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Functional artificial luciferases as an optical readout for bioassays

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ABSTRACT

This study elucidates functional artificial luciferases (ALucs) wholly synthesized for bioassays and molecular imaging. The ALucs bearing epitopes were newly created by amending the sequences of our previously reported ALucs in light of a multi-sequence alignment and hydrophobicity search. The synthesized ALucs are survived in live cells and stable in culture media for 25 days after secretion. The epitopes in ALucs are exposed during the secretion process and indeed valid for column purification and immunological assays. The ALucs exerted a 9400-times stronger optical intensity with a coelenterazine derivative (CTZ *i*), when compared with *Renilla reniformis* luciferase 8.6–535. A supersecondary structure of ALuc30 was predicted with respect to the X-ray crystallographic information of the coelenterazine-binding protein (CBP). The structure revealed that ALuc30 has a room for accommodating the iodide of CTZ *i*. This study guides on how to create functional artificial luciferases and predicts the structural details with the current bioinformatics technologies.

1. Introduction

Bioluminescence is an excellent optical readout for bioanalysis and molecular imaging, which is generated by luciferase catalysis of luciferin to oxyluciferin [1]. To date, many luciferase genes have been cloned from luminescent organisms via the great devotion of many researchers with time-consuming protocols from the reverse synthesis of cDNA from mRNA to identification of the sequence in databases [2].

As the luciferase sequences are accumulated in public databases, an alignment of many relative luciferase sequences facilitates new insights on the phylogenetic history, structural information, consensus amino acids, and much more information [3].

A sequence alignment, called "consensus sequence-driven mutagenesis strategy (CSMS)" was conventionally tried to find consensus amino acids and mutagenesis sites [4]. This approach is based on the premise that frequently occurring amino acids at a given position allow a larger thermostabilizing effect than less-frequent amino acids. Another bioinformatics analysis of multiple sequence alignments, called "statistical coupling analysis (SCA)" was successfully introduced to explain the evolutionary constraints of proteins [5].

Recently, we demonstrated an approach to artificially construct whole amino acid sequences of artificial luciferases (named ALuc), whose identities are distinctive from any existing luciferases [6]. The ALucs have been made by an extraction-and-linkage strategy of frequently occurring amino acids from an alignment of many existing copepod luciferases in public databases, where copepod luciferases were selected because they are the smallest ones among luciferases, exert busting bioluminescence, are genetically conserved, and carry two-repeated catalytic domains like a mirror image [7]. As many ALucs are created with distinctive sequential identities, the useful molecular design and functionalities of ALucs is an intriguing subject in this stage. Epitope- or affinity peptide-tagging schemes are powerful for expending the utilities of luciferases in bioassays. However, a conventional tagging scheme is invalid for copepod luciferases, because the N-terminal is naturally dissected and the C-terminal tagging invades the optical intensities.

This study first demonstrates creation of versatile ALucs embedding epitopes in the middle of the N-terminal region for the broad use in bioassays *in vivo* and *in vitro*, instead of conventional terminal-tagging schemes of an epitope. The epitope-embedding ALucs are designed to expose the epitope during the secretion process and to allow a column-affinity purification and immunological recognition besides bioluminescence imaging (BLI) in complex context of living subjects and *in vitro* assays. The long- and short-term optical stability of the luciferases after secretion is discussed. The characteristic substrate selectivity of ALucs was highlighted with respect to the chemical structures of the substrates. Supersecondary structures of the ALucs were predicted according to a template-based modeling (TBM) with the X-ray crystallographic information on a coelenterazine-binding protein (CBP) from *Renilla muelleri*.

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The structural characteristics were briefly discussed with respect to the substrate selectivity and the knowledge on the structures of photoproteins.

2. Methods

2.1. Synthesis of artificial luciferases

The cDNA constructs encoding ALucs 26–34 were custom-synthesized by Operon (Tokyo, Japan) in light of the sequences of ALuc23 and ALuc25, listed in Suppl. Fig. 1. Particularly, the sequences encoding ALucs 26–29 were created by replacing the codons encoding amino acids E^{150}, K^{179}, A^{182}, D^{197}, A^{209} in ALuc25 with codons encoding the corresponding amino acids of Suppl. Fig. 1, while the sequences of ALucs 30–34 were made by substituting the codons encoding amino acids from P20 to V30 in the N-terminal region of ALuc23 with codons encoding the epitope sequences of His×8 (HHHHHHHH, ALuc30 or ALuc31), *c*-Myc (EQKLISEEDL, ALuc32), hemagglutinin (HA) (YPYDVPDYA, ALuc33), or Flag tags (DYKDDDDK, ALuc34). The reason that we chose the region from P^{20} to V^{30} in the sequence is that the amino acids in the region share high sequential homogeneity with the epitopes according to a multiple alignment with CLUSTALW ver2.1 (NCBI). Because the made ALucs are supposed to be secreted by a secretion peptide (SP) at the N-terminal end, an endoplasmic reticulum (ER) retention signal (i.e., KDEL) was further tagged at the C-terminal end for repressing the secretion nature. The made cDNA constructs were subcloned into pcDNA 3.1(+) (Invitrogen) and the fidelity was ensured with a genetic sequence analyzer GenomeLab GeXP (Beckman Coulter). The schematic utilities of the made ALucs was briefly illustrated in Fig. 1A and B. As the internal references, the pcDNA 3.1(+) plasmids encoding *Gaussia princeps* luciferase (GLuc), a variant of *Renilla reniformis* luciferase (RLuc8.6–535), and *Metridia pacifica* luciferase 1 (MpLuc1) were obtained from our previous study [8]. They carry the same ER retention signal (KDEL) at the C-terminal end as ALucs for the fair comparison.

2.2. Identification of the synthesized ALucs

The phylogenetic correlation and maximal sequential identities of the made ALuc sequences when compared with the existing luciferases were determined using public tools provided by NCBI (Protein Blast and CLUSTALW ver2.1). The consequent phylogenetic tree with branch length was specified in Fig. 1C and the aligned sequence and the maximal similarity ranking are shown in Suppl. Fig. 1. The sequence of ALuc30 was aligned in three with CLUSTALW ver2.1 to investigate the internal sequential similarity (named single-sequence alignment (SSA); Fig. 1D). The stop codon at the 3′ end of cDNA encoding ALuc30 is sandwiched between *BamH*I and *Xho*I sites, and followed by cDNA encoding a membrane localization signal (MLS). This characteristic terminal design was previously reported by us and named "a bioluminescent capsule," which can carry any cargo proteins into the PM [9]. Owing to this terminal design, any cDNA encoding a protein of interest is easily inserted between *BamH*I and *Xho*I sites.

2.3. Relative optical intensities and stabilities of ALucs

The relative optical intensities and long-term stabilities of ALucs were determined for evaluating the utilities as an optical readout (Fig. 2A).

African green monkey kidney-derived COS-7 cells raised in a 96-well microplate (Nunc) were transiently transfected with an aliquot (0.1 μg per well) of a plasmid encoding one of ALucs

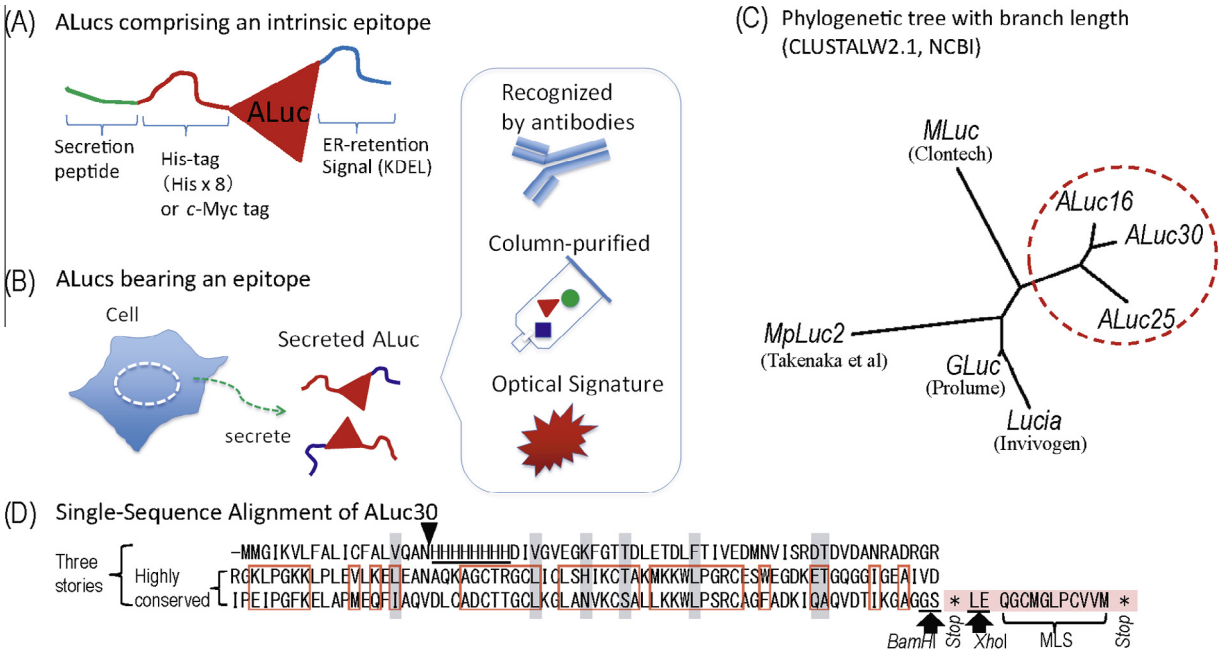

Fig. 1. Establishment of new functional artificial luciferases and the phylogenetic location in light of existing copepod luciferases. (A) Schematic diagram of ALucs 30–34. The epitopes are located in the downstream of the secretion peptide. (B) Brief illustration of the functional ALuc secreted from the host cells for immunoassay, affinity column purification, and bioluminescence assay. ALucs are naturally secreted to the extracellular compartment for column purification or immunoassays. (C) Phylogenetic tree of the existing commercial copepod luciferases and ALucs. The branch lengths reflect the relative phylogenetic distance from each other. The dotted circle indicates the relative phylogenetic location of ALucs in the tree. *Metridia pecifica* luciferase 2 (MpLuc2) was established by Takenaka et al. [17]. (D) A single-sequence alignment (SSA) of ALuc30 using CLUSTALW 2.1 (NCBI). The alignment shows a characteristic three-storey structure. The arrow head shows putative dissection site in the sequence. Red boxes and gray shadows highlight consensus amino acids. The C-terminal end is designed like a bioluminescent capsule for carrying a protein of interest to the PM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)