Met Ile Val Ser Asp Ile Met Gln
20      25

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile
1      5      10      15

Ala Lys Met Ile Val Ser Asp Ile Met
20      25
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 85

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15

Ala Lys Met Ile Val Ser Asp Ile
  20

<210> SEQ ID NO 86
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 86

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15

Ala Lys Met Ile Val Ser Asp
  20

<210> SEQ ID NO 87
<211> LENGTH: 22
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 87

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15

Ala Lys Met Ile Val Ser
  20

<210> SEQ ID NO 88
<211> LENGTH: 21
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 88

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15

Ala Lys Met Ile Val
  20

<210> SEQ ID NO 89
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 89

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
  1  5  10  15

Ala Lys Met Ile
  20

<210> SEQ ID NO 90
<211> LENGTH: 19
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 90

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile
-continued

| 1 | 5 | 10 | 15 |

Ala Lys Met

<210> SEQ ID NO 91
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 91
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys Ile

Ala Lys

<210> SEQ ID NO 92
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 92
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Ala Trp Lys Ile

Ala

<210> SEQ ID NO 93
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 93
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile

<210> SEQ ID NO 94
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 94
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp Lys

<210> SEQ ID NO 95
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 95
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Leu Ala Trp

<210> SEQ ID NO 96
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 96
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala

<210> SEQ ID NO 97
Val Gln Gly Aem Thr Asp Glu Ala Gln Glu Glu Leu
1 5 10

Val Gln Gly Aem Thr Asp Glu Ala Gln Glu Glu
1 5 10

Val Gln Gly Aem Thr Asp Glu Ala Gln Glu
1 5 10

Val Gln Gly Aem Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Llys Ile Glu Lys Met Ile Trp Ser Asp Val Met Gln Gln
20 25

Val Gln Gly Aem Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Llys Ile Ala Lys Met Ile Trp Ser Asp Val Met Gln Gln
20 25

Asp Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Val Met Gln
1 5 10 15

Gln
<210> SEQ ID NO 103
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 103

Phe Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Trp Ser Asp Val
1    5    10    15

Phe Gln Gln Cys
20

<210> SEQ ID NO 104
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 104

Phe Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Trp Ser Asp Val
1    5    10    15

Met Gln Gln Cys
20

<210> SEQ ID NO 105
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 105

Gln Glu Glu Phe Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Val
1    5    10    15

Phe Gln Gln Cys
20

<210> SEQ ID NO 106
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 106

Gln Glu Glu Phe Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Val
1    5    10    15

Phe Gln Gln Cys
20

<210> SEQ ID NO 107
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
<400> SEQUENCE: 107

Phe Glu Glu Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Val
1    5    10    15

Phe Gln Gln Cys
20

<210> SEQ ID NO 108
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile
  1   5    10   15
Ala Lys Met Ile Val Ser Asp Val Met Gln Gln Cys
  20  25

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Leu Trp Lys Ile
  1   5    10   15
Ala Lys Met Ile Val Ser Asp Val Met Gln Gln Cys
  20  25

Glu Lys Met Ile Trp Ser Asp Val Met Gln Gln Cys
  20  25

Val Gln Gly Asn Thr Asp Glu Ala Gln Glu Glu Leu Ala Trp Lys Ile
  1   5    10   15
Ala Lys Met Ile Trp Ser Asp Val Met Gln Gln Cys
  20  25

Asp Leu Ala Trp Lys Ile Ala Lys Met Ile Val Ser Asp Val Met Gln
  1   5    10   15
Gln Cys
107. An isolated nucleic acid molecule, comprising a sequence of nucleotides that encodes a polypeptide that is a D-AKAP2 mutein, or a polypeptide fragment thereof, comprising a peptide region corresponding to an A-Kinase binding (AKB) domain set forth as amino acids 623-649 of SEQ ID NO:64 or 65, wherein the sequence of the AKB domain peptide region corresponds to a peptide mutein that exhibits modified binding to a regulatory subunit of PKA compared to a native D-AKAP2.

108. A nucleic acid vector, comprising the nucleic acid molecule of claim 107.

109. A cell containing the nucleic acid vector of claim 108.

110. A method of producing a D-AKAP2 mutein by growing the cell of claim 109 under conditions whereby the D-AKAP2 mutein is expressed; and isolating the mutein.

111. The method of claim 110, wherein the cell is a mammalian cell, yeast cell, insect cell or bacterial cell.

112. The method of claim 111, wherein the mammalian cell is a human cell.

113. The isolated nucleic acid molecule of claim 107, wherein the polypeptide is a mutein of SEQ ID NO: 2, and the polypeptide exhibits modified binding to a regulatory subunit of PKA compared to a peptide having SEQ ID NO: 2.

114. The isolated nucleic acid molecule of claim 113, wherein the polypeptide is selected from the group consisting of:
   a) a peptide having the sequence SEQ ID NO: 2;
   b) a peptide having the sequence SEQ ID NO: 2 further comprising a C-terminal cysteine;
   c) a peptide having the sequence SEQ ID NO: 2 wherein residues 1-8 are deleted; and
   d) a peptide having the sequence SEQ ID NO: 2 wherein residues 1-8 are deleted further comprising a C-terminal cysteine;
   except that said peptide has an amino acid residue substitution at the residue equivalent to residue 21 of SEQ ID NO: 2 of Trp or Ile for Val, and/or an amino acid residue substitution at the residue equivalent to residue 12 of SEQ ID NO: 2 of Phe for Leu; and
   zero, one, or two amino acid residue substitutions selected from the group consisting of Phe, Ile, Leu, Val, His, Met, Arg, Thr, Trp, or Tyr at the residue equivalent to residue 9 of SEQ ID NO: 2 for Gln; and Phe, Ile, Leu, Thr, Val, Trp, or Tyr at the residue equivalent to residue 25 of SEQ ID NO: 2 for Met.

115. The isolated nucleic acid molecule of claim 107, wherein the polypeptide exhibits enhanced binding to PKA-Rlα subunits.

116. The isolated nucleic acid molecule of claim 107, wherein the polypeptide exhibits normal or reduced binding to PKA-Rlα subunits.

117. The isolated nucleic acid molecule of claim 107, wherein the polypeptide exhibits normal or increased binding to PKA-Rlα subunits.

118. The isolated nucleic acid molecule of claim 107, wherein the polypeptide exhibits a preferred or exclusive binding to PKA-Rlα subunits relative to PKA-Rlβ subunits.

119. The isolated nucleic acid molecule of claim 107, wherein the polypeptide has enhanced ability to bind to PKA-Rlα subunit, and a reduced ability to bind to PKA-Rlβ subunit, compared to the peptide of SEQ ID NOs:1 or 2, wherein the peptide is selected from the group consisting of:
   FEELAWKIAKMIISDVFQQC; (SEQ ID NO: 104; PV-37)
   FEELAWKIAKMIISDVFQQC; (SEQ ID NO: 103; PV-38)
   QEEFWKIAKMIISDVFQQC; (SEQ ID NO: 105; PV-47)
   QEEFWKIAKMIISDVFQQC; (SEQ ID NO: 106; PV-48)

120. The isolated nucleic acid molecule of claim 114, wherein the polypeptide comprises an amino acid substitution at residue 21 of Ile or Trp for Val.

121. The isolated nucleic acid molecule of claim 114, wherein the polypeptide comprises an amino acid substitution at residue 21 of Trp for Val.

122. The isolated nucleic acid molecule of claim 120, wherein the polypeptide comprises an amino acid substitution at residue 9 of Phe for Gln.

123. The isolated nucleic acid molecule of claim 107, wherein the polypeptide is a peptide having the sequence SEQ ID NO: 2 wherein amino acids 1-8 are deleted or a peptide having the sequence SEQ ID NO: 2 wherein amino acids 1-8 are deleted further comprising a C-terminal cysteine.

124. The isolated nucleic acid molecule of claim 120, wherein the polypeptide is a peptide having the sequence SEQ ID NO: 2 wherein amino acids 1-8 are deleted or a peptide having the sequence SEQ ID NO: 2 wherein amino acids 1-8 are deleted further comprising a C-terminal cysteine.

125. The isolated nucleic acid molecule of claim 121, wherein the polypeptide is a peptide having the sequence SEQ ID NO: 2 wherein amino acids 1-8 are deleted or a peptide having the sequence SEQ ID NO: 2 wherein amino acids 1-8 are deleted further comprising a C-terminal cysteine.

126. The isolated nucleic acid molecule of claim 107, wherein the polypeptide binds to the PKA-Rlα subunit but has substantially no ability to bind to the PKA-Rlβ subunit, compared to the peptide of SEQ ID NOs:1 or 2, wherein the peptide is selected from the group consisting of:
   VGQMDTDEAQEEFWKIANMISD[I/V]MQQ; (SEQ ID NO: 51)
   VGQMDTDEAQEEFWKIANMISD[I/V]MQQ; (SEQ ID NO: 52)
   VGQMDTDEAQEEFWKIANMISD[I/V]MQQ; (SEQ ID NO: 53)

127. The isolated nucleic acid molecule of claim 107, wherein the polypeptide has an enhanced ability to bind to PKA-Rlα subunit, and a reduced ability to bind to PKA-Rlβ subunit, compared to the peptide of SEQ ID NOs:1 or 2, wherein the peptide is VGQMDTDEAQEEFWKIAKMIWSD[I/V]MQQ (SEQ ID NO: 54).

128. The isolated nucleic acid molecule of claim 107, wherein the polypeptide is FEELAWKIAKMIISDVFQQC (SEQ ID NO: 107; PV-49).