PHOTOPROTEINS WITH ENHANCED BIOLUMINESCENCE AND THEIR USE AS INTRACELLULAR CALCIUM INDICATORS

The present invention relates to photoproteins with enhanced bioluminescence and to their use as intracellular calcium indicators. The photoproteins are obtained by mutagenesis of the clytin coding sequence and show enhanced bioluminescence, high affinity for calcium and long-lasting light emission. They are conveniently used in cell-based assays to determine variations of intracellular calcium concentration, particularly in assays for the screening of molecules with high and ultra-high-throughput techniques.

BACKGROUND OF THE INVENTION

Bioluminescence is the phenomenon by which visible light is emitted by living organisms or by a substance derived from them through a variety of chemiluminescent reaction systems. Bioluminescence reactions require three major components: a luciferin, a luciferase and molecular oxygen. However, other components may also be required in some reactions, including cations (Ca^{++} and Mg^{++}) and cofactors (ATP, NAD(P)H). Luciferases are enzymes that catalyse the oxidation of a substrate, luciferin, and produce an unstable intermediate. Light is emitted when the unstable intermediate decays to its ground state, generating oxyluciferin. There are many different unrelated types of luciferin, although many species from at least seven phyla use the same luciferin, known as coelenterazine. In some animals (e.g. jellyfish) the luciferin/luciferase system can be extracted in the form of a stable "photoprotein" which emits light upon calcium binding. Photoproteins differ from luciferases in that they are stabilized oxygenated intermediate complexes of luciferase and luciferin. Photoproteins are present in many marine coelenterates and allow these organisms to emit light for a variety of purposes including breeding, feeding and defense (1). There are many luminescent

organisms, but only seven photoproteins, namely Thalassicolin (2,3), Aequorin (4-6), Mitrocomin (syn. with Halistaurin) (7,8), Clytin (syn. with Phialidin) (8,9), Obelin (2,6,10,11), Mnemiopsin (12,13) and Berovin (12,13) have been isolated so far. All these proteins are complexes formed by an apoprotein, an imidazopyrazine chromophore (coelenterazine) and oxygen. Their structures are highly conserved, especially in the region containing the three calcium binding sites (EF-hand structures). The term "photoprotein" identifies the luciferin-bound polypeptide, which is capable of luminescence, while "apophotoprotein" is used to indicate the protein without luciferin.

The most studied photoproteins are Aequorin, isolated from *Aequorea victoria* (14) and Obelin, isolated from *Obelia longissima* (15). The photoprotein may be regenerated from the apophotoprotein by incubation with coelenterazine, molecular oxygen, EDTA and 2-mercaptoethanol or dithiothreitol. Since coelenterazine is the common luminescent substrate used by the photoproteins Aequorin, Mitrocomin, Clytin and Obelin, the light-emitting reaction is likely the same in these four photoproteins (16,17).

The Clytin photoprotein was cloned in 1993 by Inouye et al. (18). To date not much work has been done on this photoprotein. The primary structures of aequorin, mitrocomin, clytin and obelin were aligned and showed very strong amino acid sequence identities. The Ca^{2+}-binding sites of Clytin were also found to be highly conserved (19). It was found that hydrozoan Ca^{2+}-binding photoprotein differs from other Ca^{2+}-binding proteins such as calmodulin and troponin C by a relatively high content of cysteine, histidine, tryptophan, proline and tyrosine residues.

The analysis of the primary structure of clytin shows that it contains 198 aminoacidic residues (aa) and belongs to the family of photoproteins.

Photoproteins are widely used in reporter gene technology to monitor the cellular events associated with signal transduction and gene expression.

The study of cellular events and their regulation requires sensitive, non invasive analytic methods. Photoproteins and in general the use of bioluminescence are excellent reporter systems as they have virtually no background in contrast to fluorescence systems.

Photoproteins are expressed in mammalian cells to monitor calcium changes in response to different stimuli. Intracellular calcium concentrations can be measured by adding the cofactor coelenterazine to mammalian cells expressing the photoprotein and detecting photon emission, which is indicative of intracellular calcium concentration. The use of cells which express both a photoprotein and a receptor involved in the modulation of intracellular calcium concentration provides a valid system for the screening of compounds for their effects on the release of intracellular calcium. High throughput screening assays can also be designed using a photoprotein as reporter system. The sensitivity of the system as well as its high signal to noise ratio allow the use of small assay-volumes. Aequorin is up to now the most used photoprotein for these screening assays.

Calcium flux assays are commonly carried out in HTS format utilizing optical screening apparatuses suited for the simultaneous analysis of a high number of samples and equipped with a luminescence imaging system with a CCD Camera detector. However, one of the most used instruments in HTS is FLIPR® (Fluorometric Imaging Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA) which was developed as a high throughput optical screening tool for cell-based fluorescent assays. The apparatus is equipped with an optical detection device that allows for signal isolation on a cell-monolayer, thereby enhancing sensitivity for cell-based assays. The excitation source can be either an Argon laser or a broadband source as a Xenon lamp.

With a light-tight enclosure, extremely sensitive and fast camera, and

true simultaneous on-line liquid dispensing, the FLIPR® system most recent versions (FLIPR^3 and FLIPR^{TETRA}) have been made suitable also for luminescence assays, even if with lower sensitivity compared to CCD Camera-based equipments.

For the use of the above described instruments FLIPR®, FLIPR^3 and FLIPR^{TETRA} and in general for all instruments with a low sensitivity for luminescence assays, a photoprotein with enhanced light emission is highly desirable.

DISCLOSURE OF THE INVENTION

According to a first aspect, the invention provides an isolated photoprotein containing an amino acid sequence which:

a) is able to bind coelenterazine and calcium, producing bioluminescence;

b) has an identity of at least 90%, preferably of at least 95%, more preferably of at least 98% to SEQ ID NO: 1 (Clytin);

c) in sequence alignment with SEQ ID NO: 1 (Clytin), presents one of the following single or multiple substitutions (the residue positions are referred to SEQ ID NO: 1):

i) $C_{54}\rightarrow S$;

ii) $S_{132}\rightarrow C$;

iii) $K_{48}\rightarrow R$, $N_{195}\rightarrow D$;

iv) $Q_{68}\rightarrow R$, $A_{90}\rightarrow V$, $T_{184}\rightarrow I$;

v) $Y_{82}\rightarrow F$, $K_{110}\rightarrow N$, $F_{125}\rightarrow L$, $S_{149}\rightarrow R$;

vi) $G_{142}\rightarrow C$;

vii) $I_{53}\rightarrow V$, $S_{149}\rightarrow R$;

viii) $N_{18}\rightarrow D$, $I_{40}\rightarrow V$, $K_{56}\rightarrow R$

ix) $Gly_{58}\rightarrow Glu$, $Asp_{69}\rightarrow Val$, $Ala_{70}\rightarrow Cys$, $Lys_{76}\rightarrow Arg$, $Lys_{77}\rightarrow Gly$, $Ile_{78}\rightarrow Cys$, $Asp_{81}\rightarrow Glu$, $Val_{86}\rightarrow Ile$, $Glu_{87}\rightarrow Ala$, $Ala_{90}\rightarrow Gln$,

Val_{92}→Leu, and Glu_{97}→Gln

In a preferred embodiment, the photoprotein contains an amino acid sequence which is selected from the group of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9 and 10. Compared to known or commercially available photoproteins, the photoproteins of the invention show improved bioluminescence activity, and/or higher affinity to calcium and/or longer-lasting light emission.

Apart from the indicated residue-substitutions, which confer the desired photoprotein bioluminescence activity, the Clytin sequence can be further modified without negatively affecting the photoprotein's bioluminescence activity, especially by conservative substitutions of amino acidic residues, within the indicated sequence-identity limits. In addition, the Clytin sequence can be deleted of small portions, without altering its photoprotein activity.

In a further aspect, the invention is directed to a polynucleotide encoding a photoprotein as defined above. In a preferred embodiment, the polynucleotide sequences are optimized for mammalian codon usage according to SEQ ID NO: 11, 12, 13, 14, 15, 16, 17, 18, 19. In a further preferred embodiment, the nucleic acid molecules are fused to mitochondrial target sequences (20, 21, 22).

According to a further aspect, the invention provides expression vectors and host cells containing the indicated polynucleotides. Host cells expressing a photoprotein according to the invention produce an intense bioluminescence in response to calcium stimulation, which is much higher than that observed with natural photoproteins, in particular with the most used one, Aequorin.

In a further aspect, the invention provides a cell-based assay for determining intracellular calcium concentration by means of a photoprotein according to the invention.

In a preferred embodiment, the changes in intracellular calcium concentration are determined by:

a) providing a cell expressing a photoprotein of SEQ ID NO: 2-10, variants or fragments thereof;

b) loading of the cells with coelenterazine;

c) contacting the cells with an agent stimulating calcium influx or calcium release from intracellular stores;

d) detecting the photoprotein's bioluminescence.

The assay is preferably carried out in a high-throughput format utilizing an optical screening tool or apparatus suited for multi-sample analysis, such as a luminescence imaging system with a CCD Camera detector for high and ultra high throughput applications, or with the Fluorometric Imaging Plate Reader (FLIPR®). With both these systems, the photoproteins of the invention produce the highest signal compared to known photoproteins commonly used in automatized cell functional assays.

In a preferred embodiment, cells expressing a photoprotein and a receptor involved in intracellular calcium mobilization are used to test candidate molecules for their effects on receptor modulation. Typically, cells are transfected with an expression vector containing a photoprotein encoding sequence and when not endogenously present, a receptor or channel of interest. The positive clones are selected and plated in a suitable medium, cultured cells are loaded with the coelenterazine substrate and the assay is started by adding the test molecule or stimulus. The produced luminescence is read by a suitable detection system (CCD camera or luminometer). The assay can also be run in an automatic apparatus equipped with multi-well plate reading, in particular the FLIPR® system. In this case, photoprotein-expressing cells are plated in microplate wells, which, after addition of the test molecule/stimulus, are read simultaneously with signal recording.

High throughput screening assays set up with a photoprotein-based reporter system show improved sensitivity and signal-to-noise ratio. Cells

expressing a photoprotein of the invention produce an intense bioluminescence in response to calcium stimulation, which is generally higher than that observed with natural photoproteins.

In a further aspect, the invention provides an assay kit containing a preparation of cells expressing an invention photoprotein under the control of a stable or inducible promoter, and reagents suitable for running the assay.

In addition, the photoproteins of the invention may be used as intracellular calcium indicators in diagnostic methods based on the measurement of cellular calcium ion concentration and/or cellular calcium ion influx/outflow.

The invention will be described in more detail in the following experimental section.

MATERIALS AND METHODS

Reagents

Restriction enzymes were purchased from New England Biolabs and used according to supplier's instructions. The Ligation Independent Cloning (LIC) kit was from Novagen (Nottingham, UK). For *in vitro* Transcription and Translation we used the TNT Quick coupled kit from Promega (Madison, WI). Reagents for PCR, and competent cells of E. coli strains XL-1Blue and BL21-Gold(DE3), were from Stratagene (La Jolla, CA). Oligonucleotides were purchased from Primm (Milan). Coelenterazine was from Pharma Tech. International Inc. (Fairfield, NJ). All other chemicals were from standard sources and were of reagent grade or better.

1. Generation of a Randomly Mutagenized Library and Screening

1.1 Photoprotein optimization for expression in mammalian cells (GENEART GmbH, Regensburg, Germany)

The codon usage of the wild-type clytin gene was adapted to the codon bias of highly expressed mammalian genes. In addition regions of very high (> 80%) or very low (< 30%) GC content have been avoided where possible.

For efficient translation initiation the Kozak-consensus sequence was introduced upstream of the start codon. Two STOP codons were added to ensure efficient termination.

1.2 Random Mutagenesis

The GeneMorph II Random Mutagenesis kit (Stratagene) was used following supplier's instructions. Two different initial amounts of target DNA were used to achieve a high mutation rate, 0.1ng and 0.01ng.

PCR primers were appropriately designed to contain 5' LIC extensions (in italics) corresponding to sequences described in the Ek/LIC Cloning Kit (Novagen)

Upper: *GATGACGACGACAAG*-ATGGCCGACACCGCCAG (SEQ ID NO: 20)

Lower: *GAGGAGAAGCCCGGT*-TTATCAAGGACACGAAGT (SEQ ID NO: 21)

Amplification protocol performed in the Perkin Elmer 2400 thermocycler:

1 time the following step:

pre PCR 2' at 94°C

30 times the following steps:

denaturation 30'' at 94°C

annealing 30'' at 56°C

elongation 40'' at 72°C

1 time the following step:

elongation 10' at 72°C

Expected length of specific PCR product: 630 bp.

To quantitatively determine the amount of DNA obtained, amplification products were analysed by electrophoresis on 1% agarose gel in 1xTAE running buffer following standard procedure, as described by Maniatis et al. The

samples were compared to a DNA molecular weight marker (MWXVI, Roche).

1.3 Ek/LIC Cloning

The Novagen Ligation Independent Cloning (LIC) kit was used following suppliers instructions in order to obtain directional cloning of the PCR products without the need of restriction enzyme digestion or ligation reactions. The pET-30 Ek/LIC vector, engineered to express the target protein immediately downstream of an enterokinase cleavage site, was chosen.

1.4 Transformation

For good protein expression we chose BL21-Gold(DE3) cells (Stratagene), a derivative of *E.coli B*, an improved strain of BL21 competent cells. The genotype of the strain is: *E.coli B* F^- *ompT hsdS* (r_B^- m_B^-) dcm^+ Tet^r gal λ(DE3) endA Hte. This strain lacks both the *lon* and the *ompT* proteases, which can degrade proteins during purification. Hte phenotype increases the transformation efficiency (>$1x10^8$ cfu/μg of pUC18 DNA) In addition the *endA* gene, that encodes endonuclease I is inactivated (no degradation of plasmid DNA).

In order to obtain competent cells with a high efficiency of transformation, we followed the standard protocol for preparing and electro-transforming BL21-Gold(DE3) cells described in the *E. coli* Pulser Transformation apparatus manual (BioRad).

Transformation efficiency was tested by using the pUC18 DNA and the pET DNA vectors and the efficiencies obtained were:

- $1x10^{10}$ cfu/μg of pUC18 DNA
- $1x10^8$ cfu/μg of pET DNA

Using these highly electrocompetent cells we were able to obtain a library of approximately 84,000 colonies expressing the randomly mutated Clytin photoprotein.

1.5 Plating, induction and charging

Transformed cells were plated on an LB agar plates and grown

overnight at 37°C. After overnight colony growth, induction was obtained by adding 10 mM IPTG and 5 mM EDTA, and incubating for 4 hours at 37°C. Colonies were charged with 10 μM Coelenterazine solution and incubated overnight at 4°C in the absence of light.

1.6 CCD camera measurement

Bioluminescence is assayed by the detection of the signal over a fixed time period of 30'' at time 0, after 3 and after 5 minutes from the first measurement.

1.7 Colony picking and re-testing

The best colonies were picked and grown in 1ml of LB liquid medium and re-tested using the same experimental conditions described before.

2. *In vitro* transcription and translation

Translation of the photoproteins was carried out in the rabbit reticulocyte cell-free system (TNT Quick coupled kit, Promega), following the general instructions of the supplier. 500 ng of DNA was used for each *in vitro* transcription/translation reaction mix. The reaction volume (10 μl) included 8 μl of TNT T7 Quick Master Mix, 1.6 μl of DNA, 0.2 μl of the Methionine buffer and 0.2 μl of Coelenterazine (0.5 mM). To this end, 5 μl from each sample of the translation mixture were tested for light emission by the injection of calcium solution and by measuring at the Ascent Luminoskan (Labsystems).

3. Recombinant protein production

For the production of the recombinant protein we followed a small scale protein purification protocol under native conditions. Due to the presence of an N-terminal His tag in our constructs, we decided to purify the expressed proteins on Ni-NTA Spin Columns (Qiagen) following the suppliers' protocol.

4. Calcium concentration curve

In order to evaluate the response of the photoprotein to different calcium concentrations, 0.05 ng/well (96 MTP) of the recombinant protein were charged with Coelenterazine 10 μM overnight at 4°C.

After incubation, different concentrations of $CaCl_2$ were injected and the light released was measured at the Ascent Luminoskan with an integration time of 20 ms for a total time of 10 seconds.

5. Expression of the mutant photoprotein in mammalian cells

Reagents

Restriction enzymes were purchased from New England Biolabs (Beverly, MA) and used according to supplier's instructions. Rapid DNA ligation kit and Fugene transfection reagent were purchased from Roche (Basel, CH). Coelenterazine was from Pharma Tech. International Inc. (Fairfield, NJ). All other chemicals were from standard sources and were of reagent grade or better.

Cloning procedure

The most promising mutant photoprotein clones were subcloned for testing their expression in mammalian cells.

2 µl of plasmid were used as template in PCR analysis. In addition a negative control was performed with no template.

Standard PCR procedure were as indicated by Perkin Elmer. PCR protocol was as follows:

Primers:

Upper primer: TCGTTGGGATCCGCCACCATGGCCGACACCGCC (SEQ ID NO: 22)

Lower primer: GGGCCCTCTAGATTATCAAGGCACGAA (SEQ ID NO: 23)

PCR reaction mix:

2 µl	template
5 µl	10 x Pfx Buffer (GIBCO-LifeTechnologies)
1.5 µl	10 mM dNTPs
1 µl	50 mM $MgSO_4$ (GIBCO-LifeTechnologies)
2.5 µl	upper primer (10 µM)

2.5 µl lower primer (10 µM)

2.5 U Platinum Pfx (GIBCO-LifeTechnologies)

35 µl H_2O

Amplification protocol performed in Perkin Elmer 2400 thermocycler:

1 time the following step:

pre PCR 2' at 94°C

25 times the following steps:

denaturation 15'' at 94°C

annealing 30'' at 56°C

elongation 40'' at 68°C

1 time the following step:

elongation 10' at 68°C

Expected length of specific PCR product: 630 bp.

Amplification products were analysed by electrophoresis on 1% agarose gel in 1xTAE running buffer following the standard procedure, as described by Maniatis et al.

The PCR product was gel purified using Qiagen columns and digested with BamHI and XbaI restriction enzymes.

pcDNA3neo-/mitoMutated-clytin construction

An in-house modified pcDNA3 vector (Invitrogen) has been prepared containing the sequence encoding the mitochondrial targeting peptide from subunit VIII of human cytochrome c oxidase (20, 21, 22) so that it could be used in frame at the 5' end of the codon-usage optimized photoprotein gene. The amplification product obtained from the above mentioned PCR has been cloned into this modified pcDNA3 vector lacking the Neomycin resistance gene for expression in mammalian cell lines.

For the mitochondrial targeting the signal sequence is:

5'-ATGTCCGTCCTGACGCCGCTGCTGCTGCGGGGCTTGACAGGCTCGGC

CCGGCGGCTCCCAGTGCCGCGCGCCAAGATCCATTCGTTGGGATCCGCCA CC-3' (SEQ ID NO: 24).

pcDNA3neo-/cytoMutated-clytin construction

The Mutated-clytin gene is obtained from the pcrScript/hMutated-clytin vector by digestion with BamHI and XbaI. The pcDNA3neo- is digested with the BamHI and XbaI restriction enzymes, and purified.

The Mutated-clytin gene is then ligated into the pcDNA3neo- vector to obtain pcDNA3neo-CYTO-hMutated-clytin.

Both constructs obtained were verified by full-length dideoxy sequencing.

Cell culture

Culture medium, seeding and incubation: DMEM/F12 with Glutamax (GIBCO cod. 31331-028), 10% FBS, 1% Pen./Strep. (Invitrogen cod. 15140-122), 25 mM Hepes Buffer Solution (GIBCO cod. 15630-056), 1.0 mM Sodium Pyruvate (GIBCO cod. 11360-039), 1.5 g/L Sodium Bicarbonate (GIBCO cod. 25080-060).

Preculture conditions: Cells were seeded for experiments when 70-80% confluent.

Cell culture conditions: Split twice a week: $3.0x10^5$ cells/flask T75 (recovery: $8x10^6$ cells).

Clone Selection Process:

- CHO-K1 were transfected with pcDNA3Neo⁻/MITO-hMutated-clytin or pcDNA3Neo⁻/CYTO-hMutated-clytin.
- 48 h after transfection, the transfected cells were plated in 10x96 w/p in complete DMEM.
- At confluence the 10x96 w/p were duplicated in 10 white plates. 3 ½ hours before measurements, medium was replaced with 50 µl/well of tyrode (2 mM Ca^{2+} and coelenterazine 10 µM).
- Positive clones were selected evaluating:

- First selection was done lysing cells with TRITON X-100. CCD camera conditions: low sensitivity, integration time 1 second reading for 5 seconds.
- Five clones were chosen each diluted in 3x96 w/p.
- At confluence the 15x96 w/p were duplicated in 15 white plates. 3 ½ hours before measurements, medium was replaced with 50 µl/well of tyrode (2 mM Ca^{2+} and coelenterazine 10 µM).
- Second selection was done with 10 µM ATP monitoring the kinetics and then cells were lysed with TRITON X-100. CCD camera conditions: low sensitivity, integration time 1 second reading for 30 seconds for the ATP measurement followed by 30 seconds for TRITON X-100.
- Four limiting dilutions of the best clone selected were performed, 0.3 cells per well in 10x96 w/p.
- The final clone was chosen after the 4th LD selected with ATP 0.25 µM, coelenterazine 5 µM.
- Complete optimization of the assay was performed on the best responding clone.

CCD Camera Measurement parameters:

CHO cells are seeded at different cell densities in 384MTP (500, 750, 1,000, 1,500 cells/well) in growth media supplemented as above and measured with a CCD camera 24 hours and 48 hours after plating. Prior to experiments growth medium is removed and cells are loaded with Tyrode buffer plus coelenterazine at 37°C for 3 hours. Luminescence is finally monitored by CCD camera after the addition of the agonist (30 sec. kinetic).

Fluorometric Imaging Plate Reader (FLIPR®) Measurements

FLIPR^3 Settings for Assays standard protocol for luminescence detection

- 384 white wall clear bottom plates
- cell plating 24 hrs before the experiment

- medium removal
- addition of tyrode plus coelenterazine 25 µl/well
- incubation 4 hrs at 37°C
- experiments run at FLIPR^3: compound (2X) injection in tyrode buffer (25 µl/well).

All parameter values are the instrumentation default values, except for the following:

Pre-assay steps:

1) Camera configuration:

Exposure length = 0.7

Camera gain = 200

2) Sequence parameters:

Dispense 384 well head

Height = 30 µl

Speed = 25 µl/sec

FLIPR^{TETRA} Settings for Assays standard protocol for luminescence detection

- 384 white wall clear bottom plates
- cell plating 24 hrs before the experiment
- medium removal
- addition of tyrode plus coelenterazine 25 µl/well
- incubation for 4 hrs at 37°C
- experiments run at FLIPR^{TETRA}: compound (2X) injection in tyrode buffer (25 µl/well).

All parameter values are the instrumentation default values, except for the following:

Set up read mode:

1) Camera configuration:

Camera Gain = 200

Exposure time = 0.50

2) Sequence parameters:

Height = 30 µl

Speed = 25 µl/sec

Reference Compounds:

ATP (Sigma, A-7699) was dissolved in H_2O at a concentration of 100 mM and stored in aliquots at -20°C. Working solution was freshly prepared in tyrode buffer.

Tyrode Buffer composition: NaCl 130 mM, KCl 5 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, $NaHC0_3$ 5 mM e HEPES 20 mM, pH 7.4.

IMETIT (Sigma, I-135)

DESCRIPTION OF THE DRAWINGS

Fig. 1: Re-test of mutant colonies with three calcium concentrations.

Fig. 2: *In vitro* Transcription & Translation. Measurement of light emission upon 5 pM calcium injection.

Fig. 3: *In vitro* Transcription & Translation. Measurement of light emission upon 1 mM calcium injection.

Fig. 4: Kinetics of the measurement of light emission upon 1 mM calcium injection for the recombinant photoproteins.

Fig. 5: Calcium dose-response curve of the recombinant photoprotein 25N03b.

Fig. 6: Peak light intensity from recombinant photoproteins upon 1 mM calcium injection.

Fig. 7: Kinetics of 10 µM ATP response at the CCD camera for the CHOK1/mito25N03b cell line.

Fig. 8: ATP dose-response curve of the CHOK1/mito25N03b cell line.

Fig. 9: ATP dose-response kinetic of the CHOK1/mito12mutCly cell

line obtained at the CCD camera testing 500 cells/well 24 h after seeding.

Fig. 10: ATP dose-response curve of the CHOK1/mito12mutCly cell line obtained at the CCD camera with different number of cells/well, different coelenterazine concentrations, and incubation time.

Fig. 11: ATP dose-response curve of the CHOK1/cyto12mutCly cell line obtained at the CCD camera with different number of cells/well.

Fig. 12: IMETIT dose-response curve of the CHOK1/mito12mutCly/H3 cell line obtained at the CCD camera with different number of cells/well.

Fig. 13: IMETIT dose-response curve of the CHOK1/mito12mutCly/H3 cell line obtained at FLIPR^3.

Fig 14: Dose-response curve of the CHOK1/mito25N03b-A3 cell line with A3 agonist (IB MECA), tested on the Lumibox CCD camera, 500 cells per well, 24h after seeding. 5μM coelenterazine. Camera sensitivity 5, 60 second measurement.

Fig 15: Dose-response curve of the CHOK1/mito25N03b-A3 cell line with A3 agonist (IB MECA), tested on the CyBi Lumax HT CCD camera, 2500 cells per well, 24h after seeding. 5μM CTZ. Camera sensitivity HV 10 analog, 60 second measurement.

Fig 16: Dose-response curve of the CHOK1/mito25N03b-A3 cell line with A3 agonist (IB MECA), tested on the FLIPR^{TETRA}, 2500 cells per well, 24h after seeding. 10μM coelenterazine Exposure time 2 seconds, gain 240.

EXAMPLES

1. Generation of a Randomly Mutagenized Library and Screening

The Random mutant Library was obtained by using the GeneMorph II Random Mutagenesis kit (Stratagene). In order to achieve a high mutation rate two different initial amounts of target DNA were used: 0.1 ng and 0.01 ng. A total of 83305 bacterial colonies were tested for luminescence activity. Of these, 1089 were positive and therefore had bioluminescent characteristics.

The best colonies were picked, for a total of 289 colonies, and of these, 16 resulted best after a re-test. Finally 9 colonies were chosen and analysed.

Figure 1 shows the results obtained in a three test-point $CaCl_2$ dose-response curve obtained with the 8 mutants.

2. Photoprotein assay

5 µl of the translation mixture were directly mixed with 95 µl of PBS solution in a 96 well plate which was mounted into the Luminometer (Berthold). To trigger photoprotein light emission, a 5 pM $CaCl_2$ solution was injected into the well and luminescence recorded for 10 seconds.

The results of the in vitro transcription and translation of the DNA of the 8 mutants are shown in Fig. 2.

A new in vitro transcription and translation experiment was carried out with the best responding mutant, 25N03b (sequence ID n°16), and with the Aequorin photoprotein (Fig. 3) in order to have a comparison of the light emission upon the injection of 1mM $CaCl_2$ solution.

3. Recombinant Photoprotein and Calcium concentration curve

Recombinant photoproteins corresponding to some mutants were produced under native conditions following a small scale purification protocol. Light emission was measured upon 1 mM calcium injection and the corresponding kinetics are shown in Fig. 4.

The recombinant mutant photoprotein, corresponding to clone 25N03b, was better characterized with a complete calcium dose-response curve, which can be seen in Fig. 5.

In another experiment, the recombinant photoprotein 25N03b was compared to recombinant Aequorin. The light emission recorded upon 1 mM $CaCl_2$ injection was surprisingly higher in the case of the 25N03b mutant as shown in Fig. 6.

4. Cell-based functional assays

4.1 The CHOmito25N03b-expressing clone (CHOK1/mito25N03b) has been obtained by transfection of CHO-K1 cells (Materials and Methods). 48 hours after transfection the cells were trypsinized and plated into 10x96 MTP (Microtiter Plates) in complete MEM. At confluence, the 10x96 MTP were duplicated using MATRIX (Hudson, NH, USA) in 10x96 white MTP. 3 hours before measurement the medium was replaced with 50 µl/well of tyrode buffer 2 mM Ca^{2+} and 10 µM coelenterazine. The clones were selected on the basis of their functional response (luminescent signal) to ATP, which is known to stimulate the CHO endogenous receptor P2Y and to rise the cytoplasmic Ca^{2+} concentration. At the end of each experiment, cells were lysed by perfusion of a solution containing Triton X-100. The active photoprotein was reconstituted incubating the cells with 10 µM coelenterazine diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, at 37°C, in a 5% CO_2 atmosphere for 3 hrs. For light emission measurement, cells were lysed in the presence of calcium and the emitted luminescence recorded. The number of photons emitted during the first 30 seconds was integrated by a CCD camera and visualized on the screen. Cells transfected with an empty plasmid or untransfected did not increase photon-emission. To detect changes in calcium concentrations, 10 µM ATP was injected and the kinetics of the response determined. The curve obtained is shown in Fig. 7.

The final clone was seeded in 384 w/p and tested with increasing ATP concentrations as shown in the dose-response curve in Fig. 8.

The CHOK1/mito25N03b cell line was also transfected with a G-protein coupled receptor, the Adenosine A3 receptor, and with a chimeric Gα protein, in order to switch the signal to the PLC/IP pathway. A stable cell line was generated (from now on referred as CHOK1/mito25N03b /A3 clone).

Upon stimulation with its agonist, the A3 receptor induces an increase

in intracellular calcium concentration which is measured by mito25N03b luminescence.

The final clones of the CHO cell lines expressing mito25N03b and the human Adenosine A3 receptor were grown to 80-95% confluence in tissue culture flasks and harvested by trypsinization. Cells were dispensed at different cell densities in 384 w/p in growth medium (DMEM/F12 containing 10% Foetal Bovine Serum) and incubated for 24 h and 48h at 37°C in a humidified incubator at 5% CO_2. On the day of the experiment, the culture medium was removed and, for luminescence experiments, cells were loaded with 5 μM coelenterazine for 3h, at 37°C, 5% CO_2. Calcium response was stimulated by addition of different concentrations of ATP to each well. The kinetics of flash luminescence was followed using a CCD camera, which injects reagents and records light emission from each single well.

The agonist and antagonist were diluted in tyrode buffer at different concentrations. Approximately 25 μl of these solutions were separately injected into each well and the response measured with the CCD camera instrumentation. The emitted light was immediately recorded at different time intervals. Results shown in Fig. 14 refer to the A3 specific agonist, IB-MECA. The same experiments were performed on another CCD camera instrumentation, results are shown in Fig.15.

4.2 The CHOK1 cells were stably transfected with the mutant 12mutCly (SEQ ID NO: 19) cloned in pcDNA3neo-mito, to obtain the cell line CHOK1/mito12mutCly (Materials and Methods).

The final clone was selected on the basis of the functional response (luminescent signal) to ATP, which is known to stimulate the CHO endogenous receptor P2Y and to rise the cytoplasmic Ca^{2+} concentration. At the end of each experiment, cells were lysed by perfusion of a solution containing Triton X-100. The active photoprotein was reconstituted incubating

the cells with 2.5 or 5 μM coelenterazine diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, at 37°C, in a 5% CO_2 atmosphere for 3 hrs. For light emission measurement, cells were lysed in the presence of calcium and the emitted luminescence recorded. The number of photons emitted during the first 30 seconds was integrated by a CCD camera-based luminometer and visualized on the screen. Cells transfected with an empty plasmid or untransfected did not increase photon-emission. Different amount of cells were seeded in 384 MTP. After 24 h to detect changes in calcium concentrations, different ATP concentrations were injected and the kinetics of the response determined. Examples of kinetics obtained seeding 500 cells/well 24 h before the test are shown in Fig. 9.

The high light emission and sensitivity to Ca^{2+} observed in the CHOK1 mito12mutCly cell line allowed the use of a lower number of cells and a lower concentration of coelenterazine even for less time in comparison to the standard photoprotein-based cell assays conditions.

Examples of ATP dose-rensponse curves obtained seeding 100 to 600 cells/well 24 h before the test and incubating the cells with 2.5 and 5 μM coelenterazine diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, at 37°C, in a 5% CO_2 atmosphere for 2 and 3 h are shown in Fig.10a and 10b.

The CHOK1 cyto12mutCly cell line was obtained by transfection of CHO-K1 cells (Materials and Methods).

The final clone was selected on the basis of the functional response (luminescent signal) to ATP, which is known to stimulate the CHO endogenous receptor P2Y and to rise the cytoplasmic Ca^{2+} concentration. At the end of each experiment, cells were lysed by perfusion of a solution containing Triton X-100. The active photoprotein was reconstituted incubating the cells with 2.5 or 5 μM coelenterazine diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, at 37°C, in a 5% CO_2 atmosphere for 3 h. For light

emission measurement, cells were lysed in the presence of calcium and the emitted luminescence recorded. The number of photons emitted during the first 30 seconds was integrated by a CCD camera-based luminometer and visualized on the screen. Cells transfected with an empty plasmid or untransfected did not increase photon-emission. Different amount of cells were seeded in 384 MTP. After 24 and 48 h to detect changes in calcium concentrations, different ATP concentrations were injected and the kinetics of the responses determined. ATP dose-response curves obtained seeding 500, 1000 and 2000 cells/well 24 h before the test and incubating the cells with 2.5 and 5 μM coelenterazine diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, at 37°C, in a 5% CO_2 atmosphere for 2 and 3 h are shown in Fig. 11.

The CHOK1 mito12mutCly cell line was transfected with a G-protein coupled receptor, the Histamine-3 receptor, and with a chimeric Gα protein, in order to switch the signal to the PLC/IP pathway. A stable cell line was generated (from now on referred as CHOK1/mito12mutCly/H3 cell line) (Materials and Methods).

Upon stimulation with its agonist (IMETIT), the H3 receptor induces an increase in intracellular calcium concentration which is measured by mito12mutCly luminescence.

IMETIT dose-response curves obtained seeding 250, 500, 750, and 1000 cells/well 24 h before the test and incubating the cells with 5 μM coelenterazine diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, at 37°C, in a 5% CO_2 atmosphere for 3 h are shown in Fig. 12.

5. Cell-based assays at the FLIPR

The CHOK1/mito25N03b/A3 and CHOK1/mito12mutCly/H3 are tested at FLIPR by measuring the luminescence signal induced by the activation of the transfected receptor.

The luminescence measured with the FLIPR instrumentation is reported as Luminescence Change Units in Fig. 13, wherein are reported the results obtained by measuring the light release induced by IMETIT stimulation on CHOK1/mito12mutCly/H3. 5000 cells/well were seeded in 384 MTP 24 h before the experiment. The medium was replaced and substituted with 25 µl of 2X concentrated coelenterazine (10 µM) diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, for 3 h at 37°C, in a 5% CO_2 atmosphere. IMETIT compound (2X) at different concentrations was injected on cells (25 µl/well).

In figure 16 the luminescence measured with the FLIPR^{TETRA} instrumentation is reported as RLU (Relative Light Units), wherein are reported the results obtained by measuring the light release induced by IB-MECA stimulation on CHOK1/mito25N03b/A3. 2500 cells/well were seeded in 384 MTP 24 h before the experiment. The medium was replaced and substituted with 25 µl of 2X concentrated coelenterazine (10 µM) diluted in tyrode buffer containing 2 mM Ca^{2+}, in the dark, for 3 h at 37°C, in a 5% CO_2 atmosphere. IB-MECA compound (2X) at different concentrations was injected on cells (25 µl/well).

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CLAIMS

1. Isolated photoprotein containing an amino acid sequence which:

a) is able to bind coelenterazine and calcium, producing bioluminescence;

b) is identical by at least 90% to SEQ ID NO: 1 (Clytin);

c) in sequence alignment with SEQ ID NO: 1 (Clytin), presents one of the following single or multiple substitutions (the residue positions are referred to SEQ ID NO: 1):

i) $C_{54}\rightarrow S$;

ii) $S_{132}\rightarrow C$;

iii) $K_{48}\rightarrow R$, $N_{195}\rightarrow D$;

iv) $Q_{68}\rightarrow R$, $A_{90}\rightarrow V$, $T_{184}\rightarrow I$;

v) $Y_{82}\rightarrow F$, $K_{110}\rightarrow N$, $F_{125}\rightarrow L$, $S_{149}\rightarrow R$;

vi) $G_{142}\rightarrow C$;

vii) $I_{53}\rightarrow V$, $S_{149}\rightarrow R$;

viii) $N_{18}\rightarrow D$, $I_{40}\rightarrow V$, $K_{56}\rightarrow R$;

ix) $Gly_{58}\rightarrow Glu$, $Asp_{69}\rightarrow Val$, $Ala_{70}\rightarrow Cys$, $Lys_{76}\rightarrow Arg$, $Lys_{77}\rightarrow Gly$, $Ile_{78}\rightarrow Cys$, $Asp_{81}\rightarrow Glu$, $Val_{86}\rightarrow Ile$, $Glu_{87}\rightarrow Ala$, $Ala_{90}\rightarrow Gln$, $Val_{92}\rightarrow Leu$, and $Glu_{97}\rightarrow Gln$

a functional derivative or fragment thereof.

2. The photoprotein of claim 1, containing an amino acid sequence identical by at least 95% to SEQ ID NO: 1.

3. The photoprotein of claim 2, containing an amino acid sequence identical by at least 98% to SEQ ID NO: 1.

4. The photoprotein of claim 3, containing an amino acid sequence which is selected from the group consisting of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10.

5. A photoprotein according to claims 1-4, wherein said amino acid

sequence is fused to a mitochondrial target sequence.

6. An isolated polynucleotide encoding a photoprotein according to claims 1-5.

7. The polynucleotide of claim 6, having the sequence of SEQ ID NO: 11, 12, 13, 14, 15, 16, 17, 18, 19.

8. An expression vector containing a polynucleotide according to anyone of claims 6-7.

9. A prokaryotic or eukaryotic host cell containing the vector of claim 8.

10. A mammalian host cell according to claim 9.

11. A method in vitro for detecting changes in intracellular calcium concentration which comprises:

a) providing a cell expressing a photoprotein according to claims 1-5;

b) contacting the cell with an agent stimulating calcium influx or calcium release from intracellular stores;

c) detecting the photoprotein bioluminescence.

12. A method of screening compounds modulating intracellular calcium concentration, which comprises:

a) providing a cell expressing a photoprotein of claims 1-5;

b) contacting the cell with the candidate compound;

c) detecting the bioluminescence of the photoprotein.

13. A method according to claims 11 or 12, which is carried out in a high-throughput format.

14. A method according to claim 13, which is carried out with a high throughput optical screening apparatus suited for multi-sample analysis.

15. The use of a photoprotein according to claims 1-5 as intracellular calcium indicator.

16. The use of a photoprotein according to claim 15 in a cell-based high throughput assay.

17. The use of a photoprotein according to claims 1-5 for the preparation of a diagnostic composition.