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Truncation of *Vargula* Luciferase Still Results in Retention of Luminescence

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Significant amino acid sequence homology in two regions of *Vargula hilgendorfii* to one in apoaequorin was reported. The intra-amino acid homology in *Vargula* luciferase between residues 81-312 and 321-540 was 19.3%, and each of this intra-homologous region contained the region homologous to apoaequorin. In order to prove the possibility that only one of the homologous regions is sufficient for luminescence, we have produced a chimeric protein comprising of only the N-terminal homologous region of *Vargula* luciferase fused to protein A. Comparison of the luminescence of this truncated luciferase indicated that there was 38.5% retention in the bioluminescence of luciferase when compared to that of the mature form of luciferase. This fact may have interesting implications for further study of engineered luciferase.

Key words: bioluminescence, luciferase, protein A, truncated, *Vargula hilgendorfii*.

The light-emitting reaction of *Vargula* luciferase is simple and unique in that it consists of the oxidation of luciferin by molecular oxygen in the presence of luciferase and does not require any energy rich compounds nor any cofactors (*1-3*). It is also a cysteine rich protein which can not be produced in *Escherichia coli* (*4*). These unique features make this protein of 555 amino-acids an interesting luciferase to engineer.

Significant amino acid sequence homology in two regions of *Vargula* luciferase (residues 97-154 and 353-411) to the same amino-acid region of apoaequorin (residues 82-144) was reported (*5*), and the fact that the substrates in *Vargula* and *Aequorea* have the same imidazopyrazine backbone structure indicates that there is an internal duplication within the luciferase gene. And as such, the regions between 81-312 and 321-540 were found to be 19.3% amino acid homologous and each contained one glycosylation site. These facts may signify that one of the homologous regions is sufficient for light emission. In order to prove this possibility, we here attempted to produce a truncated *Vargula hilgendorfii* luciferase having only the N-terminal homologous region (Pro28-Cys312) in mammalian COS-1 cells.

For this purpose, we used the chimeric protein A-*Vargula* luciferase (*4*) in which the protein A (*6*) tag allowed easy activity measurement. The construction of the expression plasmids were performed with standard techniques (*7*). *E. coli* used for the propagation of DNA was XL1-Blue obtained from Stratagene.

Protein A-*Vargula* luciferase fusion vector (pRSVPALcluc) encoding a mouse V_{NP} signal sequence (*8*) and a single Fc binding domain (SpA-D) from *Staphylococcus aureus* fused upstream of the mature form of *Vargula* luciferase (Pro29-Gln555) for expression in mammalian cells was constructed previously (*4*). For expression of the truncated luciferase, vector pRSVPALclucT, shown in Fig. 1 was constructed. Primers 5′-CCTGCAGGCGGTGGCGGATCGCCGTCAAGTACACCA-3′ and 5′-ATGCGGCCGCTTATGCAGCGCATGTCTC-3′ were synthesized with *Sse*8387I site and *Not*I site with a stop codon respectively. The N-terminal fragment of luciferase containing Pro28-Cys312 was amplified by PCR using the above primers with *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA). The DNA Thermal Cycler used was from Perkin Elmer Cetus (Takara, Kyoto). Amplified fragments were ligated to pRSVPALcluc after deleting the DNA encoding the entire luciferase fragment with *Sse*8387I and *Not*I enzymes. As a result, pRSVPALclucT having the entire cDNA of *Vargula* luciferase replaced by newly amplified DNA of truncated luciferase was constructed. All enzymes were from Takara or New England BioLabs.

Simian COS-1 cells (Riken Cell Bank, Tokyo) were transfected transiently with plasmids, pRSVPALcluc and pRSVPALclucT, by DEAE-dextran method. The cells were cultured using Dulbecco's Modified Eagle Medium (Nissui Pharmaceuticals, Tokyo) containing 10% calf serum (Gibco BRL, Grand Island, NY) in a humidified 5% CO_2 incubator at 37°C. After 3-4 days of incubation, the culture supernatant and adherent cells were collected for characterization of the expressed protein.

The cytosolic extract was prepared in 300 μl of phosphate buffered saline (PBS, pH 7.2), after sonicating the cells with a Branson sonifier in the presence of protease inhibitors. 2 μl of 0.1 μg/ml luciferin in *n*-butanol, which was chemically synthesized in accordance with the method

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of Inoue *et al.* (*9*), was added per reaction volume. The luminescence measurements were taken with ATP photometer (Sai Technology, CA, USA), immediately after the addition of luciferin.

Bioluminescent analysis of the chimeric protein indicated that there was significant luciferase activity when compared to that of mock transfected cells, as shown in Table I (1st row). The results suggest that the expression of the truncated chimeric protein in COS-1 cells was successful. To confirm this, Western blot analysis using anti-*Vargula* luciferase was performed. Briefly, 50 μl of sonicated cytosolic extract was precipitated by the addition of 50 μl of 20% trichloroacetic acid. SDS-PAGE was performed, and the electrophoresed proteins were transferred to a nitrocellulose membrane, and incubated serially with rabbit anti-luciferase antiserum raised against recombinant *Vargula* luciferase and peroxidase conjugated goat anti-rabbit antibody (Tago, Burlingame, CA) in Tris buffer containing 0.1% Tween 20. The blots were visualized by enhanced chemiluminescent detection system (Amersham, Buckinghamshire, England) for peroxidase labeled antibodies. A main band of truncated luciferase fused to SpA-D was observed, as shown in Fig. 2A (lane 3) of about 45 kDa as expected, this value being higher than the calculated molecular weight (38.7 kDa) due to glycosylation. Species of that of the mature form of *Vargula* luciferase fused to SpA-D (lane 2), whose molecular weight is about 77 kDa, was also observed, as previously reported (*4*).

In order to precisely quantitate the concentration of the truncated chimeric protein and to compare the activity with that of the mature form of luciferase, we made use of the fact that SpA-D domain binds to the Fc region of IgG, and the following procedure being performed. The luciferase activity of cytosolic extracts of pRSVPALcluc and pRSVPALclucT transfected cells was measured and then the same protein was precipitated as described before and Western blotting carried out, using rabbit IgG peroxidase.

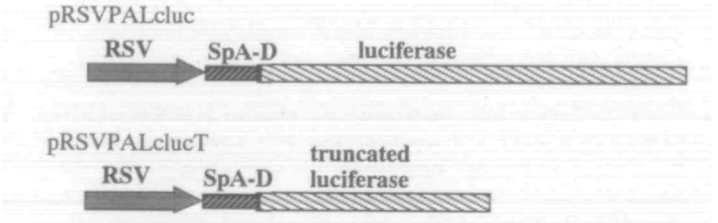

Fig. 1. **Schematic representation of the expression plasmids, pRSVPALcluc and pRSVPALclucT.** The coding region of the D-domain of protein A (SpA-D) was fused to the N-terminal region of *Vargula* luciferase (pRSVPALcluc) and to the truncated form of luciferase (pRSVPALclucT). The expression of both plasmids is driven by Rous sarcoma virus promoter (RSV).

TABLE I. **Comparison of the specific bioluminescence measurements.** Transfected COS-1 cells were sonicated in 300 μl of PBS, and then 50 μl was taken for the measurement of total bioluminescence. The band intensities of the Western blot in Fig. 2B was numerically represented for comparison with the specific bioluminescence of the truncated and the mature form of luciferase.

	Mock	pRSVPALcluc	pRSVPALclucT
Total cytosolic activity (cps)	1,000.5	18,580.8	24,913 7
Band intensity of protein	0	8,894	31,022
% Specific activity	—	100	38.5

The band intensity was further scanned with Apple Scanner (Apple Computer), and numerically represented (Table I). Figure 2B shows that distinct bands were seen at the same positions as in Fig. 2A, but the ratios of intensities were found to be slightly different. In Fig. 2A, the ratio of band intensity of lane 2 to 3 is 1:1.7, whereas that in Fig. 2B is 1:3.4, which indicates that concentration of the truncated luciferase using anti-*Vargula* luciferase resulted in 50% lower values when compared to that estimated with IgG-peroxidase. This is probably due to the decrease in binding of the anti-*Vargula* luciferase to the truncated luciferase. These results indicate that the amount of protein could be precisely determined using IgG-peroxidase. Comparison of the specific luminescence of the truncated luciferase to that of the mature form of luciferase shows that 38.5% of the activity of the mature form of luciferase is retained by the truncated form. This indicates that the N-terminal

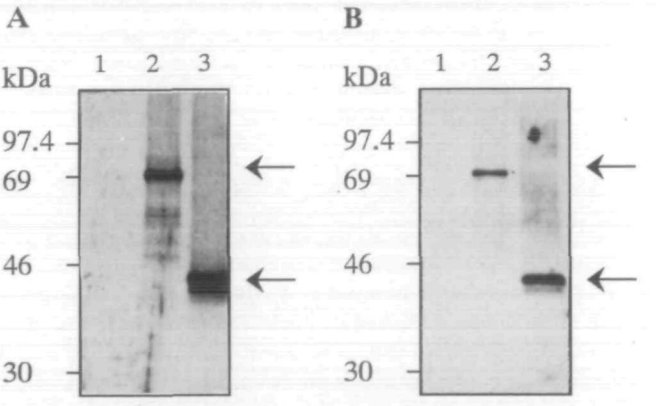

Fig. 2. **Western blot analyses of the expressed proteins.** Proteins were electrophoresed on a 7.5% reducing SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane by the semi-dry method. The expressed proteins were detected with (A) anti-*Vargula* luciferase and (B) IgG-peroxidase, as described in the text. Lanes 1, Mock-; 2, pRSVPALcluc-; and 3, pRSVPALclucT-transfected.

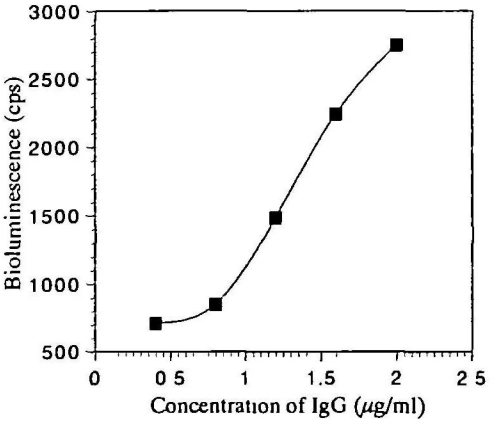

Fig 3. **Immuno-assay using the truncated chimeric luciferase.** Various amounts of IgG-Sepharose 6FF were incubated with 300 μl of culture supernatant containing the truncated chimeric luciferase at 4°C for 1 h. The unbound protein was washed out, and the bioluminescence of the luciferase bound to the beads was measured immediately after injecting luciferin.

homologous region alone may be sufficient for light emission and that the active site lies in this region. In the case of luciferase, studies have shown that the protein normally loses its luminescence when its semi-domain is deleted, due to distortion of the secondary structure. For instance, in apoaequorin, deletion of a single C-terminal proline results in retention of only 1% of the luminescence of the mature form (*10*) and in firefly luciferase, deletion of C-terminal 12 amino acids results in complete loss of activity (*11*). Therefore, it is interesting to note that the deletion of the C-terminal region of *Vargula* luciferase, still resulted in retention of luminescence.

In order to confirm that the truncated luciferase could be utilized for the quantitation of IgG, another experiment was carried out. Various amounts of IgG-Sepharose 6FF were taken and the volume of the dispersed beads was adjusted to 20 μl with Sepharose 6B. These beads were then incubated with 300 μl of the culture supernatant containing the chimeric protein at 4°C on a rotating wheel for 1 h. The beads were washed 4 to 5 times with 1 ml PBS, in order to wash away the unbound protein. The bioluminescent activity of luciferase bound to the beads was measured after dispersing the beads in 100 μl of PBS and injecting luciferin just before measurement, as described above. Figure 3 shows that there is a linear relationship between the amount of human IgG covalently immobilized on Sepharose and the bioluminescence.

In conclusion, this study has shown that the *Vargula* luciferase retained considerable activity even when the C-terminal 241 amino-acids were deleted and the N-terminal homologous region may be sufficient for the luminescence. Although further studies have to be carried out to determine the amino acids responsible for the bioluminescence, the fact that truncated luciferase retained some activity, may have important implications for the further development of engineered luciferases.

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