Cloning and Expression of cDNA for a Luciferase from the Marine Copepod Metridia longa

A NOVEL SECRETED BIOLUMINESCENT REPORTER ENZYME*

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Metridia longa is a marine copepod from which a blue bioluminescence originates as a secretion from epidermal glands in response to various stimuli. We demonstrate that Metridia luciferase is specific for coelenterazine to produce blue light (λmax = 480 nm). Using an expression cDNA library and functional screening, we cloned and sequenced the cDNA encoding the Metridia luciferase. The cDNA is an 897-bp fragment with a 656-bp open reading frame, which encodes a 219-amino acid polypeptide with a molecular weight of 23,885. The polypeptide contains an N-terminal signal peptide of 17 amino acid residues for secretion. On expression of the Metridia luciferase gene in mammalian Chinese hamster ovary cells the luciferase is detected in the culture medium confirming the existence of a naturally occurring signal peptide for secretion in the cloned luciferase. The novel secreted luciferase was tested in a practical assay application in which the activity of A2a and NPY2 G-protein-coupled receptors was detected. These results clearly suggest that the secreted Metridia luciferase is well suited as a reporter for monitoring gene expression and, in particular, for the development of novel ultra-high-throughput screening technologies.

Continuous monitoring of dynamic changes in gene expression from living cells in response to various stimuli provides important information about cell physiology. For this purpose bioluminescent and fluorescent reporters have been introduced as tools for sensitive and convenient monitoring of gene expression. A cDNA encoding a bioluminescent or fluorescent reporter such as a luciferase or green fluorescent protein (GFP)1 is fused to the promoter region of the target gene, and the construct is transfected to mammalian cells. The gene expression is monitored simply by measuring light emitted through an enzymatic reaction or fluorescence. To date several bioluminescent reporters have been widely and successfully used for optical monitoring or fluorescence. To date several bioluminescent reporters have been introduced as tools for sensitive and convenient monitoring of gene expression extracellularly without destroying the cells. This is particularly useful for time course studies of gene expression that are reported to be affected by concentrations of ATP, luciferin, and luciferin-luciferase complex (15, 16). Despite the fact that the GFP possesses excellent properties as a reporter, it has some shortcomings (toxicity for living cells (for review, see Ref. 17), slow maturation of chromophore (for review, see Ref. 18), and the long half-life of the protein in mammalian cells (for review, see Ref. 19) that limit its application. Although the Ca2+-regulated photoproteins provide high sensitivity for monitoring gene expression (20) they have one disadvantage. To measure the photoprotein activity the synthesized apoprotein needs to be charged with coelenterazine substrate over periods of several h, and this delays answering the question about gene activity. To circumvent these limitations and difficulties, fusion proteins of luciferases and of GFP were created possessing sequences that induce secretion of the protein into the culture medium (21, 22). The main advantage of using a secreted reporter protein is that it is possible to measure gene expression extracellularly without destroying the cells or tissues. This is particularly useful for time course studies of transcription activity or receptor/channel response kinetics.

There are bioluminescent organisms with naturally occurring secreted luciferases, and the cDNAs encoding some of them have been cloned. These include the marine ostraclad Vargula hilgendorfii (23), the deep sea shrimp Oplophorus gracilorostris (24), and the marine copepod Gaussia princeps (GenBank™/EBI accession number AY015993). The Vargula and Oplophorus luciferases have been expressed in mammalian cells (23–28), and the Vargula luciferase has shown promising results. For instance, in Chinese hamster ovary (CHO) cells transfected with a plasmid bearing the cDNA for Vargula luciferase it was demonstrated that the secretory process could be monitored in real time from individual cells (26).

The attractiveness of secreted luciferases as reporters is a strong stimulus for the investigation and exploitation of new.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY364164.

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‡ The abbreviations used are: GFP, green fluorescent protein; FL, firefly luciferase; CHO, Chinese hamster ovary; MLuc, Metridia luciferase; IPTG, isopropyl-β-D-thiogalactopyranoside; NPY2, human neuropeptide Y receptor 2; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine.

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bioluminescent systems. *Metridia longa* is a small (−1.5 mm) luminous marine copepod. The bioluminescence originates as a secretion from epidermal glands located in the head part and abdomen in response to mechanical, electrical, or chemical stimuli (29). Bioluminescence in *M. longa* may well serve as a defense mechanism against predators; the release of a luminous bolus from the animal is accompanied by rapid swimming that quickly carries the copepod away from its "glowing phantom." Although the bioluminescence of *M. longa* was described many years ago (30) no studies concerning the properties of the bioluminescent system were carried out until now. The present study provides information on the substrate specificity of *Metridia* luciferase (MLuc) and reports cloning, nucleotide sequence analysis of the cDNA encoding *Metridia* luciferase, expression of MLuc cDNA in *Escherichia coli* and mammalian cells, and verification of the suitability of MLuc for use as a reporter for monitoring gene expression.

**EXPERIMENTAL PROCEDURES**

**Collection of the Copepod M. longa—**We collected the animals in Chupa Bay in the region of the White Sea biological station Kartesh from aboard the research vessel "Professor Kuznetsov" using a shunting plankton net at a depth of 100–500 m. In this species dominates. Plankton were sorted with a transfer pipette under a stereomicroscope, and the *M. longa* specimens at stages IV–VI of development (0.3–1.5 mm in size) were transferred into a tank containing continuously aerated seawater at 4 °C, which was the temperature of the natural habitat of the copepod. The accumulated animals were filtered and quickly frozen with liquid nitrogen.

**Determination of Metridia Luciferase Substrate—**For studies of the substrate specificity of *Metridia* luciferase about 30 freshly collected copepods were homogenized in 2 ml of 50 mM Hepes pH 7.2 at 0 °C. The suspension was centrifuged, the pellet was discarded, and the supernatant immediately was used for measurements. The luminescence was initiated by injection of 10 μl of either coelenterazine or Vargule luciferin in methanol into 0.5 ml of the supernatant of *M. longa* extract. Preparation of the Expression cDNA Library and Screening—Total RNA was isolated from frozen whole copepods by the guanidine isothiocyanate-phenol method (31). Poly(A)+ RNA was purified on poly(U)-Sepharose 4B (Amersham Biosciences), and 1 μg of this was employed to synthesize cDNA. The expression cDNA library was constructed in a λTriplEx2 phagemid vector with the SMART cDNA library construction kit (Clontech) according to the protocol supplied with the kit. The cDNA-vector ligation mixture obtained was then packaged with GigaPack II Gold packaging extract (Stratagene), yielding about 106 independent recombinant plaques. The phage and bacterial culture plating was carried out according to the protocol for the SMART cDNA library construction kit. For screening the unamplified phage a cDNA library was carried out according to the protocol for the SMART cDNA library construction kit. The isolated cDNA was cycle-amplified in the bacterial host *E. coli* strain BL21-CodonPlus(DE3)-RIL for expression of luciferase. For protein production, the transformed *E. coli* was cultivated with vigorous shaking at 37 °C in LB medium containing 200 μg/ml ampicillin and induced with 1 μl IPTG when the culture reached an *A*600nm of 0.5−0.6. After addition of IPTG, the cultivation was continued for 3 h. For activity measurements on the expressed MLuc, *E. coli* cells were harvested by centrifugation, resuspended in 0.5 ml of the SM buffer (0.1 M NaCl, 10 mM MgSO4, 0.01% gelatin, 50 mM Tris-HCl, pH 7.5), and sonicated at 0 °C. The bioluminescence was measured by rapid injection of 10 μl of 1−3 × 10−4 M coelenterazine in methanol into a luminometer cell containing 0.5 ml of sonicated cell lysate in SM buffer at room temperature.

The bioluminescence spectra were measured with an Aminco luminometer (SpectroPhotometer, Spectronics, Madison, WI). Emission spectra were corrected with the computer program supplied with the instrument.

**Expression of MLuc and FL in CHO Cells—**The pcDNA3 (Invitrogen) and pASM (Bayer) plasmids harboring a neomycin resistance and a CAMP-sensitive mouse mammary tumor virus promoter, respectively, were used for expression. The pcDNA3-MLuc and pASM-MLuc plasmids were constructed by subcloning the full-length *Metridia* cDNA sequence from the pTriplEx2-MLuc into the EcoRV/NotI site of pcDNA3 and pASM. To produce the pcDNA3-FL plasmid, the FL (GenBank/EMBL/GenPept) sequence (Promega) was inserted into the pASM-FL plasmid (Bayer) in the same cloning site. CHO-K1 (ATCC, Manassas, VA) cells were used both for transient and for stable transfections. The transfection of CHO-K1 cells with recombinant plasmids was performed withFuGENE 6 reagent (Roche Applied Science) according to the manufacturer's protocol. The pASM plasmid was co-transfected with pcDNA3 for neomycin selection. The transfected cells were selected in medium with 2 mg/ml geneticin. The positive clones were verified by luminescence measurement of the medium for MLuc or cell lysates for FL.

**Secretion Time Course of Metridia Luciferase—**The CHO cells were stably transfected with pcDNA3-MLuc, pcDNA3-FL, or pcDNA3 and pcASM. The cells were cultured in Dulbecco's modified Eagle's F12 medium (0.1% NaCl, 10 mM MgSO4, 0.01% gelatin, 50 mM Tris-HCl, pH 7.5) at 0 °C. The crude cell lysate was assayed for bioluminescence activity with coelenterazine (PJK GmbH, Kleinblittersdorf, Germany) (final concentration, ~10−6 μM). The next replica from a positive primary plate was cut into sectors that were analyzed as above. Then the individual plaques from the positive sectors were isolated and assayed for luciferase activity. The subsequent analysis of the isolated cDNAs, the positive plaques were converted to the corresponding pTriplEx2 plasmid subclones in the *E. coli* BM25.8 strain according to the protocol supplied with the SMART cDNA library construction kit. The isolated cDNA was cycle-sequenced with the DNA cycle-sequencing kit (Medigen, Novosibirsk, Russia) and the cycle-sequence kit (ClustalW, version 1.81, was used for sequence comparison and alignment.

**Expression of Metridia Luciferase in E. coli—**Analysis of the MLuc sequence revealed a putative internal translation initiation site for bacterial expression near the C terminus of the protein (Fig. 2). To remove it we made two replacements in the nucleotide sequence with amino acid sequence conservation using a QuikChange site-directed mutagenesis kit (Stratagene). The pTriplEx2-MLuc plasmid was used for a template for mutagenesis.

All natural secreted proteins lose their signal peptide during secretion. It is reasonable to assume that this also occurs when secreted proteins are used as reporters in mammalian cells. In that case, the properties of the reporter protein without the signal peptide need to be known if the results are to be interpreted correctly. The cDNA encoding the translated part of the luciferase gene without signal peptide was amplified with a high fidelity polymerase PfuTurbo (Stratagene) according to the manufacturer's recommendations. For this we used the primers after removal of the putative internal site of translation initiation as template. The final volume of 50 μl contained 0.1 μg of DNA with 2.5 units of polymerase. The isolated PCR fragment was cloned in the correct reading frame into the pET22b+ expression vector (Novagen) in Ndel/XhoI sites. The new restriction sites for direct in-frame cloning were generated by design of the primers. The Ndel restriction site was introduced into the forward primer, and the XhoI restriction site was introduced into the reverse primer. The PCR product was digested with Ndel and XhoI, purified by agarose gel electrophoresis, and inserted into a similarly digested, dephosphorylated, and agarose gel-purified pET22b+ expression vector. The resulting plasmid was named pET22-MLuc. After verification of the nucleotide sequence, the plasmid was introduced into *E. coli* strain BL21-CodonPlus(DE3)-RIL for expression of luciferase.
Secreted Luciferase of Copepod Metridia longa

Fig. 1. Bioluminescence spectra of recombinant Metridia luciferase (dashed line) and of a crude extract of M. longa copepods (solid line). The spectra were measured after injection of 10 μl of 1–3 × 10^{-7} M coelenterazine in methanol into a spectrofluorometer cell containing 0.5 ml of 50 mM Hepes pH 7.2 with the crude extract of copepods or SM buffer containing the high purity luciferase. Both spectra are at room temperature.

RESULTS AND DISCUSSION

Substrate Specificity of Metridia Luciferase—The substrate specificity of Metridia luciferase was studied with a crude extract of Metridia. The addition of Vargula luciferin to a crude extract of copepods did not produce any detected luminescence, whereas the addition of coelenterazine produced a flash of light with fast decay of luminescence and intensity depending on the quantity of added crude extract (data not shown). A repeated injection of coelenterazine gives rise to a new luminescence flash. The bioluminescence of the extract is blue with maximum at 480 nm (Fig. 1). The color of the light emitted by the extract correlates well with the bioluminescence color observed visually from the copepods on mechanical stimulation.

Isolation of a Positive Clone, cDNA Structure, and Sequence—The M. longa cDNA library yielded ~10^6 independent recombinant plaques. After about 10,000 plaques had been screened, several positive clones revealing luminescent activity were isolated. The isolated cDNA is an 897-bp fragment (excluding poly(A) tail) with a 56-bp 5' untranslated region upstream from the first start codon (Fig. 2). The open reading frame contains 656 bp, which encode a 219-amino acid polypeptide with calculated M_r of 23,885. After the stop codon there is a 181-bp untranslated region followed by a terminal poly(A). MLuc encodes for 10 cysteine residues. This number of Cys residues indicates that cloned luciferase may contain disulfide bonds, which may be necessary for bioluminescent activity or for supporting a stable protein conformation. But unlike other coelenterazine-dependent luciferases that use coelenterazine as a substrate or the Ca^{2+}-regulated photoproteins (32–34), Metridia luciferase has only two Trp and two Tyr residues.

A homology search of the non-redundant protein data bases with the gapped Blast program and the MLuc sequence as query produced only one hit, the luciferase from another copepod, G. princeps. The degree of identity of the Gaussia luciferase sequence relative to the MLuc sequence is 60 and 68% for nucleotide and amino acid sequences, respectively. As the copepods G. princeps and M. longa belong to the same family (Metridinidae), this could account for the great similarity between the Gaussia and Metridia luciferase sequences. In contrast, the luciferase cloned from a representative of the third genus of the family Metridinidae (Pleurodomma) (GenBank®/EBI accession number AY015994) does not resemble the Metridia and Gaussia luciferases. This suggests that there is a possibility of high diversity of primary structures of luciferases among the luminous copepods, the luminous species of which are found in various genera and families (29).

To date primary sequences have been determined for several luciferases (Renilla (32), Oplophorus (24), Pleurodomma, and Gaussia luciferases) and for several Ca^{2+}-regulated photoproteins (33, 34), all of which catalyze the luminescent oxidation of the same substrate, coelenterazine (35). Despite the fact that all of them use the same substrate, it is very intriguing and possibly significant to find no sequence similarity among these bioluminescent proteins. Also unrelated are the primary structures of Metridia luciferase and Vargula luciferase (36) that catalyze the oxidation of a structurally similar imidazopyrazinone substrate.

A "neural network" trained to identify the presence and location of signal peptide cleavage sites in protein sequences (www.cbs.dtu.dk/services/SignalP/) suggests the existence of an N-terminal signal peptide of 17 amino acids with the putative cleavage site VQA-KS (Fig. 2) with probability close to 100%. Evidently, the cloned cDNA encodes the secreted luciferase, and this is in good agreement with the observed phenomenon of secreted bioluminescence.

The Metridia luciferase sequence analysis reveals the presence of repeats (www.ebi.ac.uk/Radar/) consisting of 31 residues (Fig. 2) having high identity (41.9%). This would suggest that the MLuc gene could have arisen by duplication of one precursor gene.

No potential glycosylation sites having the canonical sequence Asn-Xaa-(Ser/Thr) were found in the MLuc sequence.

The MLuc nucleotide sequence analysis also reveals that within the coding sequence there is an internal sequence (Fig. 2) that is identical to the highly efficient ribosome binding site (AAGGAGA) from the phage T7 major capsid protein and that is used in pET expression vectors. This internal site of translation initiation, the putative internal ribosome binding site, occurs with the appropriate spacing upstream of the ATG codon. For bacterial expression the putative internal site of translation initiation was eliminated from the coding sequence with amino acid sequence conservations to avoid the synthesis of additional truncated product.

To isolate cDNA encoding Metridia luciferase, we applied the expression cloning approach that we used for cloning the cDNAs encoding Ca^{2+}-regulated photoproteins from Obelia longissima (36) and Obelia geniculata (34). This approach has both advantages and disadvantages. The disadvantage of expression cloning is the difficulty (if it is even possible) in isolating cDNAs for proteins consisting of two or more subunits when activity requires all subunits folded together. In addition, the in vitro assay has to be very sensitive. The advantage of expression cloning occurs when the protein of interest consists of only one polypeptide and can be assayed with high specificity and sensitivity. Then the expression cloning approach can be applied with high efficiency. This approach allows isolation of cDNA for completely uncharacterized proteins because only an in vitro assay needs to be developed. Another advantage is that only the cDNA encoding a functional protein is cloned, and this
eliminates some laborious procedures arising on application of conventional methods. The cDNAs for the majority of uncharacterized luciferases and photoproteins, especially those derived from rare organisms, may be good candidates for applying the expression cloning approach.

Expression in E. coli and Purification of Metridia Luciferase—SDS-PAGE analysis of the induced E. coli BL21-CodonPlus(DE3)-RIL cells harboring the pET22-MLuc plasmid reveals an additional protein band with a molecular mass (22.0 kDa) approximately corresponding to that predicted for Metridia luciferase (Fig. 3). Crude cell lysate obtained from induced cells reveals only weak activity with coelenterazine in comparison with the total amount of recombinant protein in these cells. Evidently, most of the synthesized protein is accumulated in insoluble inclusion bodies.

For purification of luciferase the cell paste was resuspended in 20 mM Tris-HCl, pH 7.0 (1:5, w/v) and disrupted with ultrasound (20 s x 6) at 0 °C. The mixture was then centrifuged, and the supernatant was discarded. The pellet was washed sequentially with 0.9% NaCl, 0.5% Tween 20, and 20 mM Tris-HCl, pH 7.0. All the washing procedures were performed with centrifugation at 4 °C. According to measurements of luminescent activity, the total loss of MLuc during the washing steps was no more than 3%. The final pellet was resuspended in 6 M urea for 5 h at 4 °C and then centrifuged. The 6 M urea extract contained most of the total luminescence activity, 70–75%. The 6 M urea extract was loaded on a DEAE-Sepharose Fast Flow (Amersham Biosciences) column equilibrated previously with 20 mM Tris-HCl, pH 7.0. The proteins were eluted with a linear salt gradient (0–0.5 M NaCl in the same buffer) (Fig. 3). During this chromatography step the folding of luciferase takes place simultaneously with protein separation.

The bioluminescent spectrum from the reaction catalyzed with high purity MLuc has a maximum at 485 nm that is in good agreement with that observed for native luciferase (λ_max = 485 nm) (Fig. 1). This clearly indicates that Metridia luciferase can be correctly folded from the denatured state.
Expression of MLuc and FL in CHO Cells—Stable CHO cell lines expressing MLuc and FL were constructed to compare the secreted and non-secreted reporters. Cells were transfected with pcDNA-MLuc, pASM-MLuc, or pcDNA3 (without insert), respectively. No differences in cell morphology or cell growth between cells transfected with these plasmids or in comparison with untransfected cells were observed. This result clearly shows that Metridia luciferase is non-toxic for mammalian cells. During cell growth, MLuc and FL activities were measured both in cell lysates and in the medium. The cells expressing FL showed activity with the corresponding firefly luciferin only in the cell lysates. The cells expressing MLuc showed luciferase activity with coelenterazine both in the cell lysate and in the medium. The transfected cells secreted luciferase continuously, and it accumulated in the medium. To determine the time course of the secretion the medium was removed, the cells were washed with phosphate-buffered saline, and fresh medium was added at the beginning of the time course experiment. The MLuc luciferase activity can be detected within 1 h after the medium is changed (Fig. 4). The CHO cells transfected with pcDNA3 without cDNA inserts displayed no luciferase activity with either substrate. These results evidently indicate that the cloned cDNA encodes secreted luciferase.

Monitoring of Activity of A2a and NPY2 G-protein-coupled Receptors with MLuc as a Reporter—G-protein-coupled receptors constitute a superfamily of seven transmembrane-spanning proteins that respond to a diverse array of sensory and chemical stimuli (37). Over the past 15 years, nearly 350 therapeutic agents targeting seven transmembrane receptors have been successfully introduced for medical treatment. To identify compounds that modify the activity of G-protein-coupled receptors, it may be necessary to use ultrahigh throughput screening technologies to screen chemical libraries. The secreted Metridia luciferase has very good prospects for development of ultrahigh throughput screening protocols.

Fig. 5 demonstrates the capability of this novel luciferase for monitoring expression of genes encoding the A2a and NPY2 receptors belonging to the G-protein-coupled receptors superfamily. The receptor A2a is a G<sub>a</sub>-coupled receptor that increases the intracellular cAMP concentration through activation of adenylate cyclase. The receptor NPY2 is a G<sub>i</sub>-coupled receptor that decreases the intracellular cAMP concentration through inhibition of adenylate cyclase. The plasmids pcDNA3-A2a and pcDNA3-NPY2 were transiently transfected into stable CHO cells expressing MLuc under the control of a cAMP-sensitive promoter. After 24 h of cell growth the genes encoding receptors were activated with the corresponding agonist for 4 h, and MLuc activity was measured in the culture medium (Fig. 5). The medium of the A2a receptor-expressing cells stimulated by agonist 5'-N-ethylcarboxamidoadenosine reveals a high MLuc bioluminescent response. The medium of the A2a receptor-expressing cells that were not stimulated with agonist shows a very low level of bioluminescence similar to that observed for the medium from cells transfected with the pcDNA3 control plasmid. The MLuc activity determined in the medium of the NPY2 receptor-expressing cells pretreated with forskolin is obviously lower than for the medium of the cells stimulated by neuropeptide Y. The activation of the NPY2 receptor results in a 90% decrease of the MLuc bioluminescence.

Monitoring transcription activity provides important information about cell function. The application of secreted bioluminescent reporters is a powerful tool for this monitoring because it allows display of gene expression extracellularly without destroying the cells or tissues. Naturally occurring secreted luciferases are very suitable for this purpose, and this idea stimulates investigation of new bioluminescent systems. To date the cDNAs encoding several secreted luciferases have been cloned (23, 24). Vargula luciferase has been expressed in mammalian cells and has shown promising properties as a reporter enzyme (25–28). However, its large size (62.2 kDa) probably limits the value of Vargula luciferase as a reporter. Another secreted luciferase tested as a reporter is from the deep sea shrimp O. gracilorostris (24). This luciferase is a complex composed of 35- and 19-kDa proteins, and only the smaller one catalyzes the luminescent oxidation of coelenterazine. The cDNA encoding the 19-kDa protein was expressed in COS7 cells, and although the sequence included a natural putative signal for secretion at the N terminus, the culture medium showed very little luminescent activity. The luciferase isolated from the marine copepod O. princeps also possesses a naturally occurring putative signal for secretion, but there are no studies of it as a reporter in mammalian cells.

The cloned secreted Metridia luciferase possesses very promising properties as a reporter because the bioluminescent reaction catalyzed by this luciferase is (a) simple, involving only coelenterazine and molecular oxygen, and (b) highly specific in the sense that coelenterazine does not cross-react with any other mammalian proteins to produce background luminescence. Additionally, the assay is very sensitive because light can be detected with high sensitivity by modern devices. The low molecular mass of Metridia luciferase (23.9 kDa), 2.5 times less than for Vargula luciferase, for example, is also an advantage. Altogether, these properties clearly suggest that Metridia luciferase may find wide application both for biomedical stud-
throughput screening technologies.

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