(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 March 2006 (02.03.2006)

PCT

(10) International Publication Number
WO 2006/023972 A2

(51) International Patent Classification:
*C12Q 1/42* (2006.01)

(21) International Application Number:
PCT/US2005/030099

(22) International Filing Date: 23 August 2005 (23.08.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 60/603,623 | 23 August 2004 (23.08.2004) | US |
| 60/681,923 | 17 May 2005 (17.05.2005) | US |


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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 2006/023972 A2

(54) Title: cAMP REPORTERS

(57) Abstract: cAMP reporters useful for obtaining measurements of cAMP levels with high spatial and temporal resolution.

# cAMP REPORTERS

[01] This application claims the benefit of and incorporates by reference co-pending provisional applications Serial No. 60/603,623 filed August 23, 2004 and Serial No. 60/681,923 filed May 17, 2005.

[02] This invention was made using funds from NIH grants GM066170 and GM08763. The government retains certain rights in the invention.

## FIELD OF THE INVENTION

[03] The invention relates to detection of cAMP levels.

## BACKGROUND OF THE INVENTION

[04] Spatial and temporal control of cAMP signaling is crucial to differential regulation of cellular targets involved in various signaling cascades. Various methods exist for detecting and measuring intracellular cAMP, but none are ideally suited for monitoring spatial and temporal distributions of cAMP in living cells. For example, radioimmunoassay or enzyme immunoassays for measuring cAMP require destroying large amounts of cells or tissue, have very poor spatial and temporal resolution, and measure total rather than free cAMP. Use of engineered cyclic nucleotide-gated channels to detect free cAMP provides good temporal resolution and quantification but uses indirect calcium measurements or nontrivial patch-clamp techniques and lacks the flexibility of measuring cAMP changes within various subcellular compartments (Rich *et al.*, *Proc. Natl. Acad. Sci. USA 98*, 13049-54, 2001; Rich *et al.*, *J. Gen. Physiol. 116*, 147-61, 2000). Free cAMP can be imaged in single cells microinjected with fluorophore-labeled C and R subunits (Adams *et al.*, *Nature 349*, 694-97, 1991) or in cells expressing two colors of GFP mutants fused to the C and R subunits (Zaccolo *et al.*, *Nat. Cell Biol. 2*, 25-29, 2000), which dissociate from each other and lose fluorescence resonance energy transfer upon elevation of cAMP. However, the expression levels of the two fusions have

to be carefully matched to allow reliable measurement. Even so, mixed tetramerization may occur between the fluorophore-attached subunits and endogenous partners, reducing the number of functional reporter molecules. Furthermore, it can be difficult to target such bimolecular reporters to different subcellular locations while maintaining appropriate stoichiometry.

[05] There is a need in the art for sensitive cAMP reporters which can be used for accurate measurements of spatial and temporal cAMP distributions in living cells.

BRIEF DESCRIPTION OF THE FIGURES

[06] **FIG. 1.** Domain structure and comparison of FRET responses for cAMP reporters. Sandwiched between enhanced CFP (ECFP) and citrine are truncated forms of Epac2 or full length Epac1 (with or without an R522E mutation). The construct comprising full-length Epac1 generated the biggest FRET response and was designated as ICUE1.

[07] **FIGS. 2A-C.** Responses of ICUE1 to changes in cellular cAMP levels. **FIG. 2A**, FRET response of HEK-293 cells transfected with ICUE1. The first image is a YFP-only image. Pseudocolor images depict the FRET response of the reporter to isoproterenol (ISO) stimulation at various time points. Scale bar represents 10 μm. **FIG. 2B**, Representative emission ratio time courses of ICUE1 and the R522E mutant stimulated with 10 μM ISO followed by 10 μM propranolol and 50 μM forskolin (FsK). **FIG. 2C**, Representative emission ratio time courses of ICUE1 stimulated with 10 μM ISO, 50 μM FsK, 10 μM $PGE_1$, 300 μM 8-pCPT-2'-O-Me-cAMP, or 100 μM of DMNB-cAMP followed by UV uncaging. The flash signs indicate 5 second UV flash at two different time points.

[08] **FIGS. 3A-E.** Fusions of ICUE1 targeted to various subcellular locations. **FIG. 3A**, Domain structures of the fusion constructs. **FIG. 3B**, YFP-only images showing plasma membrane and nuclear distributions of various fusions. Scale bars represent 10 μm. Merged pseudocolor images showing co-localization of nuclear localized ICUE1 with Hoechst 33342 cell-permeable dye in nucleus and mitochondria-targeted ICUE1 with

MitoTracker at mitochondria. **FIG. 3C**, Representative emission ratio time courses for untagged (ICUE1), plasma membrane-targeted (pm ICUE1), mitochondria-targeted (MitoCOX- and MitoDAKAP1-ICUE1) and nuclear-localized cAMP reporters (NLS-ICUE1) stimulated with ISO (10 μM). **FIG. 3D**, Representative emission ratio time courses for pm ICUE1 stimulated with $PGE_1$ (10 μM), followed by the removal of $PGE_1$ and the addition of ISO (10 μM). **FIG. 3E**, Representative emission ratio time courses for NLS-ICUE1 in response to $PGE_1$ (10 μM) and ISO (10 μM) separated by a washing step.

[09] **FIGS. 4A-C.** Simultaneous imaging of cAMP reporters targeted to different subcellular locations. **FIG. 4A**, Cellular distribution of different fusions. **FIG. 4B**, Representative emission ratio time courses for the pm ICUE1 and nuclear localized PKA activity reporter (NLS-AKAR) in the same cell stimulated with ISO (10 μM). Identical results were found in four different cells. The AKAR response was plotted using normalized ratio of yellow to cyan emissions. **FIG. 4C**, Representative emission ratio time courses for pm ICUE1 and NLS-ICUE1 in the same cell stimulated with 10 μM ISO followed by 10 μM propranolol (n=4).

[10] **FIG. 5.** Graph showing emission ratio time courses for ICUE2 and targeted versions of ICUE2. Y axis, normalized emission ratio (cyan/yellow).

[11] **FIG. 6.** Graph showing emission ratio time courses for ICUE2 and ICUE3.

## DETAILED DESCRIPTION OF THE INVENTION

[12] The invention provides highly sensitive reporter molecules by which temporal and spatial distribution of cAMP can be determined in living tissues. "cAMP reporters" (also referred to as "reporters") of the invention comprise (a) a donor moiety; (b) a polypeptide linked to the donor moiety and comprising a cAMP-binding domain of an "exchange protein directly activated by cAMP" (Epac); and (c) an acceptor moiety linked to the polypeptide. In the absence of cAMP, the donor moiety and the acceptor moiety are in sufficient proximity to each other to exhibit a detectable resonance energy transfer when the donor is excited. Binding of cAMP to the cAMP-binding domain causes a conformational change which changes the distance or relative orientation between the donor and acceptor moieties and alters the resonance energy transfer between the moieties. The degree of alteration reflects cAMP levels and can be detected qualitatively or quantitatively.

[13] cAMP reporters of the invention are useful for detecting intracellular cAMP and for assessing intracellular cAMP dynamics, although they also can be used in *in vitro* assays. Nucleic acid molecules encoding cAMP reporters of the invention can be delivered to cells using standard DNA transfection techniques, thereby generating cells which express high levels of the reporters. The reporters have advantages over previous methods for assessing cAMP dynamics inside cells. The reporters are unimolecular and can be readily targeted to different subcellular locations or fused to signaling components. They can be used to examine compartmentalized Epac activities and their physiological functions. For example, as described in the Examples below, a cAMP reporter targeted to plasma membrane, mitochondria, or nucleus revealed differential dynamics of cAMP signaling in response to the activation of the β-adrenergic receptor (β-AR) or the prostanoid receptor.

[14] cAMP reporters of the invention permit simultaneous imaging of cAMP dynamics and PKA phosphorylation in single living cells using locus-specific reporters. Methods of the

invention take advantage of spatial separation of subcellular events and provide unambiguous temporal correlation of these events. This methodology complements multi-color imaging (Violin *et al.*, *J. Cell Biol. 161*, 899-909, 2003; DeBernardi & Brooker, *Proc. Natl. Acad. Sci. USA 93*, 4577-82, 1996) and is well suited for simultaneous monitoring of multiple signaling events and for evaluating the information flow within signaling cascades or crosstalk between different pathways (Zaccolo, *Cir. Res. 94*, 866-73, 2004).

*Polypeptides*

[15] Polypeptides used in cAMP reporters of the invention comprise a cAMP-binding domain of an Epac, *e.g.*, Epac1 or Epac2. Epac1 and Epac2 are well-characterized, and the locations of their cAMP-binding domains are known. See de Rooij *et al.*, *J. Biol. Chem. 275*, 20829-36, 2000. Useful polypeptides include full-length, truncated, and mutated Epac1 or Epac2 from any species which has an Epac, such as rodents (*e.g.*, mice, rats) and primates (*e.g.*, humans, orangutans). The amino acid sequences of several Epac1 and Epac2 proteins are provided in SEQ ID NOS:1, 3, and 16-20. Nucleic acid sequences which encode SEQ ID NOS:1, 3, and 20 are shown in SEQ ID NOS:2, 4, and 21, respectively. The cAMP-binding domain in a cAMP reporter typically can bind cAMP; however, polypeptides comprising non-functional cAMP-binding domains are also useful, for example, for use in control reporters. The polypeptide itself preferably does not substantially emit light or transfer energy to excite the acceptor moiety.

*Donor and acceptor moieties*

[16] As used here, a "donor moiety" is a fluorophore or a luminescent moiety. The absorption spectrum of the "acceptor moiety" overlaps the emission spectrum of the donor moiety. The acceptor moiety does not need to be fluorescent and can be a fluorophore, chromophore, or quencher. In some embodiments both the donor and acceptor moieties are fluorescent proteins. In other embodiments both the donor and acceptor moieties are luminescent moieties. In yet other embodiments, either one of the donor or acceptor

moieties can be a fluorescent protein while the other moiety is a luminescent moiety. In other embodiments, the acceptor moiety is a "quencher moiety."

[17] When both the donor and acceptor moieties are fluorophores, resonance energy transfer is detected as "fluorescence resonance energy transfer" (FRET). If a luminescent moiety is involved, resonance energy transfer is detected as "luminescent resonance energy transfer "(LRET). LRET includes "bioluminescent resonance energy transfer" (BRET; Boute *et al., Trends Pharmacol. Sci. 23*, 351-54, 2002; Ayoub *et al., J. Biol. Chem. 277*, 21522-28, 2002). Because excitation of the donor moiety does not require exogenous illumination in an LRET method, such methods are particularly useful in live tissue and animal imaging, because penetration of the excitation light is no longer a concern. LRET methods have a high contrast and high signal-to-noise ratio; 2) no photobleaching occurs; and 3) quantification is simplified because the acceptor moiety is not directly excited.

[18] Suitable acceptor moieties include, for example, a coumarin, a xanthene, a fluorescein, a fluorescent protein, a circularly permuted fluorescent protein, a rhodol, a rhodamine, a resorufin, a cyanine, a difluoroboradiazaindacene, a phthalocyanine, an indigo, a benzoquinone, an anthraquinone, an azo compound, a nitro compound, an indoaniline, a diphenylmethane, a triphenylmethane, and a zwitterionic azopyridinium compound.

[19] Suitable donor moieties include, but are not limited to, a coumarin, a xanthene, a rhodol, a rhodamine, a resorufin, a cyanine dye, a bimane, an acridine, an isoindole, a dansyl dye, an aminophthalic hydrazide, an aminophthalimide, an aminonaphthalimide, an aminobenzofuran, an aminoquinoline, a dicyanohydroquinone, a semiconductor fluorescent nanocrystal, a fluorescent protein, a circularly permuted fluorescent protein, and fluorescent lanthanide chelate.

*Fluorescent proteins*

[20] In some preferred embodiments either or both of the donor and acceptor moieties is a fluorescent protein. Suitable fluorescent proteins include green fluorescent proteins (GFP), red fluorescent proteins (RFP), yellow fluorescent proteins (YFP), and cyan fluorescent proteins (CFP). Useful fluorescent proteins also include mutants and spectral variants of these proteins which retain the ability to fluoresce.

[21] RFPs include *Discosoma* RFPs, such *Discosoma* DsRed (SEQ ID NO:9) or a mutant thereof which includes an Ile125Arg mutation, or a non-oligomerizing tandem DsRed containing, for example, two RFP monomers linked by a peptide linker. For example, a non-oligomerizing tandem RFP can contain two DsRed monomers or two mutant DsRed-I125R monomers linked by a peptide (having, for example, the amino acid sequence shown in SEQ ID NO:10).

[22] Useful GFPs include an *Aequorea* GFP (*e.g.*, SEQ ID NO:11), a *Renilla* GFP, a *Phialidium* GFP, and related fluorescent proteins for example, a cyan fluorescent protein (CFP), a yellow fluorescent protein (YFP), or a spectral variant of the CFP or YFP. CFP (cyan) and YFP (yellow) are color variants of GFP. CFP and YFP contain 6 and 4 mutations, respectively. They are Tyr66Try, Phe66Leu, Ser65Thr, Asn145Ile, Met153Thr, and Val163Ala in CFP and Ser65Gly, Val168Leu, Ser72Ala, and Thr203Tyr. Spectral variants include an enhanced GFP (EGFP; SEQ ID NO:12), an enhanced CFP (ECFP; SEQ ID NO:13), an enhanced YFP (EYFP; SEQ ID NO:14), and an EYFP with V68L and Q69K mutations. Other examples of fluorescent proteins comprising mutations are *Aequorea* GFP with one or more mutations at amino acid residues A206, L221 or F223 of SEQ ID NO:11 (*e.g.*, mutations A206K, L221K, F223R, Q80R); mutations L221K and F223R of ECFP (SEQ ID NO:12), and EYFP-V68L/Q69K of SEQ ID NO:11. See also US 2004/0180378; U.S. Patents 6,150,176; 6,124,128; 6,077,707; 6,066,476; 5,998,204; and 5,777,079; Chalfie *et al.*, *Science 263*:802-805, 1994.

[23] Other useful GFP-related fluorescent proteins include those having one or more folding mutations, and fragments of the proteins that are fluorescent, for example, an *A. victoria* GFP from which the two N-terminal amino acid residues have been removed. Several of these fluorescent proteins contain different aromatic amino acids within the central chromophore and fluoresce at a distinctly shorter wavelength than the wild type GFP species. For example, the engineered GFP proteins designated P4 and P4-3 contain, in addition to other mutations, the substitution Y66H; and the engineered GFP proteins designated W2 and W7 contain, in addition to other mutations, Y66W.

[24] Folding mutations in *Aequorea* GFP-related fluorescent proteins improve the ability of the fluorescent proteins to fold at higher temperatures and to be more fluorescent when expressed in mammalian cells, but have little or no effect on the peak wavelengths of excitation and emission. If desired, these mutations can be combined with additional mutations that influence the spectral properties of GFP to produce proteins with altered spectral and folding properties, and, particularly, with mutations that reduce or eliminate the propensity of the fluorescent proteins to oligomerize. Folding mutations, with respect to SEQ ID NO:11, include the substitutions F64L, V68L, S72A, T44A, F99S, Y145F, N1461, M153T, M153A, V163A, I167T, S175G, S205T, and N212K.

*Luminescent moieties*

[25] Luminescent moieties useful in a cAMP reporter include lanthanides, which can be in the form of a chelate, including a lanthanide complex containing the chelate (*e.g*, β-diketone chelates of cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, or ytterbium). Lanthanide chelates are well known in the art. *See* Soini and Kojola, *Clin. Chem. 29*, 65, 1983; Hemmila *et al.*, *Anal. Biochem. 137*, 335 1984; Lovgren *et al.*, In: Collins & Hoh, eds., Alternative Immunoassays, Wiley, Chichester, U.K., p. 203, 1985; Hemmila, *Scand. J. Clin. Lab. Invest. 48*, 389, 1988; Mikola *et al.*, *Bioconjugate Chem. 6*, 235, 1995; Peruski *et al.*, *J. Immunol. Methods 263*, 35-41, 2002; U.S. Patent 4,374,120; and U.S. Patent

6,037,185. Suitable β-diketones are, for example, 2-naphthoyltrifluoroacetone (2-NTA), 1-naphthoyltrifluoroacetone (1-NTA), p-methoxybenzoyltrifluoroacetone (MO-BTA), p-fluorobenzoyltrifluoroacetone (F-BTA), benzoyltrifluoroacetone (BTA), furoyltrifluoroacetone (FTA), naphthoylfuroylmethane (NFM), dithenoylmethane (DTM), and dibenzoylmethane (DBM). See also US 20040146895.

[26] Luminescent proteins include, but are not limited to, lux proteins (*e.g.*, luxCDABE from *Vibrio fischerii*), luciferase proteins (*e.g.*, firefly luciferase, *Gaussia* luciferase, *Pleuromamma* luciferase, and luciferase proteins of other beetles, Dinoflagellates (*Gonylaulax; Pyrocystis*;), Annelids (*Dipocardia*), Molluscs (*Lativa*), and Crustacea (*Vargula; Cypridina*), and green fluorescent proteins of bioluminescent coelenterates (*e.g.*, *Aequorea Victoria*, *Renilla mullerei*, *Renilla reniformis*; *see* Prendergast *et al.*, *Biochemistry 17*, 3448-53, 1978; Ward *et al.*, *Photochem. Photobiol. 27*, 389-96, 1978; Ward *et al.*, *J. Biol. Chem. 254*, 781-88, 1979; Ward *et al.*, *Photochem. Photobiol. Rev 4*, 1-57, 1979; Ward *et al.*, *Biochemistry 21*, 4535-40, 1982). Many of these proteins are commercially available. Firefly luciferase is available from Sigma, St. Louis, MO, and Boehringer Mannheim Biochemicals, Indianapolis, IN. Recombinantly produced firefly luciferase is available from Promega Corporation, Madison, WI. Jellyfish aequorin and luciferase from *Renilla* are commercially available from Sealite Sciences, Bogart, GA.

[27] The DNA sequences of the aequorin and other luciferases employed for preparation of some cAMP reporters of the invention can be derived from a variety of sources. For example, cDNA can be prepared from mRNA isolated from the species disclosed above. *See* Faust, *et al.*, *Biochem. 18*, 1106-19, 1979; De Wet *et al.*, *Proc. Natl. Acad. Sci. USA 82*, 7870-73, 1985.

[28] Luciferase substrates (luciferins) are well known and include coelenterazine (available from Molecular Probes, Eugene, OR) and ENDUREN™. These cell-permeable reagents can be directly administered to cells, as is known in the art. Luciferin compounds can be

prepared according to the methods disclosed by Hori *et al.*, *Biochemistry 14*, 2371-76, 1975; Hori *et al.*, *Proc. Natl. Acad. Sci. USA 74*, 4285-87, 1977).

*Dark quenchers*

[29] In some embodiments the acceptor moiety is a quencher moiety, preferably a "dark quencher" (or "black hole quencher") as is known in the art. In this case, the change in conformation which occurs upon cAMP binding eliminates quenching, resulting in an increase in energy emission from the donor moiety. "Dark quenchers" themselves do not emit photons. Use of a "dark quencher" reduces or eliminates background fluorescence or luminescence which would otherwise occur as a result of energy transfer from the donor moiety. Suitable quencher moieties include dabcyl (4-(4'-dimethylaminophenylazo)-benzoic acid), QSY™-7 carboxylic acid, succinimidyl ester (N,N'-dimethyl-N,N'-diphenyl-4-((5-t-butoxycarbonylaminopentyl)aminocarbon yl) piperidinylsulfone-rhodamine (a diarylrhodamine derivative from Molecular Probes, Eugene, OR). Suitable quencher moieties are disclosed, for example, in US 2005/0118619; US 20050112673; and US 20040146959.

[30] Any suitable fluorophore may be used as the donor moiety provided its spectral properties are favorable for use with the chosen dark quencher. The donor moiety can be, for example, a Cy-dye, Texas Red, a Bodipy dye, or an Alexa dye. Typically, the fluorophore is an aromatic or heteroaromatic compound and can be a pyrene, anthracene, naphthalene, acridine, stilbene, indole, benzindole, oxazole, thiazole, benzothiazole, cyanine, carbocyanine, salicylate, anthranilate, coumarin, a fluorescein (*e.g.*, fluorescein, tetrachlorofluorescein, hexachlorofluorescein), rhodamine, tetramethyl-rhodamine, or other like compound. Suitable fluorescent moieties for use with dark quenchers include xanthene dyes, such as fluorescein or rhodamine dyes, including 6-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), tetrachlorofluorescein (TET), 6-carboxyrhodamine (R6G), N,N,N;N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX). Suitable fluorescent reporters also include

the naphthylamine dyes that have an amino group in the alpha or beta position. For example, naphthylamino compounds include 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate and 2-p-toluidinyl-6-naphthalene sulfonate, 5-(2'-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS).

[31] Other suitable fluorescent moieties include coumarins, such as 3-phenyl-7-isocyanatocoumarin; acridines, such as 9-isothiocyanatoacridin- e and acridine orange; N-(p-(2-benzoxazolyl)phenyl)maleimide; cyanines, such as indodicarbocyanine 3 (Cy3), indodicarbocyanine 5 (Cy5), indodicarbocyanine 5.5 (Cy5.5), 3-1-carboxy-pentyl)-3'-ethyl-5,5'-dimethy- loxacarbocyanine (CyA); 1H,5H,1H,15H-Xantheno[2,3,4-ij:5,6,7-i'j']diquinol- izin-18-ium, 9-[2(or 4)-[[[6-[2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]amino]sulfonyl]-4(or 2)-sulfophenyl]-2,3,6,7,12,13,16,17-octahyd- ro-inner salt (TR or Texas Red); BODIPY™ dyes; benzoxaazoles; stilbenes; pyrenes; and the like.

*Subcellular targeting sequences*

[32] cAMP reporters of the invention optionally can include a subcellular targeting sequence which can target a cAMP reporter to a subcellular domain such as a plasma membrane, a nuclear membrane, a cytosol, an endoplasmic reticulum, a mitochondria, a mitochondrial matrix, a chloroplast, a medial trans-Golgi cisternae, a lumen of a lysosome, or a lumen of an endosome. Many such targeting sequences are known in the art. Examples include the plasma membrane targeting sequence shown in SEQ ID NO:6, the nuclear localization signal sequence shown in SEQ ID NO:5, the mitochondrial localization sequence shown in SEQ ID NO:7, and the mitochondrial matrix targeting signal shown in SEQ ID NO:8. Targeting sequences can be linked to cAMP reporters using, for example, a tetracysteine motif such as Cys Cys Xaa Xaa Cys Cys (SEQ ID NO:15). Targeting sequences can be linked at either the N- or C-terminus of a cAMP reporter or at intermediate points in the reporter.

[33] In some embodiments, cAMP reporters of the invention do not include those which consist of YFP which is not circularly permuted, CFP which is not circularly permuted,

and any of the following polypeptides: amino acids 1-443 of SEQ ID NO:3 (a mouse Epac2), amino acids 1-149 of SEQ ID NO:3, amino acids 29-149 of SEQ ID NO:3, amino acids 285-443 of SEQ ID NO:3, amino acids 304-443 of SEQ ID NO:3, amino acids 310-443 of SEQ ID NO:3, amino acids 285-454 of SEQ ID NO:3, amino acids 285-460 of SEQ ID NO:3, and amino acids157-316 of SEQ ID NO:1 (human Epac1).

*Assembly of cAMP reporters*

[34] cAMP reporters which are fusion proteins preferably can be expressed recombinantly, and the invention provides nucleic acid molecules for this purpose. A nucleic acid molecule encoding a cAMP reporter can comprise any nucleotide sequence which encodes the amino acid sequence of the reporter. Nucleic acid molecules of the invention include single- and double-stranded DNA (including cDNA) and mRNA. Many kits for constructing fusion proteins are available from companies such as Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), CLONTECH (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

[35] In some embodiments the nucleic acid molecules are expression constructs which contain the necessary elements for the transcription and translation of an inserted coding sequence encoding a cAMP reporter. Expression constructs can be used as vectors for introducing cAMP reporters into cells. Methods which are well known to those skilled in the art can be used to construct expression vectors containing sequences encoding cAMP reporters and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook *et al.* (1989) and in Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, N.Y., 1989.

[36] Expression vectors of the invention can be expressed in a variety of host cells. These include, but are not limited to, microorganisms, such as bacteria transformed with

recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors, insect cell systems infected with virus expression vectors (*e.g.*, baculovirus), plant cell systems transformed with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmids), or animal cell systems, particularly mammalian systems, including human systems. See WO 01/98340, which is incorporated herein by reference in its entirety. The choice of vector components and appropriate host cells is well within the capabilities of those skilled in the art.

[37] Alternatively, protein or non-protein donor and/or acceptor moieties can be linked to the polypeptide by covalent attachment. There are a variety of methods known in the art which are useful for this purpose. For example, the attachment can be direct, via a functional group on the polypeptide (*e.g.*, amino, carboxyl and sulfhydryl groups) and a reactive group on the fluorophore. Free amino groups in the polypeptide can be reacted with fluorophores derivatized with isothiocyanate, maleic anhydride, N-hydroxysuccinimide, tetrafluorylphenyl and pentafluoryl esters. Free carboxyl groups in the polypeptide can be reacted with carbodiimides such as 1-ethyl-3-[dimethylaminopropyl]carbodiimide hydrochloride to create a reactive moiety that will react with an amine moiety on the donor or acceptor moiety. Sulfhydryl groups can be attached to donor or acceptor moities modified with maleimide and iodoacetyl groups, although such linkages are more susceptible to reduction than linkages involving free amino groups. The polypeptide can also be linked indirectly via an intermediate linker or spacer group, using chemical groups such as those listed above.

[38] It is also possible to produce cAMP reporters of the invention using chemical methods to synthesize the amino acid sequence of the polypeptide and, optionally, one or more fluorescent or luminescent proteins. Methods include direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc. 85*, 2149-2154, 1963; Roberge *et al.*, *Science 269*, 202-204, 1995). Protein synthesis can be performed using manual

techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Optionally, fragments of polypeptide portions of cAMP reporters can be separately synthesized and combined using chemical methods to produce a full-length reporter molecule. See WO 01/98340.

*Delivery of cAMP reporters to cells*

[39] All cAMP reporters of the invention can be introduced into cells *in vitro* using reversible permeabilization techniques. See U.S. Patent 6,127,177; U.S. Patent 6,902,931; Russo *et al.*, *Nature Biotechnology 15*, 278-82, March 1997; Santangelo *et al.*, *Nucleic Acids Res. 32*, 1-9, April 14, 2004.

[40] If the cAMP reporter is a fusion protein, expression vectors comprising a cAMP reporter-encoding nucleotide sequence can be transfected into any cell *in vitro* in which it is desired to monitor cAMP levels or distribution. Any transfection method known in the art can be used, including, for example, including, but not limited to, transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, "gene gun," and DEAE- or calcium phosphate-mediated transfection.

[41] Useful vectors and methods of delivering the vectors to cells *in vivo* are disclosed, for example, in U.S. Patent 6,825,012; U.S. Patent 6,878,549; U.S. Patent 6,645,942; U.S. Patent 6,692,737; U.S. Patent 6,689,758; U.S. Patent 6,669,935; and U.S. Patent 6,821,957.

*Methods of detecting cAMP*

[42] The invention provides various methods for detecting cAMP by detecting conformational changes in a cAMP reporter. Broadly, the methods involve detecting a change in resonance energy transfer of a cAMP reporter of the invention when the reporter is subjected to a change in cAMP concentration. cAMP binding to the reporter induces a

conformational change that changes resonance energy transfer from the donor moiety to the acceptor moiety.

[43] A change in resonance energy transfer can readily be detected using methods well known in the art. *See, e.g.*, US 2005/0118619; US 2002/0137115; US 2003/0165920; US 2003/0186229; US 2004/0137479; US 2005/0026234; US 2005/0054573; US 2005/0118619; U.S. Patent 6,773,885; U.S. Patent 6,803,201; U.S. Patent 6,818,420; Ayoub *et al.*, 2002; Boute *et al.*, 2002; Domin *et al.*, *Prog. Biomed. Optics and Imaging, Proc. SPIE, vol 5139*, 2003, pp238-242; Evellin *et al.*, *Methods Mol. biol. 284*, 259-70, 2004; Honda *et al.*, *Proc. Natl. Acad. Sci. USA 98*, 437-42, February 27, 2001; Honda *et al.*, *Methods Mol. Biol. 3*, 27-44, 1005; Mongillo *et al.*, *Cir. Res. 95*, 67-75, July 9, 2004; Mongillo *et al.*, *Methods Mol. Biol. 307*, 1-14, 2005; Nagai *et al.*, *Proc. Natl. Acad. Sci. USA 101*, 10554-59, July 20, 2004; Nikolaev *et al.*, *J. Biol. Chem. 279*, 37215-18, 2004; Polit *et al.*, *Eur. J. Biochem. 270*, 1413-23, 2003; Ponsioen *et al.*, *EMBO Rep. 5*, 1176-80, 2004; Santangelo *et al.*, *Nucl. Acids Res. 32*, 1-9, e-published April 14, 2004; and Warrier *et al.*, *Am. J. Physiol. Cell Phiol. 289*, C455-61, August 2005. Properties which can be detected as resonance energy transfer (RET) measurements include a molar extinction coefficient at an excitation wavelength, a quantum efficiency, an excitation spectrum, an emission spectrum, an excitation wavelength maximum, an emission wavelength maximum, a ratio of excitation amplitudes at two wavelengths, a ratio of emission amplitudes at two wavelengths, an excited state lifetime, anisotropy, a polarization of emitted light, resonance energy transfer, and a quenching of emission at a wavelength.

[44] cAMP reporters of the invention can be used in cell-free systems, in isolated cells (for example, in primary cell culture or a cell line) or in cells *in situ* (*e.g.*, in an isolated tissue sample, an isolated whole organ, or in a mammal). Subcellular distribution of cAMP or changes in cAMP concentration can be detected, for example, as described in Example 2, below. Absolute cAMP levels can be detected by obtaining a RET measurement in the assay system and comparing it to a standard curve obtained *in vitro*.

[45] In some embodiments, steady-state RET measurements are first obtained and then measurements are taken after addition of a test compound to the assay system. Test compounds can be used, for example, to increase cAMP concentration to make it easier to detect cAMP in a particular subcellular compartment or to monitor the effect of the test compound on cAMP concentration (*e.g.*, in drug-screening methods). Test compounds can be pharmacologic agents already known in the art to affect cAMP levels or can be compounds previously unknown to have such an activity. Compounds known to affect cAMP levels include, for example, β-adrenergic receptor agonists (*e.g.*, norepinephrine, epinephrine, isoproterenol, sulfonterol, metaproterenol, SB-251023), β-adrenergic receptor antagonists (*e.g.*, propranolol, butoxamine, practolol, alprenolol, pindolol, nadolol, metaprolol, SR-59230A), direct or indirect activators of adenylate cyclase (*e.g.*, forskolin, prostalglandin $E_1$), cAMP analogs (*e.g.*, 8-(4-chloro-phenylthio)-2′-O-methyl adenosine 3′,5′-monophosphate; $N^6$,2′-*O*-dibutyryl cyclic adenosine 3′,5′monophosphate ($Bt_2$cAMP)), and photolytic release agents (*e.g.*, *P*-(4,5-dimethoxy-2-nitrobenzyl) adenosine 3′,5′-monophosphate, and phosphodiesterase inhibitors such as 3-isobutyl-1-methylxanthine).

[46] Test compounds can be naturally occurring or designed in the laboratory. They can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art. If desired, test compounds can be obtained using any of the numerous combinatorial library methods known in the art, including but not limited to, biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection.

[47] All patents, patent applications, and references cited in this disclosure are expressly incorporated herein by reference. The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following

specific examples, which are provided for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

*Preparation and function of cAMP reporters*

[48] We generated a number of proteins by fusing the amino terminus of various Epac truncations to ECFP and the carboxyl terminus to citrine, an improved version of YFP (FIG. 1). Full length Epac1 (1-881, SEQ ID NO:1) and truncated forms of Epac2, T316-A501 (amino acids 316-501 of SEQ ID NO:20) and P350-A501 (amino acids 350-501 of SEQ ID NO:20), were created by PCR using Epac1 (de Rooij *et al.*, *Nature 396,* 474-77, 1998.) or Epac2 (SEQ ID NO:20; Ozaki *et al.*, *Nature Cell Biology 2*, 805-11, 2000) as the templates. In one construct, ECFP and citrine were fused together with a domain (amino acids P350-A501 of SEQ ID NO:20) containing the second cyclic nucleotide monophosphate-binding domain from Epac2 and a C-terminal lid (the α-helix that stabilizes the cAMP-binding site) (FIG. 1). Mutation R522E was incorporated by the QUICKCHANGE® method (Stratagene). Enhanced cyan fluorescent protein (ECFP) and citrine were fused to the N and C terminal ends of the individual gene constructs (FIG. 1). The constructs were first generated in pRSET B (Invitrogen) and subcloned into pcDNA3 (Invitrogen) behind a Kozak sequence for mammalian expression.

[49] For nuclear targeting, the nuclear localization signal PKKKRKVEDA (SEQ ID NO:5) was added to the C terminus. Localization to the mitochondrial matrix was achieved by fusing the first 12 amino acids of subunit IV of human cytochrome oxidase *c* and a four-residue linker (SEQ ID NO:8) to the N terminal of the construct. For plasma membrane targeting of ICUE1, the sequence KKKKKSKTKCVIM (SEQ ID NO:6) was inserted at the C terminus. The signal sequence MAIQLRSLFPLALPGMLALLGWWWFFSRKK (SEQ ID NO:7) was inserted at the N terminus for targeting ICUE to mitochondria.

EXAMPLE 2

*Cell culture and imaging*

[50] *Cell Culture.* HEK-293, HeLa and PC12 cells were plated onto sterilized glass coverslips in 35mm dishes and grown to 50-90% confluency in DMEM (10% FBS at 37°C, 5% $CO_2$). Cells were then transfected with FuGENE-6 transfection reagent (Roche) or calcium phosphate and allowed to grow for 12-24 hours before imaging. Colocalization studies were performed by incubating transfected HEK-293 cells with MitoTracker Red 580 or Hoechst 33342 cell-permeable dyes (Molecular Probe) for staining mitochondria or nucleic acids, respectively.

[51] *Imaging.* Cells were washed twice with Hanks' balanced salt solution buffer after 12- to 24-h incubation at 37 °C culture medium. Cells were maintained in buffer in the dark at room temperature with addition of isoproterenol (Aldrich), forskolin (Calbiochem), Prostaglandin $E_1$ ($PGE_1$) (Sigma), and 8-(4-chloro-phenylthio)-2'-O-methyl adenosine 3',5'-monophosphate (8-pCPT-2'-O-Me-cAMP) (Axxora Biolog) as indicated. Cells were also treated with *P*-(4,5-dimethoxy-2-nitrobenzyl) adenosine 3',5'-monophosphate (DMNB-cAMP) (Molecular Probe). Uncaging of cAMP was performed as previously described ( Zhang *et al.*, *Proc. Natl. Acad. Sci. USA 98*, 14997-15002, 2001.).

[52] Cells were imaged on a Zeiss Axiovert 200M microscope with a cooled charge-coupled device camera MicroMAX BFT512 (Roper Scientific) controlled by METAFLUOR 6.2 software (Universal Imaging). Dual-emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror and two emission filters (475DF40 for ECFP, 535DF25 for citrine) alternated by a filter changer Lambda 10-2 (Sutter Instruments). Exposure time was 100-500 ms and images were taken every 8-30 s. Fluorescent images were background-corrected by subtracting autofluorescence intensities of untransfected cells (or background with no cells) from the emission intensities of fluorescent cells expressing reporters. The ratios of cyan to yellow emissions were then calculated at

different time points and normalized by dividing all ratios by the emission ratio just prior to stimulation therefore setting the basal emission ratio to 1.

[53] FRET efficiency was determined by acceptor photobleaching as reported (Miyawaki & Tsien, *Methods Enzymol. 327*, 472-500, 2000). Briefly, citrine was photobleached at the end of the experiment by intense illumination with a 525DF40 filter. ECFP fluorescence intensities before ($F_{da}$) and after citrine photobleaching ($F_d$) and the equation $E = 1 - (F_{da}/F_d)$ were then used to calculate the FRET efficiency.

EXAMPLE 3

*Function of cAMP reporters*

[54] A cAMP reporter in which ECFP and citrine were fused together with a domain (P350-A501; amino acids 350-501 of SEQ ID NO:20) containing the second cyclic nucleotide monophosphate-binding domain from Epac2 and a C-terminal lid was expressed in HEK-293 cells. This reporter showed variable ratios of cyan to yellow emissions which are inversely correlated with expression level of the protein. This concentration dependence indicates intermolecular FRET between different reporter molecules that may occur due to oligomerization or aggregation (Zacharias *et al.*, *Science 296*, 913-16, 2002). Upon cAMP elevations, this protein did not show a cAMP-dependent FRET change. We incorporated a larger portion of Epac2 sequence N-terminal to the binding domain (T316-A501; amino acids 316-501 of SEQ ID NO:20) (FIG. 1) and obtained a construct that showed more homogeneous emission ratios and a 5% increase in emission ratio of cyan to yellow upon cAMP elevations.

[55] To improve the dynamic range of the response and to develop a reporter for Epac activation, we sandwiched the full-length Epac1 between ECFP and citrine (FIG. 1). When this reporter (designated as ICUE1) was transfected in HEK-293 cells, the fluorescence was uniformly distributed in the cytosolic compartment in 60% of the cells (FIG. 2A, leftmost image). In the remaining 40% of the cells, the protein was localized

to perinuclear region or mitochondria, consistent with our previous observation using full-length Epac1 fused to YFP (Qiao *et al.*, *J. Biol. Chem. 277*, 26581-86, 2002). A similar expression pattern was also observed in HeLa and PC12 cells.

[56] Stimulation of endogenous β-adrenergic receptor (β-AR) with isoproterenol generated a FRET decrease in HEK293 cells expressing ICUE1, resulting in an increase in the ratio of cyan to yellow emissions (FIG. 2A and 2B). The change in emission ratios was detectable within several seconds and reached a plateau of 16.8% ± 1.0 (average value ± S.E.M, n=8) signal increase within 1.5-3 min. This FRET change consisted of reciprocal decreases in yellow and increases in cyan emission and the FRET efficiencies were measured by acceptor photobleaching to be 29% ± 3 and 21% ± 1 (n=3), respectively, before and after isoproterenol stimulation. The presence of propranolol, a β-adrenergic-receptor antagonist, prevented the isoproterenol-stimulated response.

[57] We next tested if the FRET response was reversible. Addition of 10 μM propranolol after the isoproterenol-stimulated response reached the plateau resulted in an initial decrease in emission ratio of cyan to yellow in 2-3 minutes, and a full recovery over 6-8 minutes. Removal of isoproterenol had the same effect. Finally, a second rise in emission ratio was induced by addition of forskolin to activate adenylate cyclase (AC) and elevate cAMP. The change in emission ratio reached a plateau in 3-5 minutes (FIG. 2B).

[58] To verify the FRET response is due to cAMP binding, we generated a variant of the reporter that carries a point mutation in the cAMP binding domain. Arginine 279 in Epac1 is a conserved residue that contributes to cAMP binding (Rehmann *et al.*, *Nat. Struc. Biol. 10*, 26-32, 2003). EpacR279E is defective in cAMP binding (de Rooij *et al.*, *Nature 396*, 474-77, 1998; Mei *et al.*, *J. Biol. Chem. 277*, 11497-504, 2002), and mutation of the same Arginine to Glutamate (R522E) in the reporter completely abolished the FRET change induced by isoproterenol and forskolin (FIG. 2B, n=7).

[59] Different means of elevating intracellular cAMP revealed different kinetics for the FRET response (FIG. 2C). As shown in FIG. 2B and 2C, activation of β-AR with a selective agonist such as isoproterenol (10 μM) induced decreases in FRET in 1.5-3 minutes, while stimulation of adenylate cyclase required slightly longer treatment with forskolin (3-5 minutes) to produce a maximal increase in emission ratios (19.6% ± 1.0 response, n=8) (FIG. 2C). Addition of 10 μM prostaglandin E1 ($PGE_1$) to increase cAMP levels (Rich *et al., Proc. Natl. Acad. Sci. USA 98*, 10349-54, 2001) induced a FRET response (13.3% ± 0.9, n=7) within 3-5 minutes, noticeably delayed compared to the response induced by isoproterenol (FIG. 2C). A newly characterized analogue of cAMP, 8-pCPT-2'-O-Me-cAMP, specifically activates Epac but not PKA (Enserink *et al., Nat. Cell Biol. 4*, 901-06, 2002). This cAMP analog, when administrated at 300 μM, required 10-15 mininutes to produce a half-maximal increase in emission ratios ($t_{1/2}$) (FIG. 2C, 12.8% ± 0.9, n=6).

[60] The fastest intracellular responses were generated by photolytic release ("uncaging") of cAMP from a membrane-permeant ester, DMNB-cAMP (Nerbonne *et al., Nature 310*, 74-76, 1984). Cells expressing ICUE1 were first incubated with 100 μM DMNB-cAMP for 3 minutes and were exposed to UV to uncage the cAMP intracellularly. Flash of 5 seconds acutely increased the emission ratio by 4.7% ± 0.7 (n=8) in just 15-30 seconds (FIG. 2C). The response was then quickly reversed due to the degradation of uncaged cAMP by phosphodiesterase (PDE). The slower time courses of the other responses are presumably due to rate-limiting steps in activating adenylate cyclase and accumulating sufficient cAMP, rather than the kinetics of cAMP binding to Epac1, or the FRET response of the reporter.

EXAMPLE 4

*cAMP dynamics within subcellular compartments*

[61] To directly monitor cAMP dynamics at different subcellular locations inside cells, we prepared several fusions of ICUE1 to various specific targeting motifs (FIG. 3A). To localize the reporter to the plasma membrane, we fused the plasma membrane-targeting

signal of small guanosine triphosphatase K-ras4B (Roy *et al.*, *Biochem. 39*, 8298-307, 2000) to the C terminus of ICUE1. This targeting motif combined a farnesylated cysteine residue with a strongly polybasic sequence and effectively targeted the reporter to the plasma membrane (FIG. 3B).

**[62]** As shown in FIG. 3C, plasma membrane targeted ICUE1 generated a FRET response of 18.3% ± 1.2 (n=8) upon stimulation with isoproterenol. The response time ($t_{1/2}$ = 24.9 s ± 2.8, n= 8) was shortened by 40% compared to the time course for the cytoplasmically-distributed ICUE1 ($t_{1/2}$= 40.5 s ± 3.3, n=8), while both plasma membrane-targeted and cytoplasmically-distributed ICUE1 generated rapid responses upon whole-cell cAMP uncaging. These results indicate that this delay in response of untargeted ICUE1 is not due to the intrinsic kinetic properties of the localized reporters, but most likely due to restricted release of cAMP from the plasma membrane to cytosol (Rich *et al.*, *J. Gen. Physiol. 116*, 147-61, 2000).

EXAMPLE 5

*cAMP dynamics and Epac activation in mitochondria*

**[63]** Epac localizes to mitochondria in a subpopulation of cells, but monitoring of cAMP accumulation at mitochondria has not been possible with previous methods. To examine the cAMP dynamics and Epac activation at this subcellular location, we fused two different mitochondria targeting motifs to ICUE1 (FIG. 3A). The first MitoCOX-ICUE1 was generated by fusing the targeting sequence of subunit IV of cytochrome c oxidase (COX) to the N-terminus of ICUE1. This COX sequence delivers fused proteins to the mitochondrial matrix (Hurt *et al.*, *EMBO J. 4*, 2061-68). As shown in FIG. 3B, MitoCOX-ICUE1 was partially targeted to mitochondria (Filippin *et al.*, *J. Biol. Chem. 278*, 39224-34, 2003), showing partial colocalization with a cell permeable mitochondrial dye, MitoTracker.

[64] Activation of β-AR by isoproterenol generated a FRET response (19.0% ± 1.6, n=5) in the punctate mitochondria structure within 2-3 minutes ($t_{1/2}$= 40.4 s ± 7.3, n=5), indicating that cAMP can enter mitochondria and accumulate in the matrix. In a second mitochondria-targeted ICUE1 (MitoDAKAP1-ICUE1), a mitochondria targeting motif taken from the N-terminal sequence of DAKAP1a (Ma & Taylor, *J. Biol. Chem. 277*, 27328-36, 2002) effectively targeted ICUE1 to mitochondria (FIG. 3B), where the isoproterenol stimulated FRET response (14.5% ± 1.5, $t_{1/2}$= 42.4 s ± 2.5, n=6) is similar to the cytosolic response (FIG. 3C).

[65] When fused to a nuclear localization signal (NLS), ICUE1 was appropriately targeted to the nucleus (FIG. 3B), where its response to isoproterenol stimulation was smaller (5.6% ± 0.5, n=12) than the 16.8% ± 1.0 FRET change for cytoplasmically-distributed ICUE1 (FIG. 3C). Stimulation with bicarbonate to activate endogenous soluble adenylate cyclase in HEK-293 cells did not generate a cAMP-dependent response in the nucleus, possibly due to limited copy numbers of soluble AC in this cell type or the sensitivity of the detection. Interestingly, the isoproterenol-stimulated response in the nucleus is not delayed ($t_{1/2}$= 38.5 s ± 3.5, n=12) compared to that from untargeted ICUE1. This indicates that the available pool of cAMP in the nucleus, while possibly smaller, is not kinetically crippled due to the fast diffusion of cAMP from cytosol to nucleus.

[66] To test if activation of different receptors leads to production of different pools of cAMP, we compared the cAMP responses induced by $PGE_1$ and isoproterenol at different subcellular sites. At the plasma membrane, addition of $PGE_1$ generated a 12.6% ± 0.9 (n=8) emission ratio increase within 2-3 minutes. Surprisingly, sustained stimulation with 10 μM $PGE_1$ did not produce a transient response as observed previously using cyclic nucleotide-gated ion channels (Rich *et al.*, *Proc. Natl. Acad. Sci. USA 98*, 13049-54, 2001). In contrast, the response at the plasma membrane is sustained until removal of $PGE_1$ (FIG. 3D). Variable cellular PDE activities may be responsible for this discrepancy.

[67] After removal of $PGE_1$, a second response of similar amplitude was induced by stimulation with isoproterenol. Both untargeted and mitochondria targeted ICUE1 showed similar responses to $PGE_1$ and isoproterenol. In the nucleus, $PGE_1$ also stimulated a small response (3.4% ± 0.4, n=13), noticeably a few percentages smaller than that induced by isoproterenol in the same cell (FIG. 3E).

EXAMPLE 6

*Simultaneous imaging of cAMP dynamics and PKA phosphorylation*

[68] Soluble AC and regulatory and catalytic subunits of protein kinase A (PKA) coexist in the nucleus of mammalian cells (Zippin *et al., J. Cell Biol. 164*, 527-34, 2004). The activation of bicarbonate-responsive soluble AC in the nucleus led to a rapid increase in PKA-dependent phosphorylation, which was detectable within two minutes. The immediate presence of a nuclear pool of cAMP following β-AR activation raised the question whether this pool of cAMP could produce functional PKA responses in the nucleus. Here, we took advantage of the targeted cAMP indicators and a PKA activity reporter, AKAR (Zhang *et al., Proc. Natl. Acad. Sci. USA 98*, 14997-5002, 2001), to examine the temporal correlation of cAMP dynamics and PKA activation within single living cells.

[69] We co-expressed the plasma membrane-targeted ICUE1 and nuclear-localized AKAR in HEK-293 cells (FIG. 4A). An immediate increase in emission ratios of cyan to yellow occurred at the plasma membrane upon stimulation with isoproterenol, indicating an acute rise in cAMP. The emission ratio in the nucleus did not increase in the same time frame. After a delay of 5-10 minutes, in the presence of the sustained cAMP response a gradual increase in ratio of yellow to cyan emissions occurred and reached a plateau in 20-30 minutes. Untargeted AKAR generated acute responses in 2-3 minutes in the cytosol of HEK-293 cells upon stimulation with isoproterenol. This is consistent with acute cytosolic AKAR responses upon cAMP elevations we previously reported (Zhang *et al.*, 2001). Therefore, the delay in response indicates that PKA phosphorylation in the

nucleus does not occur immediately following cAMP production (FIG. 4B). This delayed nuclear response of PKA phosphorylation is consistent with a slow diffusional translocation of the catalytic (C) subunit of PKA into the nucleus following the dissociation of catalytic and regulatory subunits in the cytoplasm upon cAMP elevation (Harootunian *et al.*, *Mol. Cell Biol. 4*, 993-1002, 1993; Meinkoth *et al.*, *Proc. Natl. Acad. Sci. USA 87*, 9595-99, 1990).

[70] As a control experiment, we also recorded cAMP responses from single cells co-expressing both plasma membrane-targeted and nuclear-localized ICUE1 for direct comparison of the cAMP dynamics at the plasma membrane and in the nucleus. As shown in FIG. 4C, isoproterenol stimulated an acute cAMP response at the plasma membrane followed by a response in the nucleus, which reached the plateau in 2-3 minutes. This is consistent with data obtained from separate cells expressing either targeted reporter indicating that ICUE reporter molecules do not notably perturb cAMP distribution throughout the cell. This acute nuclear cAMP response is in sharp contrast to the delayed response of PKA phosphorylation in the nucleus, which requires 20-30 minutes to reach the maximum. Thus, the presence of this nuclear pool of cAMP immediately following cAMP production is not sufficient to generate a detectable phosphorylation of AKAR by PKA within the nucleus. This lack of immediate PKA response could be due to either the absence of the PKA holoenzyme in the nucleus or insufficient activation of soluble AC-coupled PKA by this pool of cAMP. In this case, the slow diffusion of the C subunit rather than the fast diffusion of cAMP as the rate-limiting step may provide the temporal control of β-AR-stimulated PKA-dependent phosphorylation in the nucleus.

EXAMPLE 7

*Preparation and function of a cAMP reporter comprising a truncated Epac1 (ICUE2)*

[71] In about 40% of transfected cells, untargeted ICUE1 localizes to mitochondria and perinuclear region, which has been documented as the subcellular localization of

endogenous Epac1. To create a more uniformly expressed reporter, we deleted the disheveled, Egl-10, and pleckstrin homology (DEP) domain (amino acids 1-148) which is responsible for this localization. The truncation was generated by PCR amplification of ICUE1 in pRSETB bacterial vector starting from glycine149 of Epac1 through to the end of citrine using the forward primer shown in SEQ ID NO:15:

5'-CGCGGTACCCCCGTGGGAACTCATGAGATGG-3'

and a pRSETB reverse primer. The PCR fragment was then ligated into pcDNA3 mammalian vector containing ECFP. As a result, the truncated reporter, ICUE2, is more diffusible, showing no specific subcellular targeting.

[72] Imaging with ICUE2 in HEK-293 cells revealed a 40-50% increase in cyan/yellow emission ratio upon stimulation of cAMP production with forskolin, compared to a 15-30% response generated by ICUE1. Maximum FRET response was reached in 1.5-3 minutes upon stimulation with isoproterenol, which is on the same time scale as the ICUE1 response. Targeted versions of ICUE2 exhibited the increased dynamic range in cyan/yellow emission ratio as well, therefore improving the signal-to-noise ratio.

[73] ICUE2 also responded to lower concentrations of isoproterenol. We observed FRET responses upon the addition of 0.1μM, 1μM, as well as 10μM isoproterenol, which was the lowest concentration of isoproterenol that generated a FRET response of ICUE1. The ICUE2 response reverses in an average of 9 minutes once it reaches maximum without addition of β-AR antagonist, propranolol, or washing out of agonist.

EXAMPLE 8

*Preparation and function of a cAMP reporter comprising a circularly permuted acceptor moiety (ICUE3)*

[74] The dynamic range of ICUE2 was increased by replacing citrine with a circularly permuted YFP, cpVenus L195 to form a cAMP reporter termed ICUE3. Circular

permutation introduces new N and C termini to a protein and can improve the dynamic range of FRET-based reporters by altering the relative orientation of fluorescent proteins (Nagai *et al.*, 2004).

[75] Upon stimulation with the adenylate cyclase activator forskolin, ICUE3 produced a maximum 93% increase in cyan/yellow emissions ratio (average 78% ± 0.05, N= 8) in HEK-293 cells, which is approximately double the average response measured by ICUE2. Like ICUE2, the ICUE3 response is also reversible when cells are stimulated by the β-adrenergic receptor agonist, isoproterenol.

CLAIMS

1. A cAMP reporter comprising:

(a) a donor moiety;

(b) a polypeptide comprising a cAMP-binding domain of an exchange protein directly activated by cAMP (Epac), wherein the polypeptide is linked to the donor moiety;

(c) an acceptor moiety linked to the polypeptide, wherein the donor moiety and the acceptor moiety exhibit a detectable resonance energy transfer when the donor is excited.

2. The cAMP reporter of claim 1 wherein the polypeptide is Epac1 or Epac2.

3. The cAMP reporter of claim 1 wherein the polypeptide:

(a) comprises SEQ ID NO:1;

(b) comprises SEQ ID NO:2;

(c) comprises SEQ ID NO:1 but for an R522E mutation;

(d) comprises SEQ ID NO:1 but for an R279E mutation;

(e) comprises of amino acids 149-318 of SEQ ID NO:1;

(d) consists of amino acids 149-881 of SEQ ID NO:1;

(e) comprises amino acids 1-160 of SEQ ID NO:20;

(f) consists of amino acids 1-160 of SEQ ID NO:20;

(g) comprises amino acids 280-463 of SEQ ID NO:20;

(h) consists of amino acids 280-463 of SEQ ID NO:20;

(i) comprises amino acids 315-501 of SEQ ID NO:20;

(j) consists of amino acids 315-501 of SEQ ID NO:20;

(k) comprises amino acids 350-501 of SEQ ID NO:20;

(l) consists of amino acids 350-501 of SEQ ID NO:20;

(m) comprises SEQ ID NO:16;

(n) comprises SEQ ID NO:17;

(o) comprises SEQ ID NO:18; or

(p) comprises SEQ ID NO:19.

4. The cAMP reporter of claim 1 wherein at least one of the donor and acceptor moieties is a fluorescent protein.

5. The cAMP reporter of claim 1 wherein each of the donor and the acceptor moieties is a fluorescent protein.

6. The cAMP reporter of claim 1 wherein at least one of the donor and acceptor moieties is a luminescent moiety.

7. The cAMP reporter of claim 6 wherein the luminescent moiety is selected from the group consisting of a luminescent protein and a lanthanide chelate.

8. The cAMP reporter of claim 1 wherein the acceptor moiety is selected from the group consisting of a coumarin, a xanthene, a fluorescein, a fluorescent protein, a circularly permuted fluorescent protein, a rhodol, a rhodamine, a resorufin, a cyanine, a difluoroboradiazaindacene, a phthalocyanine, an indigo, a benzoquinone, an anthraquinone, an azo compound, a nitro compound, an indoaniline, a diphenylmethane, a triphenylmethane, and a zwitterionic azopyridinium compound.

9. The cAMP reporter of claim 1 wherein the acceptor moiety is a dark quencher.

10. The cAMP reporter of claim 1 wherein the donor moiety is selected from the group consisting of a coumarin, a xanthene, a rhodol, a rhodamine, a resorufin, a cyanine dye, a bimane, an acridine, an isoindole, a dansyl dye, an aminophthalic hydrazide, an aminophthalimide, an aminonaphthalimide, an aminobenzofuran, an aminoquinoline, a dicyanohydroquinone, a semiconductor fluorescent nanocrystal, a fluorescent protein, a circularly permuted fluorescent protein, and fluorescent lanthanide chelate.

11. The cAMP reporter of claim 1 wherein the donor moiety is a fluorescent protein selected from the group consisting of a green fluorescent protein (GFP), a red fluorescent protein (RFP), a yellow fluorescent protein (YFP), a blue fluorescent protein (BFP), a cyan fluorescent protein (CFP), and fluorescent mutants thereof.

12. The protein of claim 1 wherein the acceptor moiety is a luminescent protein.

13. The protein of claim 1 wherein the acceptor moiety is a luminescent protein selected from the group consisting of an aequorin, an obelin, a lux protein, a luciferase protein, a phycobiliprotein, a pholasin, and a green fluorescent protein.

14. The cAMP reporter of claim 1 further comprising a subcellular targeting sequence.

15. The cAMP reporter of claim 14 wherein the subcellular targeting sequence targets the reporter to a subcellular location selected from the group consisting of a plasma membrane, a nuclear membrane, a cytosol, an endoplasmic reticulum, a mitochondria, a mitochondrial matrix, a chloroplast, a medial trans-Golgi cisternae, a lumen of a lysosome, and a lumen of an endosome.

16. The cAMP reporter of claim 1 which comprises a subcellular targeting sequence selected from the group consisting of a plasma membrane targeting sequence comprising SEQ ID NO:6, a nuclear localization signal sequence comprising SEQ ID NO:5, a mitochondrial localization sequence comprising SEQ ID NO:7, and a mitochondrial matrix targeting signal comprising SEQ ID NO:8.

17. A nucleic acid molecule which encodes the cAMP reporter of claim 1 wherein each of the donor and acceptor moieties is a protein.

18. The nucleic acid molecule of claim 17 which is an expression vector.

19. A host cell comprising the nucleic acid molecule of claim 17.

20. A method for cAMP, comprising:

detecting a change in resonance energy transfer of a cAMP reporter subjected to a change in cAMP concentration, wherein the cAMP reporter comprises:

(a) a donor moiety;

(b) a polypeptide comprising a cAMP-binding domain of an exchange protein directly activated by cAMP (Epac), wherein the polypeptide is linked to the donor moiety;

(c) an acceptor moiety linked to the polypeptide, wherein the donor moiety and the acceptor moiety exhibit a detectable resonance energy transfer when the donor is excited.

wherein a change in resonance energy transfer indicates a change in cAMP concentration.

21. The method of claim 20 wherein the cAMP concentration is changed by addition of a test compound.

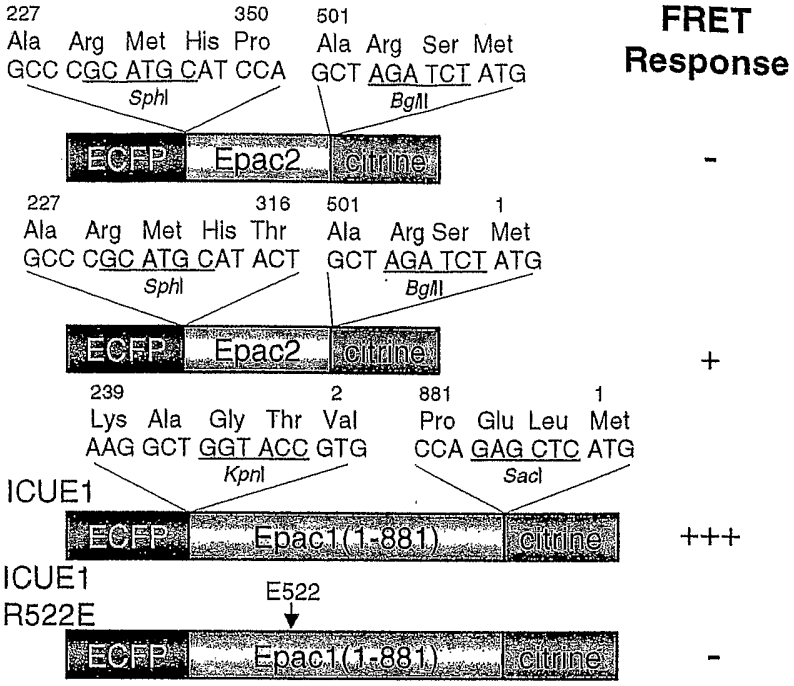
22. The method of claim 20 wherein the cAMP reporter is in a cell-free system.

23. The method of claim 20 wherein the cAMP reporter is in a cell.

24. The method of claim 24 wherein the cell is in a tissue sample.

25. The method of claim 24 wherein the cell is in a whole organ.

26. The method of claim 20 wherein the change in resonance energy is detected by determining a property selected from the group consisting of a molar extinction coefficient at an excitation wavelength, a quantum efficiency, an excitation spectrum, an emission spectrum, an excitation wavelength maximum, an emission wavelength maximum, a ratio of excitation amplitudes at two wavelengths, a ratio of emission amplitudes at two wavelengths, an excited state lifetime, anisotropy, a polarization of emitted light, resonance energy transfer, and a quenching of emission at a wavelength.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Ala Arg Met His Pro Ala Arg Ser Met
GCC CGC ATG CAT CCA GCT AGA TCT ATG
SphI
BglII
ECFP
Epac2
citrine
-
227 316 501 1
Ala Arg Met His Thr Ala Arg Ser Met
GCC CGC ATG CAT ACT GCT AGA TCT ATG
SphI
BglII
ECFP
Epac2
citrine
+
239 2 881 1
Lys Ala Gly Thr Val Pro Glu Leu Met
AAG GCT GGT ACC GTG CCA GAG CTC ATG
KpnI
SacI
ICUE1
ECFP
Epac1(1-881)
citrine
+++
ICUE1
R522E
E522
ECFP
Epac1(1-881)
citrine
-

Figure 1

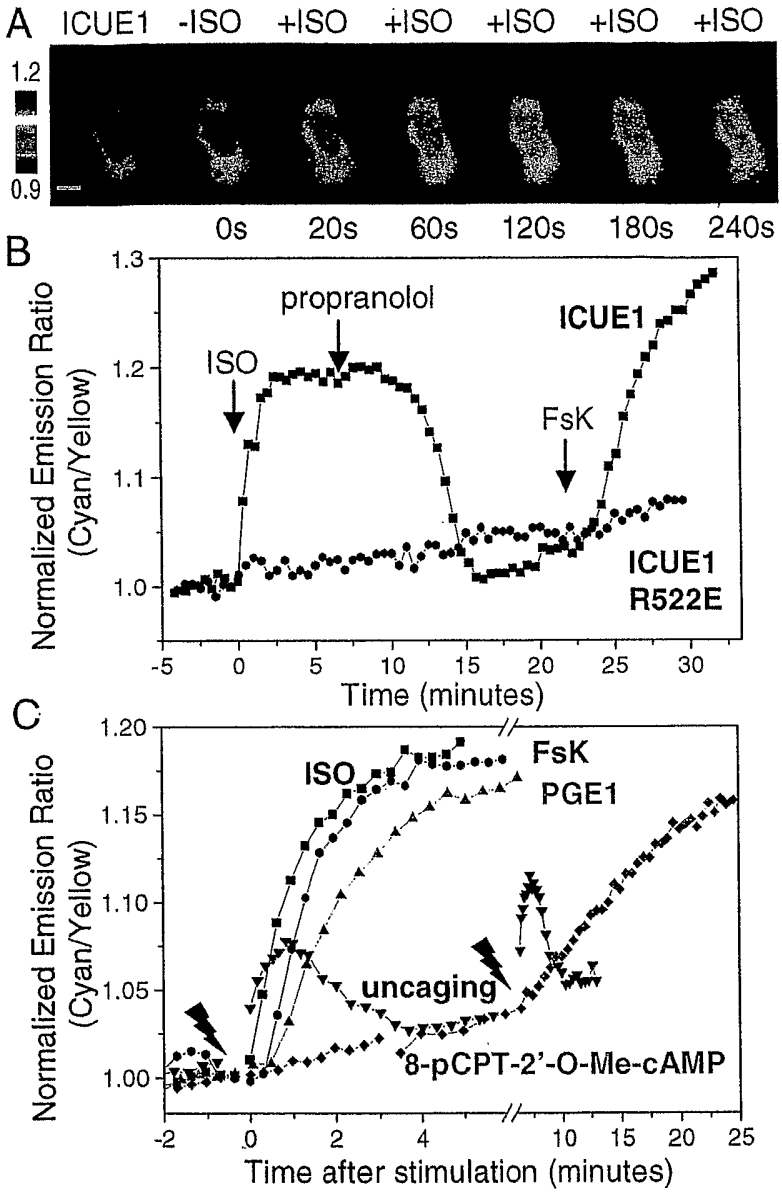

A
ICUE1
-ISO
+ISO
+ISO
+ISO
+ISO
+ISO
1.2
0.9
0s
20s
60s
120s
180s
240s
B
Normalized Emission Ratio (Cyan/Yellow)
propranolol
ISO
ICUE1
FsK
ICUE1 R522E
Time (minutes)
C
Normalized Emission Ratio (Cyan/Yellow)
ISO
FsK
PGE1
uncaging
8-pCPT-2'-O-Me-cAMP
Time after stimulation (minutes)

FIG. 2

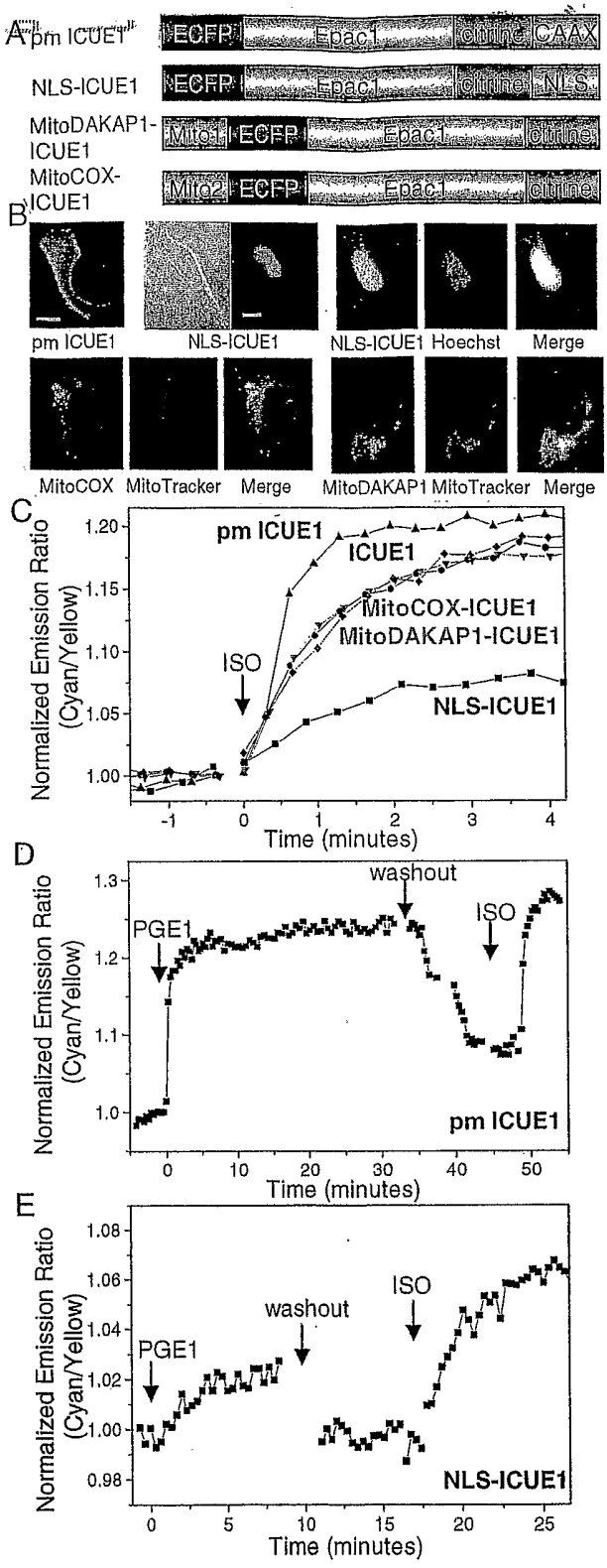

A
pm ICUE1
ECFP
Epac1
citrine
CAAX
NLS-ICUE1
NLS
MitoDAKAP1-ICUE1
Mito1
MitoCOX-ICUE1
Mito2
B
pm ICUE1
NLS-ICUE1
NLS-ICUE1
Hoechst
Merge
MitoCOX
MitoTracker
Merge
MitoDAKAP1
MitoTracker
Merge
C
Normalized Emission Ratio (Cyan/Yellow)
pm ICUE1
ICUE1
MitoCOX-ICUE1
MitoDAKAP1-ICUE1
NLS-ICUE1
ISO
Time (minutes)
D
Normalized Emission Ratio (Cyan/Yellow)
PGE1
washout
ISO
pm ICUE1
Time (minutes)
E
Normalized Emission Ratio (Cyan/Yellow)
PGE1
washout
ISO
NLS-ICUE1
Time (minutes)

Figure 3

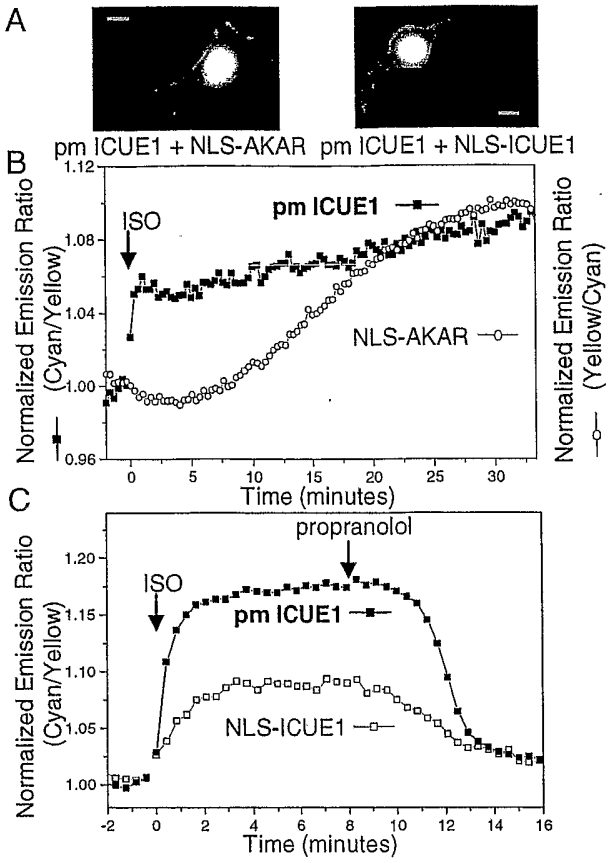

A
pm ICUE1 + NLS-AKAR
pm ICUE1 + NLS-ICUE1
B
Normalized Emission Ratio (Cyan/Yellow)
Normalized Emission Ratio (Yellow/Cyan)
ISO
pm ICUE1
NLS-AKAR
1.12
1.08
1.04
1.00
0.96
0
5
10
15
20
25
30
Time (minutes)
C
Normalized Emission Ratio (Cyan/Yellow)
propranolol
ISO
pm ICUE1
NLS-ICUE1
1.20
1.15
1.10
1.05
1.00
-2
0
2
4
6
8
10
12
14
16
Time (minutes)

Figure 4

FIGURE 5

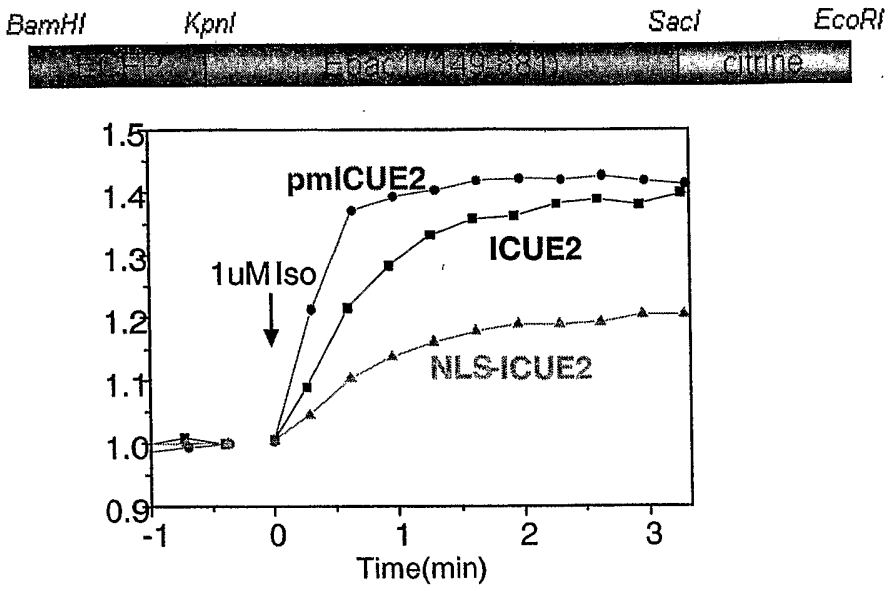
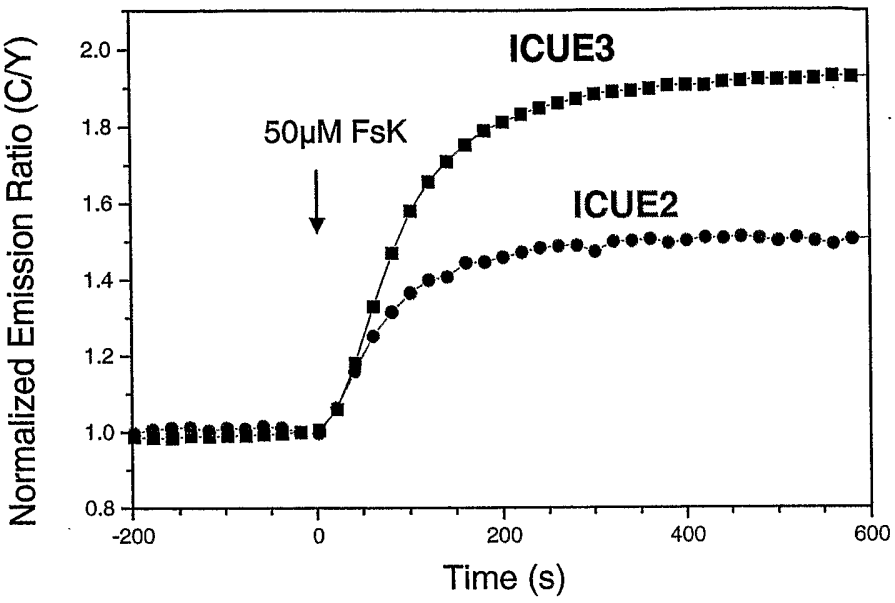

BamHI
KpnI
SacI
EcoRI
citrine
pmICUE2
ICUE2
NLS-ICUE2
1uM Iso
1.5
1.4
1.3
1.2
1.1
1.0
0.9
-1
0
1
2
3
Time(min)

FIGURE 6


ICUE3
50μM FsK
ICUE2
Normalized Emission Ratio (C/Y)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
-200
0
200
400
600
Time (s)

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<110> Zhang, Jin
DiPilato, Lisa Marie
The Johns Hopkins University

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Pro Leu Thr Asn Ser Glu Glu Ser Leu Asp Phe Ser Glu Ser Leu Glu
        35                  40                  45
Gln Ala Ser Thr Glu Arg Val Leu Arg Ala Gly Arg Gln Leu His Arg
    50                  55                  60
His Leu Leu Ala Thr Cys Pro Asn Leu Thr Arg Asp Arg Lys Tyr His
65                  70                  75                  80
Leu Arg Leu Tyr Arg Gln Cys Cys Ser Gly Arg Glu Leu Val Asp Gly
                85                  90                  95
Ile Leu Ala Leu Gly Leu Gly Val His Ser Arg Ser Gln Val Val Gly
            100                 105                 110
Ile Cys Gln Val Leu Leu Asp Glu Gly Ala Leu Cys His Val Lys His
        115                 120                 125
Asp Trp Ala Phe Gln Asp Arg Asp Ala Gln Phe Tyr Arg Phe Pro Gly
    130                 135                 140
Pro Glu Pro Glu Pro Val Gly Thr His Glu Met Glu Glu Glu Leu Ala
145                 150                 155                 160
Glu Ala Val Ala Leu Leu Ser Gln Arg Gly Pro Asp Ala Leu Leu Thr
                165                 170                 175
Val Ala Leu Arg Lys Pro Pro Gly Gln Arg Thr Asp Glu Glu Leu Asp
            180                 185                 190
Leu Ile Phe Glu Glu Leu Leu His Ile Lys Ala Val Ala His Leu Ser
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        195                 200                 205
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    210                 215                 220
Ser Lys Ala Gly Thr Val Leu Phe Ser Gln Gly Asp Lys Gly Thr Ser
225                 230                 235                 240
Trp Tyr Ile Ile Trp Lys Gly Ser Val Asn Val Val Thr His Gly Lys
                245                 250                 255
Gly Leu Val Thr Thr Leu His Glu Gly Asp Asp Phe Gly Gln Leu Ala
            260                 265                 270
Leu Val Asn Asp Ala Pro Arg Ala Ala Thr Ile Ile Leu Arg Glu Asp
        275                 280                 285
Asn Cys His Phe Leu Arg Val Asp Lys Gln Asp Phe Asn Arg Ile Ile
    290                 295                 300
Lys Asp Val Glu Ala Lys Thr Met Arg Leu Glu Glu His Gly Lys Val
305                 310                 315                 320
Val Leu Val Leu Glu Arg Ala Ser Gln Gly Ala Gly Pro Ser Arg Pro
                325                 330                 335
Pro Thr Pro Gly Arg Asn Arg Tyr Thr Val Met Ser Gly Thr Pro Glu
            340                 345                 350
Lys Ile Leu Glu Leu Leu Leu Glu Ala Met Gly Pro Asp Ser Ser Ala
        355                 360                 365
His Asp Pro Thr Glu Thr Phe Leu Ser Asp Phe Leu Leu Thr His Arg
    370                 375                 380
Val Phe Met Pro Ser Ala Gln Leu Cys Ala Ala Leu Leu His His Phe
385                 390                 395                 400
His Val Glu Pro Ala Gly Gly Ser Glu Gln Glu Arg Ser Thr Tyr Val
                405                 410                 415
Cys Asn Lys Arg Gln Gln Ile Leu Arg Leu Val Ser Gln Trp Val Ala
            420                 425                 430
Leu Tyr Gly Ser Met Leu His Thr Asp Pro Val Ala Thr Ser Phe Leu
        435                 440                 445
Gln Lys Leu Ser Asp Leu Val Gly Arg Asp Thr Arg Leu Ser Asn Leu
    450                 455                 460
Leu Arg Glu Gln Trp Pro Glu Arg Arg Arg Cys His Arg Leu Glu Asn
465                 470                 475                 480
Gly Cys Gly Asn Ala Ser Pro Gln Met Lys Ala Arg Asn Leu Pro Val
                485                 490                 495
Trp Leu Pro Asn Gln Asp Glu Pro Leu Pro Gly Ser Ser Cys Ala Ile
            500                 505                 510
Gln Val Gly Asp Lys Val Pro Tyr Asp Ile Cys Arg Pro Asp His Ser
        515                 520                 525
Val Leu Thr Leu Gln Leu Pro Val Thr Ala Ser Val Arg Glu Val Met
    530                 535                 540
Ala Ala Leu Ala Gln Glu Asp Gly Trp Thr Lys Gly Gln Val Leu Val
545                 550                 555                 560
Lys Val Asn Ser Ala Gly Asp Ala Ile Gly Leu Gln Pro Asp Ala Arg
                565                 570                 575
Gly Val Ala Thr Ser Leu Gly Leu Asn Glu Arg Leu Phe Val Val Asn
            580                 585                 590
Pro Gln Glu Ala His Glu Leu Ile Pro His Pro Asp Gln Leu Gly Pro
        595                 600                 605
Thr Val Gly Ser Ala Glu Gly Leu Asp Leu Val Ser Ala Lys Asp Leu
    610                 615                 620
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625                 630                 635                 640
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Val Thr Thr Ala Asn Leu Glu Arg Phe Met Arg Arg Phe Asn Glu Leu
            660                 665                 670
Gln Tyr Trp Val Ala Thr Glu Leu Cys Leu Cys Pro Val Pro Gly Pro
        675                 680                 685
Arg Ala Gln Leu Leu Arg Lys Phe Ile Lys Leu Ala Ala His Leu Lys
    690                 695                 700
Glu Gln Lys Asn Leu Asn Ser Phe Phe Ala Val Met Phe Gly Leu Ser
705                 710                 715                 720
Asn Ser Ala Ile Ser Arg Leu Ala His Thr Trp Glu Arg Leu Pro His
                725                 730                 735
Lys Val Arg Lys Leu Tyr Ser Ala Leu Glu Arg Leu Leu Asp Pro Ser
            740                 745                 750
Trp Asn His Arg Val Tyr Arg Leu Ala Leu Ala Lys Leu Ser Pro Pro
        755                 760                 765
Val Ile Pro Phe Met Pro Leu Leu Leu Lys Asp Met Thr Phe Ile His
    770                 775                 780
Glu Gly Asn His Thr Leu Val Glu Asn Leu Ile Asn Phe Glu Lys Met
785                 790                 795                 800
Arg Met Met Ala Arg Ala Ala Arg Met Leu His His Cys Arg Ser His
                805                 810                 815
Asn Pro Val Pro Leu Ser Pro Leu Arg Ser Arg Val Ser His Leu His
            820                 825                 830
Glu Asp Ser Gln Val Ala Arg Ile Ser Thr Cys Ser Glu Gln Ser Leu
        835                 840                 845
Ser Thr Arg Ser Pro Ala Ser Thr Trp Ala Tyr Val Gln Gln Leu Lys
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ctggatttca gcgagagcct ggagcaggcc tccacagagc gggtgctcag ggctgggagg     180
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atcaaggctg tggcccacct ctccaactcg gtgaagcgag aattagcggc tgttctgctc     660
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Pro Asn Leu Leu Arg Gln Ile Cys Leu Cys Gly Tyr Tyr Glu Asn Leu
    50                  55                  60
Glu Lys Gly Ile Thr Leu Phe Arg Gln Gly Asp Ile Gly Thr Asn Trp
65                  70                  75                  80
Tyr Ala Val Leu Ala Gly Ser Leu Asp Val Lys Val Ser Glu Thr Ser
                85                  90                  95
Ser His Gln Asp Ala Val Thr Ile Cys Thr Leu Gly Ile Gly Thr Ala
```

```
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Pro Tyr Gly Val Met Glu Thr Gly Ser Asn Asn Asp Arg Ile Pro Asp
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Lys Glu Asn Val Pro Ser Glu Lys Ile Leu Arg Ala Gly Lys Ile Leu
            180                 185                 190
Arg Ile Ala Ile Leu Ser Arg Ala Pro His Met Ile Arg Asp Arg Lys
        195                 200                 205
Tyr His Leu Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr Glu Leu Val
    210                 215                 220
Asp Trp Met Ile Gln Gln Thr Ser Cys Val His Ser Arg Thr Gln Ala
225                 230                 235                 240
Val Gly Met Trp Gln Val Leu Leu Glu Asp Gly Val Leu Asn His Val
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Asp Gln Glu Arg His Phe Gln Asp Lys Tyr Leu Phe Tyr Arg Phe Leu
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        275                 280                 285
Glu Cys Asp Glu Glu Leu Gln Asp Thr Met Leu Leu Leu Ser Gln Met
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Gly Pro Asp Ala His Met Arg Met Ile Leu Arg Lys Pro Pro Gly Gln
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Arg Thr Val Asp Asp Leu Glu Ile Ile Tyr Asp Glu Leu Leu His Ile
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Lys Ala Leu Ser His Leu Ser Thr Thr Val Lys Arg Glu Leu Ala Gly
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Gln Lys Arg Gln Pro Ile Arg Gly Ser Asp Glu Val Leu Phe Lys Val
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Tyr Cys Ile Asp His Thr Tyr Thr Thr Ile Arg Val Pro Val Ala Ala
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Leu Lys Ser Asn Asp Val Ser Val Phe Thr Thr Leu Thr Ile Asn Gly
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Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu Thr Pro Leu
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Pro Glu Gln Glu Gly Pro Thr Thr Gly Thr Val Gly Thr Phe Glu Leu
            740                 745                 750
Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Thr Tyr Asp Trp Glu
        755                 760                 765
Leu Phe Asn Cys Val His Glu Leu Glu Leu Ile Tyr His Thr Phe Gly
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Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu Phe Leu Arg
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Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Phe Ile Lys Ile
            820                 825                 830
Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe Ala Ile
        835                 840                 845
Val Met Gly Leu Ser Asn Val Ala Val Ser Arg Leu Ala Leu Thr Trp
    850                 855                 860
Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe Glu Ser
865                 870                 875                 880
Leu Met Asp Pro Ser Arg Asn His Arg Ala Tyr Arg Leu Thr Ala Ala
                885                 890                 895
Lys Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu Ile Lys Asp
            900                 905                 910
Met Thr Phe Thr His Glu Gly Asn Lys Thr Phe Ile Asp Asn Leu Val
        915                 920                 925
Asn Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg Thr Val Arg
    930                 935                 940
Tyr Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln Ala Asn Lys
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Pro

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gcggtgacca tctgcactct gggaattggg acagcctttg gagagtccat tctggataac 360
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ccttcagaga agatcctcag agctggaaaa attttacgaa ttgccattct ctctcgagct 600
ccccacatga taagagacag aaagtaccac ctaaagacat acagacaatg ctgtgttggg 660
actgagctgg tagactggat gatacagcag acatcctgtg ttcactcgcg gactcaagct 720
gttggcatgt ggcaagtctt gctggaagat ggtgtcctca accatgtgga ccaggagcgc 780
catttccaag acaaatattt attttatcga tttctggatg acgagcgtga ggatgcccct 840
ttgcctactg aggaagagaa gaaggagtgt gatgaagaac ttcaggacac catgctgctg 900
ctctcacaga tgggccctga cgcccacatg agaatgatcc tgcgaaaacc acctggccag 960
aggactgtgg atgacctaga gattatctac gacgagctcc ttcatattaa agccttatcc 1020
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aaaggaggaa ctgtgttgtt taaccagggg gaagaaggta cctcctggta catcattctg 1140
aaaggatccg tgaatgtagt catttatggc aagggtgtgg tctgcaccct gcacgaagga 1200
gatgactttg gcaagttagc tctagtgaac gatgctccaa gagctgcctc cattgttctt 1260
cgggaagata attgtcactt cctaagagtc gacaaggaag acttcaatcg gattctgagg 1320
gacgttgagg cgaatacagt cagacttaaa gaacatgacc aagatgtctt ggtactggag 1380
aaggtcccag cagggaacag agctgctaat caaggaaact cacagcctca gcaaaagtat 1440
actgtgatgt caggaacacc tgaaaagatt ttagagcatt ttctagaaac aatacgcctt 1500
gagccatcgt tgaatgaagc aacagattcg gttttaaatg actttgttat gatgcactgt 1560
gtttttatgc caaataccca gctttgccct gcccttgtgg cccattacca cgcacagcct 1620
tctcaaggta ccgagcagga gagaatggat tatgccctca acaacaagag gcgggtcatc 1680
cgcttggtcc tgcagtgggc ggccatgtat ggcgatctcc tccaagaaga tgatgtggcc 1740
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ttcaaggagc agctgccaga gctggagaag attgtcaagc aaatctcaga agacgcaaaa 1860
gctccacaga agaagcacaa ggtgcttttg caacagttca acacaggtga cgagagggcc 1920
cagaagcgtc agcctattcg tggctctgat gaggttttgt tcaaggtcta ctgcatcgac 1980
cacacctata ctaccattcg tgtgccggta gctgcctcgg tgaaggaagt catcagtgca 2040
gtagctgaca aactgggctc aggggaaggc ctgatcatcg tcaagatgaa ctctggagga 2100
gaaaaggtgg tgctgaaatc taatgatgtt tcagtattta cgacgctcac cattaatgga 2160
cgcctgtttg cctgcccgag agagcaattc gactcactga ctcccttgcc ggaacaggaa 2220
ggcccgacca ctgggacagt gggaacattt gagctgatga gctcgaaaga cctggcgtac 2280
cagatgacaa cctacgattg ggaactcttc aactgtgtgc atgagctgga gctaatctac 2340

cacacatttg gaaggcataa ttttaaaaag accacggcaa acttggattt gttcctgagg 2400
aggtttaatg aaattcagtt ttgggttgtc actgaggtct gcctttgttc ccagctcagc 2460
aaacgtgttc agcttttgaa aaaatttatc aagatagcgg ctcactgcaa ggagtacaaa 2520
aatctaaatt ccttttcgc catcgtcatg ggactcagca acgtggccgt gagccgcttg 2580
gcactaacgt gggagaaact gccgagcaag tttaagaagt tctatgcgga gtttgagagc 2640
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cctctcatcc ctttcatgcc cttgcttatt aaagatatga catttactca tgaggggaac 2760
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agaacagtac ggtactacag gagccagccc ttcaatccgg atgccgctca agctaataag 2880
aaccatcagg atgtccggag ttatgtacgg caattaaatg tgattgacaa ccagagaact 2940
ttatcacaga tgtcacacag attagagcct cgaaggccat ag 2982

<210> 5
<211> 10
<212> PRT
<213> Homo sapiens

<220>
<223> peptide linker

<400> 5
Pro Lys Lys Lys Arg Lys Val Glu Asp Ala
1 5 10

<210> 6
<211> 13
<212> PRT
<213> Homo sapiens

<400> 6
Lys Lys Lys Lys Lys Ser Lys Thr Lys Cys Val Ile Met
1 5 10

<210> 7
<211> 30
<212> PRT
<213> Homo sapiens

<400> 7
Met Ala Ile Gln Leu Arg Ser Leu Phe Pro Leu Ala Leu Pro Gly Met
1 5 10 15
Leu Ala Leu Leu Gly Trp Trp Trp Phe Phe Ser Arg Lys Lys
20 25 30

<210> 8
<211> 16
<212> PRT
<213> Homo sapiens

<400> 8
Met Leu Ser Leu Arg Gly Ser Ile Arg Phe Phe Lys Arg Ser Gly Ile
1 5 10 15

<210> 9
<211> 225
<212> PRT
<213> Discosoma sp.

<400> 9
Met Arg Ser Ser Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys Val
1 5 10 15
Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Glu Gly Glu
20 25 30
Gly Glu Gly Arg Pro Tyr Glu Gly His Asn Thr Val Lys Leu Lys Val
35 40 45
Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp Ile Leu Ser Pro Gln
50 55 60
Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His Pro Ala Asp Ile Pro
65 70 75 80
Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly Phe Lys Trp Glu Arg Val
85 90 95
Met Asn Phe Glu Asp Gly Gly Val Val Thr Val Thr Gln Asp Ser Ser
100 105 110
Leu Gln Asp Gly Cys Phe Ile Tyr Lys Val Lys Phe Ile Gly Val Asn
115 120 125
Phe Pro Ser Asp Gly Pro Val Met Gln Lys Lys Thr Met Gly Trp Glu
130 135 140
Ala Ser Thr Glu Arg Leu Tyr Pro Arg Asp Gly Val Leu Lys Gly Glu
145 150 155 160
Ile His Lys Ala Leu Lys Leu Lys Asp Gly Gly His Tyr Leu Val Glu
165 170 175
Phe Lys Ser Ile Tyr Met Ala Lys Lys Pro Val Gln Leu Pro Gly Tyr
180 185 190
Tyr Tyr Val Asp Ser Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr
195 200 205
Thr Ile Val Glu Gln Tyr Glu Arg Thr Glu Gly Arg His His Leu Phe
210 215 220
Leu
225

<210> 10
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> peptide linker

<400> 10
Gly Ser Thr Ser Gly Ser Gly Lys Pro Gly Ser Gly Glu Gly Ser Thr
1 5 10 15
Lys Gly

<210> 11
<211> 238

<212> PRT
<213> Aequorea victoria

<400> 11
Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu Val
1 5 10 15
Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly Glu
20 25 30
Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile Cys
35 40 45
Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr Phe
50 55 60
Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys Gln
65 70 75 80
His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu Arg
85 90 95
Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu Val
100 105 110
Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly Ile
115 120 125
Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr Asn
130 135 140
Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly
145 150 155 160
Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val
165 170 175
Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro
180 185 190
Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser
195 200 205
Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val
210 215 220
Thr Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
225 230 235

<210> 12
<211> 239
<212> PRT
<213> Aequorea victoria

<400> 12
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60
Leu Thr Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65 70 75 80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95

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Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145                 150                 155                 160
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
        195                 200                 205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225                 230                 235
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<210> 13
<211> 239
<212> PRT
<213> Aequorea victoria

<400> 13

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Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
 1               5                  10                  15
Val Glu Leu Asp Gly Asp Val Asn Gly His Arg Phe Ser Val Ser Gly
            20                  25                  30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
        35                  40                  45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
    50                  55                  60
Leu Thr Trp Gly Val Gln Cys Phe Ser Arg Tyr Pro Asp His Met Lys
65                  70                  75                  80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
                85                  90                  95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
            100                 105                 110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
        115                 120                 125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
    130                 135                 140
Asn Tyr Ile Ser His Asn Val Tyr Ile Thr Ala Asp Lys Gln Lys Asn
145                 150                 155                 160
Gly Ile Lys Ala His Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
                165                 170                 175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
            180                 185                 190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu
        195                 200                 205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
    210                 215                 220
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Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225 230 235

<210> 14
<211> 239
<212> PRT
<213> Aequorea victoria

<400> 14
Met Val Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
1 5 10 15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser Gly
20 25 30
Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys Phe Ile
35 40 45
Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu Val Thr Thr
50 55 60
Phe Gly Tyr Gly Leu Lys Cys Phe Ala Arg Tyr Pro Asp His Met Lys
65 70 75 80
Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu Gly Tyr Val Gln Glu
85 90 95
Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn Tyr Lys Thr Arg Ala Glu
100 105 110
Val Lys Phe Glu Gly Asp Thr Leu Val Asn Arg Ile Glu Leu Lys Gly
115 120 125
Ile Asp Phe Lys Glu Asp Gly Asn Ile Leu Gly His Lys Leu Glu Tyr
130 135 140
Asn Tyr Asn Ser His Asn Val Tyr Ile Met Ala Asp Lys Gln Lys Asn
145 150 155 160
Gly Ile Lys Val Asn Phe Lys Ile Arg His Asn Ile Glu Asp Gly Ser
165 170 175
Val Gln Leu Ala Asp His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly
180 185 190
Pro Val Leu Leu Pro Asp Asn His Tyr Leu Ser Tyr Gln Ser Ala Leu
195 200 205
Ser Lys Asp Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe
210 215 220
Val Thr Ala Ala Gly Ile Thr Leu Gly Met Asp Glu Leu Tyr Lys
225 230 235

<210> 15
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> tetracysteine motif

<221> VARIANT
<222> (1)...(6)
<223> Xaa = Any Amino Acid

<221> VARIANT

<222> (1)...(6)
<223> Xaa = Any Amino Acid

<221> VARIANT
<222> (1)...(6)
<223> Xaa = Any Amino Acid

<221> VARIANT
<222> (1)...(6)
<223> Xaa = Any Amino Acid

<400> 15
Cys Cys Xaa Xaa Cys Cys
1 5

<210> 16
<211> 31
<212> DNA
<213> Homo sapiens

<400> 16
cgcggtaccc ccgtgggaac tcatgagatg g 31

<210> 17
<211> 436
<212> PRT
<213> Rattus norvegicus

<220>
<221> misc_feature
<222> (1)...(0)
<223> n = A,T,C or G

<400> 17
Arg Val Ile Arg Leu Val Leu Gln Trp Ala Ala Met Tyr Gly Asp Leu
1 5 10 15
Leu Gln Glu Asp Asp Val Ala Met Ala Phe Leu Glu Glu Phe Tyr Val
20 25 30
Ser Val Ser Asp Asp Ala Arg Met Met Val Ala Phe Lys Glu Gln Leu
35 40 45
Ala Glu Leu Glu Lys Thr Val Lys Gln Ile Ser Glu Asp Ala Lys Ala
50 55 60
Pro Gln Lys Lys His Lys Val Leu Leu Gln Gln Phe Asn Thr Gly Asp
65 70 75 80
Glu Arg Ala Gln Lys Arg Gln Pro Ile Arg Gly Ser Asp Glu Val Leu
85 90 95
Phe Lys Val Tyr Cys Ile Asp His Thr Asp Thr Thr Ile Arg Val Pro
100 105 110
Val Ala Ala Ser Val Lys Glu Val Ile Ser Ala Val Ala Asp Lys Leu
115 120 125
Gly Ser Gly Glu Gly Leu Ile Ile Val Lys Met Asn Ser Gly Gly Glu
130 135 140
Lys Val Val Leu Lys Pro Asn Asp Val Ser Val Phe Thr Thr Leu Thr

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145                 150                 155                 160
Ile Asn Gly Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu
                165                 170                 175
Thr Pro Leu Pro Glu Gln Glu Gly Pro Thr Thr Gly Thr Val Gly Thr
            180                 185                 190
Phe Glu Leu Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Thr Tyr
        195                 200                 205
Asp Trp Glu Leu Phe Asn Cys Val Leu Glu Leu Glu Leu Ile Tyr His
    210                 215                 220
Thr Phe Gly Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu
225                 230                 235                 240
Phe Leu Arg Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile
                245                 250                 255
Cys Leu Cys Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Cys
            260                 265                 270
Ile Lys Ile Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe
        275                 280                 285
Phe Gly Ile Val Met Gly Leu Ser Asn Val Ala Glu Ser Arg Leu Ala
    290                 295                 300
Leu Thr Trp Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu
305                 310                 315                 320
Phe Glu Ser Leu Met Asp Pro Ser Arg Asn His Lys Ala Tyr Arg Leu
                325                 330                 335
Thr Ala Ala Lys Leu Glu Pro Pro Leu Ile Pro Phe Met Pro Leu Leu
            340                 345                 350
Ile Lys Asp Met Thr Phe Thr His Glu Gly Asn Lys Thr Phe Ile Asp
        355                 360                 365
Asn Leu Val Asn Phe Glu Lys Met Arg Met Ile Ala Asn Thr Ala Arg
    370                 375                 380
Thr Val Arg Tyr Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln
385                 390                 395                 400
Ala Asn Lys Asn His Gln Asp Val Arg Ser Tyr Val Arg Gln Leu Asn
                405                 410                 415
Val Ile Asp Asn Gln Arg Thr Leu Ser Gln Met Ser His Arg Leu Glu
            420                 425                 430
Pro Arg Arg Pro
        435
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<210> 18
<211> 931
<212> PRT
<213> Pongo pygmaeus

<400> 18

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Met Val Ala Thr His Ala Ala His Ser Ser Ser Ser Ala Glu Trp Ile
 1               5                  10                  15
Ala Cys Leu Asp Lys Arg Pro Leu Glu Arg Ser Ser Glu Asp Val Asp
            20                  25                  30
Ile Ile Phe Thr Arg Leu Lys Glu Val Lys Ala Phe Glu Lys Phe His
        35                  40                  45
Pro Asn Leu Leu His Gln Ile Cys Leu Cys Gly Tyr Tyr Glu Asn Leu
    50                  55                  60
Glu Lys Gly Ile Thr Leu Phe Arg Gln Gly Asp Ile Gly Thr Asn Trp
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65 70 75 80
Tyr Ala Val Leu Ala Gly Ser Leu Asp Val Lys Val Ser Glu Thr Ser
85 90 95
Ser His Gln Asp Ala Val Thr Ile Cys Thr Leu Gly Ile Gly Thr Ala
100 105 110
Phe Gly Glu Ser Ile Leu Asp Asn Thr Pro Arg His Ala Thr Ile Val
115 120 125
Thr Arg Glu Ser Ser Glu Leu Leu Arg Ile Glu Gln Lys Asp Phe Lys
130 135 140
Ala Leu Trp Glu Lys Tyr Arg Gln Tyr Met Ala Gly Leu Leu Ala Pro
145 150 155 160
Pro Tyr Gly Val Met Glu Thr Gly Ser Asn Asn Asp Arg Ile Pro Asp
165 170 175
Lys Glu Asn Val Pro Ser Glu Lys Ile Leu Arg Ala Gly Lys Ile Leu
180 185 190
Arg Asn Ala Ile Leu Ser Arg Ala Pro His Met Ile Arg Asp Arg Lys
195 200 205
Tyr His Leu Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr Glu Leu Val
210 215 220
Asp Trp Met Met Gln Gln Thr Pro Cys Val His Ser Arg Thr Gln Ala
225 230 235 240
Val Gly Met Trp Gln Val Leu Leu Glu Asp Gly Val Leu Asn His Val
245 250 255
Asp Gln Glu His His Phe Gln Asp Lys Tyr Leu Phe Tyr Arg Phe Leu
260 265 270
Asp Asp Glu Asn Glu Asp Ala Pro Leu Pro Thr Glu Glu Glu Lys Lys
275 280 285
Glu Cys Asp Glu Glu Leu Gln Asp Thr Met Leu Leu Leu Ser Gln Met
290 295 300
Gly Pro Asp Ala His Met Arg Met Ile Leu Arg Lys Pro Pro Gly Gln
305 310 315 320
Arg Thr Val Asp Asp Leu Glu Ile Ile Tyr Glu Glu Leu Leu His Ile
325 330 335
Lys Ala Leu Ser His Leu Ser Thr Thr Val Lys Arg Glu Leu Ala Gly
340 345 350
Val Leu Ile Phe Glu Ser His Ala Lys Gly Gly Thr Val Leu Phe Asn
355 360 365
Gln Gly Glu Glu Gly Thr Ser Trp Tyr Ile Ile Leu Lys Gly Ser Val
370 375 380
Asn Val Val Ile Tyr Gly Lys Gly Val Val Cys Thr Leu His Glu Gly
385 390 395 400
Asp Asp Phe Gly Lys Leu Ala Leu Val Asn Asp Ala Pro Arg Ala Ala
405 410 415
Ser Ile Val Leu Arg Glu Asp Asn Cys His Phe Leu Arg Val Asp Lys
420 425 430
Glu Asp Phe Asn Arg Ile Leu Arg Asp Val Glu Ala Asn Thr Val Arg
435 440 445
Leu Lys Glu His Asp Gln Asp Val Leu Val Leu Glu Lys Val Pro Ala
450 455 460
Gly Asn Arg Ala Ser Asn Gln Gly Asn Ser Gln Pro Gln Gln Lys Tyr
465 470 475 480
Thr Val Met Ser Gly Thr Pro Glu Lys Ile Leu Glu His Phe Leu Glu
485 490 495

```
Thr Ile Arg Leu Glu Ala Thr Leu Asn Glu Ala Thr Asp Ser Val Leu
                500                 505                 510
Asn Asp Phe Ile Met Met His Cys Val Phe Met Pro Asn Thr Gln Leu
            515                 520                 525
Cys Pro Ala Leu Val Ala His Tyr His Ala Gln Pro Ser Gln Gly Thr
        530                 535                 540
Glu Gln Glu Lys Met Asp Tyr Ala Leu Asn Asn Lys Arg Arg Val Ile
545                 550                 555                 560
Arg Leu Val Leu Gln Trp Ala Ala Met Tyr Gly Asp Leu Leu Gln Glu
                565                 570                 575
Asp Asp Val Ser Met Ala Phe Leu Glu Glu Phe Tyr Val Ser Val Ser
            580                 585                 590
Asp Asp Ala Arg Met Ile Ala Ala Leu Lys Glu Gln Leu Pro Glu Leu
        595                 600                 605
Glu Lys Ile Val Lys Gln Ile Ser Glu Asp Ala Lys Ala Pro Gln Lys
    610                 615                 620
Lys His Lys Val Leu Leu Gln Gln Phe Asn Thr Gly Asp Glu Arg Ala
625                 630                 635                 640
Gln Lys Arg Gln Pro Ile Arg Gly Ser Asp Glu Val Leu Phe Lys Val
                645                 650                 655
Tyr Cys Met Asp His Thr Tyr Thr Thr Ile Arg Val Pro Val Ala Ala
            660                 665                 670
Ser Val Lys Glu Val Ile Ser Ala Val Ala Asp Lys Leu Gly Ser Gly
        675                 680                 685
Glu Gly Leu Ile Val Val Lys Met Ser Ser Gly Gly Glu Lys Val Val
    690                 695                 700
Leu Lys Pro Asn Asp Val Ser Val Phe Thr Thr Leu Thr Ile Asn Gly
705                 710                 715                 720
Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu Thr Pro Leu
                725                 730                 735
Pro Glu Gln Glu Gly Pro Thr Val Gly Thr Val Gly Thr Phe Glu Leu
            740                 745                 750
Met Ser Ser Lys Asp Leu Ala Tyr Gln Met Thr Ile Tyr Asp Trp Glu
        755                 760                 765
Leu Phe Asn Cys Val His Glu Leu Glu Leu Ile Tyr His Thr Phe Gly
    770                 775                 780
Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu Phe Leu Arg
785                 790                 795                 800
Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile Cys Leu Cys
                805                 810                 815
Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Phe Ile Lys Ile
            820                 825                 830
Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe Phe Ala Ile
        835                 840                 845
Ala Met Gly Leu Ser Asn Val Ala Val Ser Arg Leu Ala Leu Thr Trp
    850                 855                 860
Glu Lys Leu Pro Ser Lys Phe Lys Lys Phe Tyr Ala Glu Phe Glu Ser
865                 870                 875                 880
Leu Met Asp Pro Ser Arg Asn His Arg Ala Tyr Arg Leu Thr Val Ala
                885                 890                 895
Lys Leu Glu Pro Pro Val Ile Pro Phe Met Pro Leu Leu Ile Lys Ala
            900                 905                 910
His Asp Cys Lys Tyr Gly Gln Asn Ser Glu Ile Leu Gln Glu Pro Thr
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915 920 925
Leu Gln Ser
930

<210> 19
<211> 944
<212> PRT
<213> Pongo pygmaeus

<400> 19
Pro Met Val Ala Ala His Ala Ala His Ser Ser Ser Ser Ala Glu Trp
1 5 10 15
Ile Ala Cys Leu Asp Lys Arg Pro Leu Glu Arg Ser Ser Glu Asp Val
20 25 30
Asp Ile Ile Phe Thr Arg Leu Lys Glu Val Lys Ala Phe Glu Lys Phe
35 40 45
His Pro Asn Leu Leu His Gln Ile Cys Leu Cys Gly Tyr Tyr Glu Asn
50 55 60
Leu Glu Lys Gly Ile Thr Leu Phe Arg Gln Gly Asp Ile Gly Thr Asn
65 70 75 80
Trp Tyr Ala Val Leu Ala Gly Ser Leu Asp Val Lys Val Ser Glu Thr
85 90 95
Ser Ser His Gln Asp Ala Val Thr Ile Cys Thr Leu Gly Ile Gly Thr
100 105 110
Ala Phe Gly Glu Ser Ile Leu Asp Asn Thr Pro Arg His Ala Thr Ile
115 120 125
Val Thr Arg Glu Ser Ser Glu Leu Leu Arg Ile Glu Gln Lys Asp Phe
130 135 140
Lys Ala Leu Trp Glu Lys Tyr Arg Gln Tyr Met Ala Gly Leu Leu Ala
145 150 155 160
Pro Pro Tyr Gly Val Met Glu Thr Gly Ser Asn Asn Asp Arg Ile Pro
165 170 175
Asp Lys Glu Asn Thr Pro Leu Ile Glu Pro His Val Pro Leu Arg Pro
180 185 190
Ala Asn Thr Ile Thr Lys Val Pro Ser Glu Lys Ile Leu Arg Ala Gly
195 200 205
Lys Ile Leu Arg Asn Ala Ile Leu Ser Arg Ala Pro His Met Ile Arg
210 215 220
Asp Arg Lys Tyr His Leu Lys Thr Tyr Arg Gln Cys Cys Val Gly Thr
225 230 235 240
Glu Leu Val Asp Trp Met Met Gln Gln Thr Pro Cys Val His Ser Arg
245 250 255
Thr Gln Ala Val Gly Met Trp Gln Val Leu Leu Glu Asp Gly Val Leu
260 265 270
Asn His Val Asp Gln Glu His His Phe Gln Asp Lys Tyr Leu Phe Tyr
275 280 285
Arg Phe Leu Asp Asp Glu Asn Glu Asp Ala Pro Leu Pro Thr Glu Glu
290 295 300
Glu Lys Lys Glu Cys Asp Glu Glu Leu Gln Asp Thr Met Leu Leu Leu
305 310 315 320
Ser Gln Met Gly Pro Asp Ala His Met Arg Met Ile Leu Arg Lys Pro
325 330 335
Pro Gly Gln Arg Thr Val Asp Asp Leu Glu Ile Ile Tyr Glu Glu Leu

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            340                 345                 350
Leu His Thr Lys Ala Leu Ser His Leu Ser Thr Thr Val Lys Arg Glu
        355                 360                 365
Leu Ala Gly Val Leu Ile Phe Glu Ser His Ala Lys Gly Gly Thr Val
    370                 375                 380
Leu Phe Asn Gln Gly Glu Glu Gly Thr Ser Trp Tyr Ile Ile Leu Lys
385                 390                 395                 400
Gly Ser Val Asn Val Val Ile Tyr Gly Lys Gly Val Val Cys Thr Leu
                405                 410                 415
His Glu Gly Asp Asp Phe Gly Lys Leu Ala Leu Val Asn Asp Ala Pro
            420                 425                 430
Arg Ala Ala Ser Ile Val Leu Arg Glu Asp Asn Cys His Phe Leu Arg
        435                 440                 445
Val Asp Lys Glu Asp Phe Asn Arg Ile Leu Arg Asp Val Glu Ala Asn
    450                 455                 460
Thr Val Arg Leu Lys Glu His Asp Gln Asp Val Leu Val Leu Glu Lys
465                 470                 475                 480
Val Pro Ala Gly Asn Arg Ala Ser Asn Gln Gly Asn Ser Gln Pro Gln
                485                 490                 495
Gln Lys Tyr Thr Val Met Ser Gly Thr Pro Glu Lys Ile Leu Glu His
            500                 505                 510
Phe Leu Glu Thr Ile Arg Leu Glu Ala Thr Leu Asn Glu Ala Thr Asp
        515                 520                 525
Ser Val Leu Asn Asp Phe Ile Met Met His Cys Val Phe Met Pro Asn
    530                 535                 540
Thr Gln Leu Cys Pro Ala Leu Val Ala His Tyr His Ala Gln Pro Ser
545                 550                 555                 560
Gln Gly Thr Glu Gln Glu Lys Met Asp Tyr Ala Leu Asn Asn Lys Arg
                565                 570                 575
Arg Val Ile Arg Leu Val Leu Gln Trp Ala Ala Met Tyr Gly Asp Pro
            580                 585                 590
Leu Gln Glu Asp Asp Val Ser Met Ala Phe Leu Glu Glu Phe Tyr Val
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Ser Val Ser Asp Asp Ala Arg Met Ile Ala Ala Leu Lys Glu Gln Leu
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Pro Glu Leu Glu Lys Ile Val Lys Gln Ile Ser Glu Asp Ala Lys Ala
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Pro Gln Lys Lys His Lys Val Leu Leu Gln Gln Phe Asn Thr Gly Asp
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Glu Arg Ala Gln Lys Arg Gln Pro Ile Arg Gly Ser Asp Glu Val Leu
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Phe Lys Val Tyr Cys Met Asp His Thr Tyr Thr Thr Ile Arg Val Pro
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Val Ala Ala Ser Val Lys Glu Val Ile Ser Ala Val Ala Asp Lys Leu
    690                 695                 700
Gly Ser Gly Glu Gly Leu Ile Val Val Lys Met Ser Ser Gly Gly Glu
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Lys Val Val Leu Lys Pro Asn Asp Val Ser Val Phe Thr Thr Leu Thr
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Ile Asn Gly Arg Leu Phe Ala Cys Pro Arg Glu Gln Phe Asp Ser Leu
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Thr Pro Leu Pro Glu Gln Glu Gly Pro Thr Val Gly Thr Val Gly Thr
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Asp Trp Glu Leu Phe Asn Cys Val His Glu Leu Glu Leu Ile Tyr His
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Thr Phe Gly Arg His Asn Phe Lys Lys Thr Thr Ala Asn Leu Asp Leu
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Phe Leu Arg Arg Phe Asn Glu Ile Gln Phe Trp Val Val Thr Glu Ile
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Cys Leu Cys Ser Gln Leu Ser Lys Arg Val Gln Leu Leu Lys Lys Phe
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Ile Lys Ile Ala Ala His Cys Lys Glu Tyr Lys Asn Leu Asn Ser Phe
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Phe Ala Ile Ala Met Gly Leu Ser Asn Val Ala Val Ser Arg Leu Ala
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Leu Thr Trp Glu Arg Met Ile Ala Asn Thr Ala Arg Thr Val Arg Tyr
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Tyr Arg Ser Gln Pro Phe Asn Pro Asp Ala Ala Gln Ala Asn Lys Asn
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His Gln Asp Val Trp Ser Tyr Val Arg Gln Leu Asn Val Ile Asp Asn
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Tyr Ala Val Leu Ala Gly Ser Leu Asp Val Lys Val Ser Glu Thr Ser
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Ser His Gln Asp Ala Val Thr Ile Cys Thr Leu Gly Ile Gly Thr Ala
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Phe Gly Glu Ser Ile Leu Asp Asn Thr Pro Arg His Ala Thr Ile Val
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Thr Arg Glu Ser Ser Glu Leu Leu Arg Ile Glu Gln Glu Asp Phe Lys
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Lys Glu Asn Thr Pro Leu Ile Glu Pro His Val Pro Leu Arg Pro Ala
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