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High-active truncated luciferase of copepod *Metridia longa*

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ABSTRACT

The technology of real-time imaging in living cells is crucial for understanding of intracellular events. For this purpose, bioluminescent reporters have been introduced as sensitive and convenient tools. *Metridia* luciferase (MLuc) from the copepod *Metridia longa* is a coelenterazine-dependent luciferase containing a natural signal peptide for secretion. We report the high-active MLuc mutants with deletion of the N-terminal variable part of amino acid sequence. The MLuc variants were produced in *Escherichia coli* cells, converted to an active protein, and characterized. We demonstrate that the truncated MLucs have significantly increased bioluminescent activity as against the wild type enzyme but substantially retain other properties. One of the truncated variants of MLuc was transiently expressed in HEK 293 cells. The results clearly suggest that the truncated *Metridia* luciferase is well suited as a secreted reporter ensuring higher detection sensitivity in comparison with a wild type enzyme.

1. Introduction

The technology of real-time imaging in living cells is crucial for understanding of biological events. For this purpose, bioluminescent reporters have been introduced as the sensitive and convenient tools [1]. While a number of luciferase–luciferin pairs have been identified in nature, only a handful has been sufficiently characterized for use in cells and small mammals. These are firefly and click beetle luciferases from insects [2,3], bacterial luciferases [4], Ca^{2+}-regulated photoproteins of coelenterates [5,6], *Renilla* luciferase from soft coral [7], *Gaussia* and *Metridia* luciferases of marine copepods [8,9]. The size and structure of these components vary between species, but each pair utilizes a common mechanism to produce light. The luciferase catalyzes the oxidation of small cognate luciferin molecule in course of which an electronically excited intermediate is generated that emits light upon relaxation to the ground state. Firefly luciferase and other insect luciferases catalyze the oxidation of D-luciferin in the presence of ATP and magnesium ions [10,11], whereas *Renilla* luciferase and copepod luciferases oxidize a common substrate, coelenterazine, in a co-factor-independent manner [12] and can therefore function in extracellular environments and spaces [1].

Although the real-time imaging involving bioluminescent reporters is successfully employed, intracellular assays present difficulties for continuous measurements in some cases; it is not easy to keep an intracellular concentration of luciferin at a constant level, for example, in the case of application of insect and *Renilla* luciferases. The secreted luciferases are free from these drawbacks because they enable monitoring of intracellular events with a high sensitivity without destroying cells or tissues. Hence the secreted coelenterazine-dependent luciferases are widely employed now in the optical imaging in cells and small animals [13,14].

The coelenterazine-dependent luciferases possessing apparent advantages as against other luciferases owing to simple bioluminescent reaction nevertheless have some shortcomings that limit their application. The wild type *Renilla* luciferase has low bioluminescent activity and stability, for instance; and all of them emit blue light (~480 nm) which is largely absorbed by hemoglobin and other pigmented molecules in mammals making difficult their application for imaging in the whole organisms. Recently, however, with a random mutagenesis applied, the variants of *Renilla* luciferase with increased bioluminescent activity and stability as well as remarkable red-shifted bioluminescence, especially with coelenterazine-v, have been produced [15–19]. The similar successful optimization has been conducted for *Gaussia* luciferase [20,21].

Commonly, the enzymes are composed of different functional domains some of which are not directly involved into catalysis. In this study we demonstrate that a catalytic domain of luciferase from *Metridia longa* can be isolated by simple truncating of variable

Abbreviations: MLuc, the variant of *Metridia longa* luciferase; ML164, a wild type luciferase 164 of copepod *Metridia longa*; ML164M3, *Metridia longa* luciferase truncated at Met54; ML164M4, *Metridia longa* luciferase truncated at Thr73; ML164M5, *Metridia longa* luciferase truncated at Met80.

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part of N-terminal sequence. The truncated variants reveal ~5-fold increase of bioluminescent activity and can be expressed in mammalian cell ensuring significantly higher sensitivity of detection as compared to a wild type enzyme.

2. Material and methods

2.1. Materials

Coelenterazine was obtained from Prolume Ltd. (Pinetop, USA). A stock coelenterazine solution was prepared by its dissolving in methanol, and stored at −20 °C for several days. Its concentration was calculated by absorption at 435 nm using the $\varepsilon_{435\ nm}$ = 9800 cm^{-1} M^{-1} [12]. The appropriate purification kits (QIAGEN) were used to prepare a high-purity recombinant DNA (plasmid DNA and DNA fragments). All other reagents were purchased from Sigma–Aldrich, unless otherwise stated, were of analytical reagent grade or better and used as received.

2.2. Expression constructions for truncated Metridia luciferase ML164

For expression in *Escherichia coli* cells, the truncated variants of *Metridia* luciferase ML164 were produced by mutagenesis using pET22-MLuc plasmid [8] for bacterial expression of the ML164 cDNA coding sequence without natural signal peptide as a template. Deletion mutants were constructed using the QuickChange site-directed mutagenesis kit (Stratagene) and primers: the forward 5′-GGAGATATACATATGATCAAAGCAGATATTGC-3′ for ML164M3, the forward 5′-GGAGATATACATATGACCGATGCTAACCG TGG-3′ for ML164M4, and the forward 5′-GAAGGAGATATACATAT GCCTGGCAAAAAACTG-3′ for ML164M5 variant. The reverse primers were complimentary according to the accompanying protocol. The first obligatory Met in expression construction was added in case of the ML164M4 variant started with Thr. After verification of the nucleotide sequences, the obtained plasmids were introduced into *E. coli* strain BL21-CodonPlus(DE3)-RIPL (Stratagene) for expression of *Metridia* luciferase variants.

2.3. Expression in E. coli, purification, and preparation of monomeric MLuc luciferases

Since secreted proteins lose the signal peptide during secretion, the constructs for expression in *E. coli* cells of ML164 luciferase and its truncated variants were produced without signal peptide. The *E. coli* cells BL21 CodonPlus (DE3)-RIPL (Stratagene) transformed with the plasmids pET22-ML164, pET22-ML164M3, pET22-ML164M4 and pET22-ML164M5, were cultivated with vigorous shaking at 37 °C in LB medium containing 200 μg/ml ampicillin. When the culture reached an OD_{600} of 1.0, the luciferase synthesis was induced with 1 mM IPTG. After induction, the cultivation was continued for 2 h. Cells were harvested by centrifugation at 4 °C.

The cell paste was resuspended (1:5, w/v) in a buffer 20 mM Tris–HCl pH 8.8, disrupted by sonication (20 s × 6) at 0 °C, and centrifuged thereafter. The pellet was sequentially washed with 0.15 M NaCl, 20 mM Tris–HCl pH 8.8, 0.1% Triton X-100 (×3), 2 M urea, 0.1% Triton X-100, and 20 mM Tris–HCl pH 8.8. All the washing procedures were followed by centrifugation at 4 °C. The final pellet was extracted with 6 M guanidine–HCl in 1 mM EDTA, 20 mM Tris–HCl pH 8.8.

The truncated variants and wild type MLuc were refolded in a buffer containing 1 mM EDTA, 50 mM NaCl, 5 mM cysteine, 0.5 mM cystine, 20 mM Tris–HCl pH 8.8 at 4 °C during 18 h, concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore), and purified by gel-filtration on Superdex 200 column (GE Healthcare) equilibrated with 0.5 M NaCl, 1 mM EDTA, 20 mM Tris–HCl pH 7.5 to produce monomeric forms (Fig. S1). After gel-filtration the monomeric fractions retained ~77–97% of the total activity. The final products were of high purity according to SDS–PAGE (Fig. S2).

2.4. Bioluminescence assay

Bioluminescence was measured by rapid injection of 5 μl of coelenterazine methanol solution into a luminometer cell containing a protein sample in 0.2 ml of the assay buffer 0.1 M NaCl, 50 mM Tris–HCl pH 7.5 at 10 °C. The temperature of the assay tube was supported by water circulation through a jacketed photometer.

2.5. Spectral measurements

The bioluminescence and fluorescence spectra were measured with a Cary Eclipse spectrofluorimeter (Varian) in a 0.1 M NaCl, 50 mM Tris–HCl buffer pH 7.5 at 17 °C. The bioluminescence was initiated by injection of coelenterazine methanol solution. The concentration of coelenterazine was ~0.15 mM (protein/coelenterazine molar ratio ~1:100). The bioluminescence spectra were recorded at approximately constant level of light signal. The fluorescence spectra of ML164, ML164M3, and ML164M4 were determined in half an hour after addition of coelenterazine (~2.5 μM, protein/coelenterazine molar ratio ~1:1.5) after the bioluminescence signal ceased. The bioluminescence and fluorescence spectra were corrected for spectral sensitivity of the instrument.

2.6. Expression of MLucs in mammalian cells

The pcDNA3.1(+) (Invitrogen) plasmid harboring a neomycin resistance was used for expression of MLuc luciferases in mammalian cells under constitutive cytomegalovirus (CMV) enhancer-promoter. The expression pcDNA3m-ML164 plasmid was generated by subcloning the fragment encoding full-size cDNA gene of ML164 luciferase from original plasmid pTriplEx2-MLuc [8] into the KpnI/XbaI sites of pcDNA3.1(+). The expression plasmid pcDNA3m-ML164M3 harboring the deletion mutant ML164M3 luciferase was obtained by the QuickChange site-directed mutagenesis kit (Stratagene) by using pcDNA3m-ML164 as a template and the forward 5′-CATTGGTTCAGGCACAGAAGACCGATATTGCAGATACTG-3′ and complimentary reverse primers. For better secretion of truncated ML164M3 luciferase at mammalian expression, the first and the third amino acid residues after natural signal peptide were replaced; the sequence IKA was changed to QKT (the new cleavage site QA-QKT). The efficacy of cleavage site was evaluated with SignalP 3.0 program (www.cbs.dtu.dk/services/SignalP/).

HEK 293 cells were grown in 96-well plates in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FCS at 37 °C, 5% CO_2. The transient transfection of HEK 293 cells with pcDNA3m-ML164, pcDNA3m-ML164M3, and pcDNA3.1(+) plasmids was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells (~5000 cells/well) were continued to grow in DMEM medium supplemented with 10% FCS during 7 h. After 7 h the medium was replaced by fresh medium to monitor the secretion time course of MLucs.

The medium aliquots and cells were separately assayed at regular time intervals of 2 h. For cell assay after medium removing, the cells were trypsinized with 20 μl of 0.25% trypsin/EDTA (GIBCO) for a minute and 180 μl of the appropriate neutralization medium was added, the detached cells were immediately suspended and transferred to 96-well plates. Then the cells were disrupted by twice freezing-thawing and assayed. The freezing-thawing was applied because this method had no affect on MLuc activity (data not shown) in contrast to the commonly used Triton X-100

which presence resulted in a considerable drop of MLuc light yield (~20 times at 1.5% and ~10 times at 0.5% for total light) and decrease of the light signal decay rate (data not shown). In addition, Triton X-100 increased a background signal in cell lysates apparently because of an unspecific oxidizing of coelenterazine.

The luciferase activity was assayed by rapid injection of 5 μl of coelenterazine methanol solution into a luminometer cell containing the 20-μl aliquot of the cell-free medium (or the disrupted cells) in 180 μl of the assay buffer 0.1 M NaCl, 50 mM Tris–HCl pH 7.5 at 10 °C. The coelenterazine concentration in cuvette was 4×10^{-7} M.

3. Results and discussion

M. longa is a small luminous marine copepod. The bioluminescence originates as a secretion from epidermal glands located in the head part and abdomen in response to various stimuli. Its bioluminescence is conditioned by secreted coelenterazine-dependent luciferases. With a functional screening applied, the cDNAs encoding two luciferase isospecies were cloned from the expression cDNA library of *Metridia longa* [8,22] and successfully used as a secreted reporter enzyme in mammalian cells [8,23] and *in vitro* assay [22].

The amino acid sequences of two isoforms ML164 [8] and ML39 [22] of *M. longa* and homologous *Gaussia princeps* (GpLuc) (GenBank Accession No. AAG54095) luciferases are presented in Fig. 1. The degree of identity of the ML164 sequence relative to those of ML39 and GpLuc is 80.8% and 68.6%, respectively. In essence, the difference between ML164 and other luciferases is determined by gaps in the N-terminal part of amino acid sequences (Fig. 1). It assumes that this region might be unimportant for bioluminescence. To test the supposition, the three truncated ML164 variants (ML164M3, ML164M4, and ML164M5) with deleting 53, 72, and 79 amino acid residues from N-terminus including signal peptide of 17 amino acids were constructed. The first variant was chosen to delete approximately 2/3 of the variable part of N-terminal domain and the position at Met54 was selected to express ML164M3 (166 aa) in *E. coli* cells without additional insertion of start codon (Fig. 1). The second truncated variant ML164M4 (147 aa) was constructed shorter than both ML39 and GpLuc (Fig. 1) and the position for truncating was selected before the first conservative domain of copepod luciferase sequences. The shortest variant ML164M5 (140 aa) was with additional deletion of 7 amino acids of the conservative region. The calculated molecular masses of ML164M3, ML164M4, and ML164M5 are 17.9, 16.0, and 15.1 kDa, respectively.

The preliminary testing of the effect of deletions on bioluminescent activity was performed in crude *E. coli* cell lysates. We have found that the removal of variable part leads to significant increase of luciferase activity of ML164M3 and ML164M4, approximately by a factor of 6 and 10, respectively. The deletion affecting conservative region (ML164M5) results in a 5-fold drop of bioluminescent activity as compared to ML164M4. However the activity of ML164M5 exceeded that of MLuc164 wild type luciferase more than twice. With a view of these results, the thorough studies have been carried out only for ML164M3 and ML164M4 variants.

Since at refolding from inclusion bodies the *Metridia* luciferase also forms the inactive aggregates [22], the bioluminescent properties of ML164 luciferase and its truncated variants ML164M3 and ML164M4 (Fig. 1) were studied using only monomeric forms of these proteins (see Section 2).

The temperature optimum was determined by injecting 5 μl of luciferase into 500 μl of the assay buffer (0.1 M NaCl, 50 mM Tris–HCl pH 7.5) held at a given temperature. The temperature of the assay tube was regulated by water circulation through a jacketed photometer. The temperature optima for light intensity and total light of both ML164 and its truncated variants are at 10–12 °C (Fig. 2A). The thermal inactivation of ML164, ML164M3, and ML164M4 was determined by incubating a luciferase sample in a buffer 0.5 M NaCl, 1 mM EDTA, 50 mM Tris–HCl pH 7.5 at 4 and 37 °C. Aliquots for testing the residual bioluminescence were taken in 5, 30, and 120 min. Under these conditions the ML164 retains its activity at all tested temperatures. Both truncated variants are stable only at 4 °C; ML164M3 and ML164M4 respectively lose 65% and 50% of activity at 37 °C after 2-h incubation (Fig. 2B). However the ML164M3 is more stable than ML164M4 during the first 30 min of incubations; the loss of activity was 29% and 40% respec-

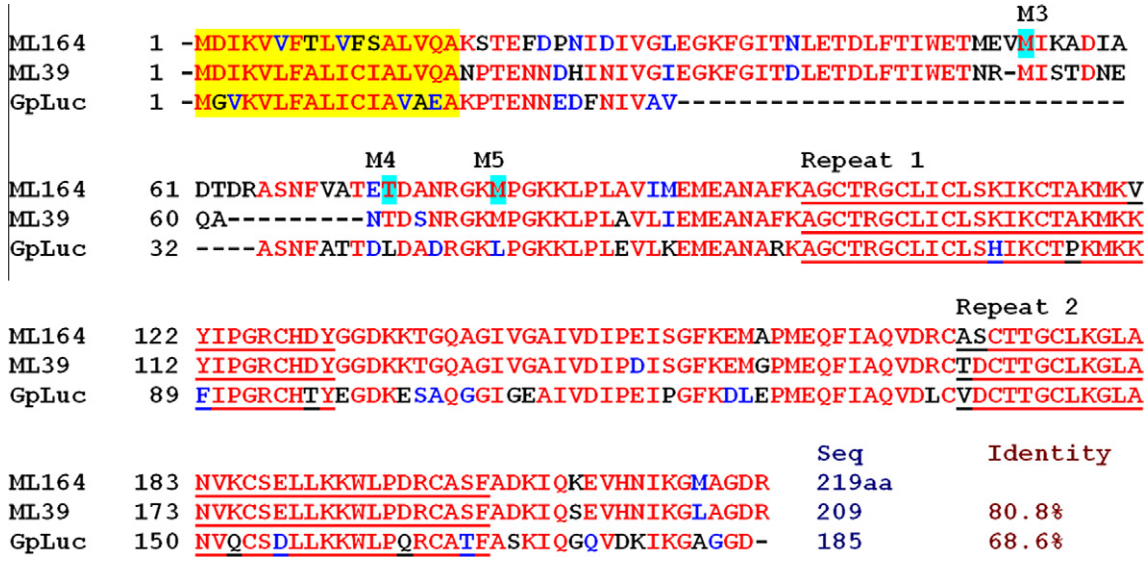

Fig. 1. Sequence alignment of ML164 and ML39 *Metridia longa* luciferase isoforms, and *Gaussia princeps* luciferase (GpLuc). The alignment was produced by ClustalW and adjusted manually using nucleotide sequence information. Red letters indicate the sequence positions in which the amino acid residues are identical, blue letters represent similar residues, and black letters show nonidentical residues. Signal peptide sequences are marked by yellow. Repeats are underlined. Gaps are shown by dashes. The amino acid residues shadowed with cyan indicate the truncating positions.

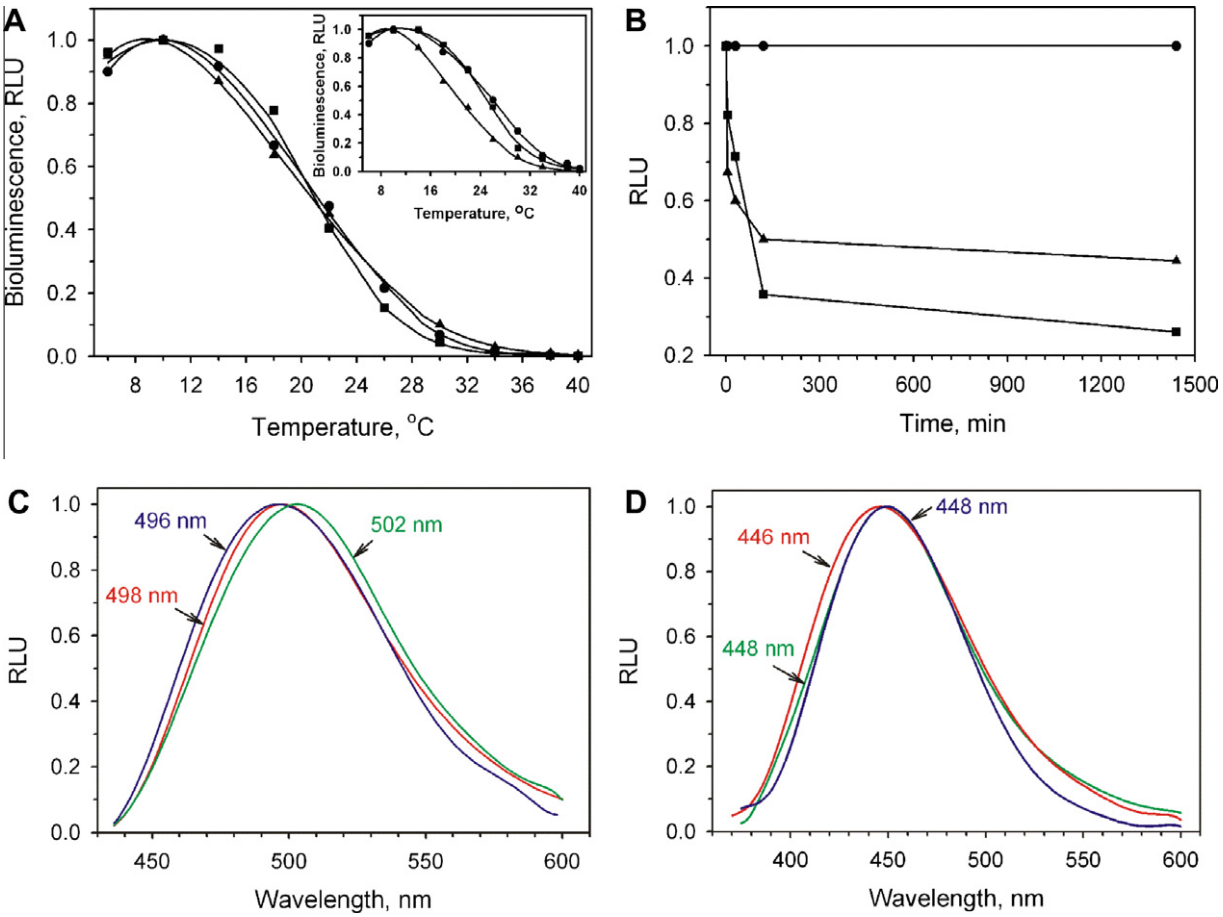

Fig. 2. Bioluminescent properties of ML164 and its truncated variants. (A) Effect of temperature on light intensity and total light (insert) of ML164 (•), ML164M3 (■), and ML164M4 (▲). (B) Thermal inactivation at 37 °C of ML164 (•), ML164M3 (■), and ML164M4 (▲). Protein concentration was 0.03 mg/ml. (C and D) Normalized bioluminescence (C) and fluorescence (D) spectra of ML164 (red), MLuc164M3 (blue), and ML164M4 (green). RLU, relative light units(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

tively. It needs to note that the temperature increase leads to decrease of bioluminescence decay rate for both MLuc164 and its truncated variants (Fig. S3).

The pH profiles of light intensities of the ML164, ML164M3, and ML164M4 are shown in Fig. S4. The truncated variants of the *Metridia* luciferase display pH optima for bioluminescence at more acidic pH as against the wild type ML164.

The light emission spectra of ML164, ML164M3, and ML164M4 reveal peaks in the range of 496–502 nm with a full width at half-maximum intensity of 80 nm (Fig. 2C). These values are very close to those which were published earlier for different preparations of *M. longa* luciferases [8,22,24]. The *M. longa* luciferase like other proteins containing no chromophores, has no fluorescence in the visible range. However, after the bioluminescence signal ceased, ML164 and its truncated variants display fluorescence (Fig. 2D). Although fluorescence intensity is low it demonstrates that coelenteramide remains bound with protein for some time after bioluminescent reaction. The fluorescence maxima of all *Metridia* luciferases are blue-shifted (λ_{max} = 446 – 448 nm) unlike bioluminescence maxima being evidence of less polar environment of coelenteramide after reaction [25–28].

Fig. 3A shows the concentration dependence on bioluminescence intensity maximum for ML164, ML164M3, and ML164M4. The bioluminescence intensity maxima reach a constant value at approximately 2 and 5 μM for truncated variants and ML164, respectively. The bioluminescent intensities for ML164M3 and ML164M4 are several times higher than that of ML164 over the whole range of coelenterazine concentrations. Fig. 3B shows the bioluminescence kinetics at substrate concentrations in the mid-range of Fig. 3A. Under exactly the same conditions of measurements (substrate and luciferase concentrations, pH, temperature, buffer) the truncated variants produce higher intensities but decay faster than ML164, so that the area under the ML164M3 and ML164M4 curves, a measure of total bioluminescence yield, is about 2.4 and 4.8 times higher than that under ML164 curve.

Fig. 3C presents a Lineweaver–Burk plot using the initial maximum bioluminescence intensity as a measure of the luciferase velocity, *V*. From this plot, the apparent Michaelis constants (K_m) and the maximal rates (V_{max}) can been determined as 2.2, 3, 1.56 μM and 2.7×10^5, 5×10^5, 1.4×10^5 RLU, for ML164M3, ML164M4, and ML164, respectively. Michaelis constants of other coelenterazine-dependent luciferases from scyphozoan medusa *Periphylla periphylla*, soft coral *Renilla reniformis* and *Renilla muelleri* are 1.1 μM [29], 0.3 μM [30], and 10 μM [31] respectively, i.e. determined K_m values for wild type ML164 and its truncated variants are in the same range as for other coelenterazine-dependent luciferases.

To characterize an efficiency of ML164 and its truncated variants we estimated the turnover numbers ($k_{cat} = V_{max}/[E]$, where [E] is luciferase concentration) and rate constants (k_{cat}/K_m) with the assumption that the total bioluminescence emitted light is in proportion to the formed product, coelenteramide, and so we will use the terms "apparent k_{cat}" and "apparent rate constant" [31]. Then the apparent k_{cat} for ML164 is 4667 s^{-1} and for ML164M3 and ML164M4 7357 and 13,333 s^{-1}, i.e. the truncated *Metridia* luciferases turn over approximately 1.6 and 2.9 times faster than

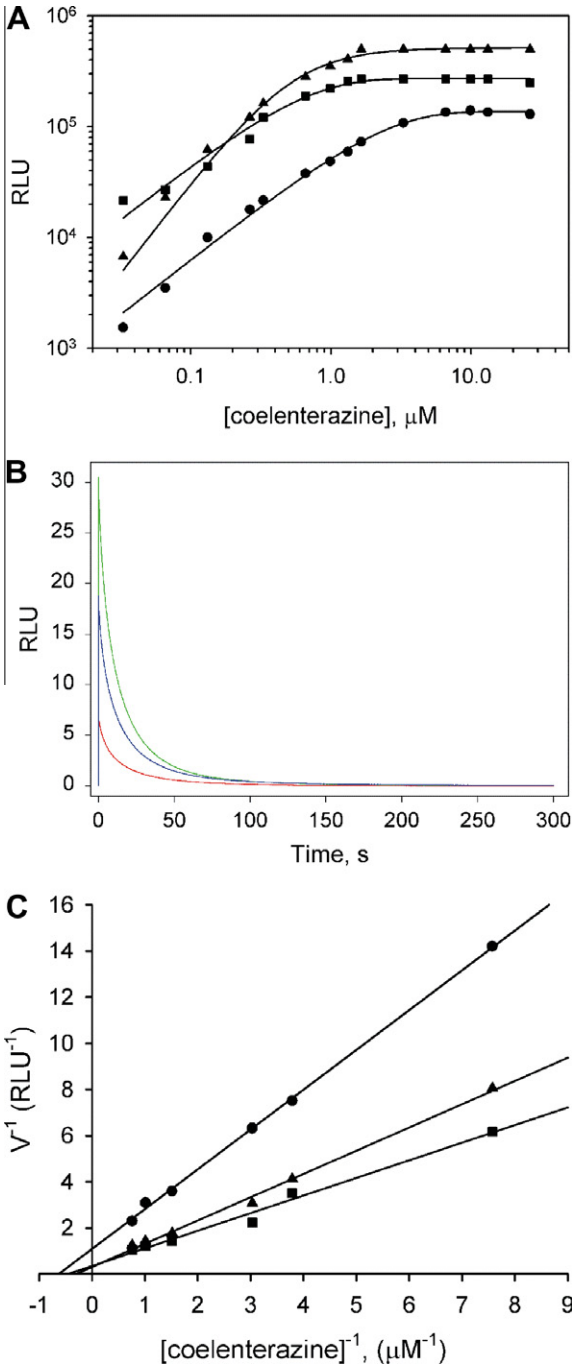

Fig. 3. Kinetics analysis of ML164, ML164M3, and ML164M4. (A) Log–log plots of ML164 (●), ML164M3 (■), and ML164M4 (▲) bioluminescence assay. The luciferase concentrations are 30 nM for ML164, 36.7 nM for ML164M3 and 37.5 nM for ML164M3. The points on the plots are average of three measurements. (B) Bioluminescent signal records for ML164 (red line), ML164M3 (blue line), and ML164M4 (green line). The concentrations of luciferases and coelenterazine were 0.5 and 12.5 nM respectively. (C) Lineweaver–Burk plot for ML164 (●), ML164M3 (■), and ML164M4 (▲). The luciferase concentration is 30 nM for ML164, 36.7 nM for ML164M3 and 37.5 nM for ML164M4, i.e. the substrate concentrations are much higher than enzyme concentration over the whole range. RLU, initial bioluminescence intensity in relative light units. The measurements were carried out at 10 °C(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

the wild type luciferase. The apparent rate constant, which characterizes the enzyme efficiency is 2991, 3344, and 4444 $\mu M^{-1}\,s^{-1}$ for ML164, ML164M3, and ML164M4 respectively; in other words the ML164M4, for example, oxidizes coelenterazine approximately 1.5

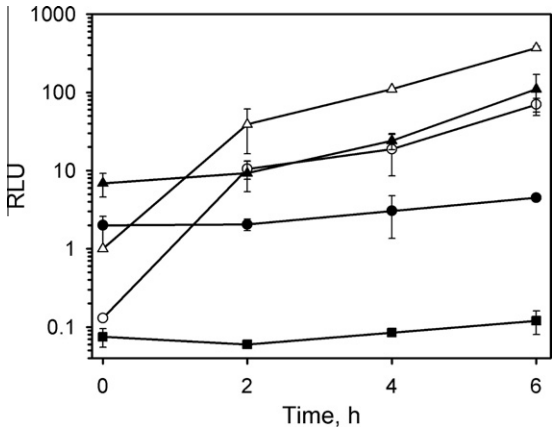

Fig. 4. Time course of ML164 (circles) and ML164M3 (triangles) activities in culture media (open symbols) and cells (filled symbols). The squares show the light signals from culture medium of cells transfected with pcDNA3. Data are the mean ± SD. RLU, relative light units.

times more effectively than the enzyme of a wild type. Of course, we realize that the values calculated for k_{cat} and k_{cat}/K_m do not correspond to those generally accepted in enzymatic kinetics since we used "relative light units" instead real product concentrations.

Application of secreted bioluminescent reporters is a powerful tool for the real-time monitoring because it allows display of intracellular events without destroying cells or tissues. To test the truncated MLuc as a secreted bioluminescent reporter, we transiently transfected HEK 293 cells with pcDNA3m-ML164, pcDNA3m-ML164M3, and pcDNA3.1 (without insert). The ML164M3 variant was selected for these experiments because although its bioluminescent activity is less than that of ML164M4, this truncated variant is slightly more stable during the first hours of incubation at 37 °C as against ML164M3. The transfected cells secrete luciferase continuously, and it is accumulated in the medium. To determine the time course of the secretion, the medium was removed and fresh medium was added at the beginning of the time course experiment. The bioluminescence for cells expressing both luciferases is detected, however, for cells expressing truncated luciferase ML164M3 the bioluminescence signal is five times higher than in cells expressing wild type ML164 luciferase (Fig. 4). This result is in a good agreement with observation *in vitro* that the bioluminescent activity of truncated luciferases is higher than that of wild type luciferase. The HEK 293 cells transfected with pcDNA3.1 without inserts displayed no luciferase activity with coelenterazine.

In summary, in this study we for the first time demonstrate that the simple truncation of N-terminal variable part of *Metridia* luciferase ML164 sequence results in the increase of light intensity and efficiency of bioluminescent reaction. At that, the truncated variants substantially retain other properties. In addition, we show that ML164 luciferase truncated at Met54 is well suited as a secreted reporter when expressed in mammalian cells ensuring significantly higher sensitivity of detection in comparison with a wild type enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.063.

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