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Unconventional secretion of the mutated 19 kDa protein of *Oplophorus* luciferase (nanoKAZ) in mammalian cells

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ABSTRACT

The putative amino-terminal signal peptide of the catalytic 19 kDa protein (KAZ) of *Oplophorus* luciferase was found to be a functional secretory peptide in mammalian cells. A 16 amino acid substituted mutant of KAZ (nanoKAZ) could be secreted from mammalian cells using the amino-terminal signal peptide of KAZ, but KAZ could not be secreted at all. Notably, nanoKAZ lacking the amino-terminal signal peptide could be secreted from mammalian cells, and the distribution of nanoKAZ on the cell membrane was confirmed by video-rate bioluminescence imaging. Thus, nanoKAZ lacking the amino-terminal signal peptide was expressed in the cytoplasm, translocated to the cell membrane, and released into the culture medium through an endoplasmic reticulum–Golgi-independent pathway.

1. Introduction

Oplophorus luciferase of the deep sea shrimp *Oplophorus gracilorostris* is a secretory protein [1] that catalyzes the oxidation of coelenterazine (a luciferin) to emit light (λ_{max} = ~460 nm) [2]. The luciferase consists of the 19 kDa and 35 kDa proteins with a total molecular weight of 106 kDa [2], and the cDNA cloning of the 19 kDa and 35 kDa proteins showed that both proteins have a putative secretory signal peptide at the amino terminus [2]. The independent expression of the genes encoding the 19 kDa and 35 kDa proteins indicated that the 19 kDa protein (designated as KAZ) has the catalytic function of oxidizing coelenterazine [2–5], but the function of the 35 kDa protein is still unclear. When KAZ possessing a putative amino-terminal signal peptide was expressed in mammalian cells, KAZ was not secreted into the culture medium, and the luminescence activity of KAZ was detected in cell extracts [2]. In contrast, a KAZ mutant substituted at 16 amino acid positions, named "nanoLuc" [6] or "nanoKAZ" [7,8], could be secreted into the culture medium using the secretory signal peptide of human interleukin-6 [6] and *Gaussia* luciferase (GLase) [7,8]. Furthermore, functional analysis by site-specific mutagenesis suggested that all 16 amino acid substitutions in wild-type KAZ might be necessary for efficient secretion in mammalian cells [8]. In mammalian cells, proteins possessing a signal peptide at the amino terminus are transported to the plasma membrane or the extracellular medium through an endoplasmic reticulum (ER)/Golgi-dependent pathway [9,10]. On the other hand, the protein secretion by the amino-terminal signal peptide-independent pathway was known as "unconventional protein secretion" and has also been reported in mammalian cells [11,12].

Here we characterized the amino-terminal signal peptide of wild-type KAZ for protein secretion in mammalian cells. We also demonstrated that nanoKAZ lacking the amino-terminal signal peptide was secreted into the culture medium, and the distribution of nanoKAZ to the cell membrane was confirmed by video-rate bioluminescence imaging. Our results suggested that nanoKAZ lacking the amino-terminal signal peptide can be secreted from mammalian cells through the ER/Golgi-independent pathway.

2. Materials and methods

2.1. Construction of expression vectors in mammalian and bacterial cells

For expression in mammalian cells, vectors for KAZ and nanoKAZ with or without the signal peptide sequences of *Gaussia*

Abbreviations: KAZ, the catalytic 19 kDa protein of *Oplophorus* luciferase; nanoKAZ, a KAZ mutant with 16 amino acid residue substitutions; GLase, *Gaussia* luciferase; GLsp, the amino-terminal signal peptide of *Gaussia* luciferase for secretion; GLsp-nanoKAZ, nanoKAZ with the signal peptide of GLase; KZsp, the amino-terminal signal peptide of the catalytic 19 kDa protein of *Oplophorus* luciferase; ER/Golgi, endoplasmic reticulum–Golgi; ZZ domain, synthetic IgG-binding domain; I_{max}, maximum intensity of luminescence; rlu, relative light units.

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luciferase (GLsp) and the 19 kDa protein of *Oplophorus* luciferase (KZsp) were constructed as follows (Fig. 1A). The *Asp*718/*Xba*I and *Eco*RI/*Xba*I fragments of *KAZ* and *nanoKAZ* were ligated to the corresponding sites of pcDNA3 (Invitrogen, Carlsbad, CA) and pcDNA3-GLsp [7] to produce the vectors of pcDNA3-KAZ, pcDNA3-nanoKAZ, pcDNA3-GLsp-KAZ, pcDNA3-GLsp-dnKAZ, pcDNA3-KZsp-KAZ, and pcDNA3-KZsp-dnKAZ, respectively (Supplementary Fig. S1). To identify the signal peptide of KZsp, pcDNA3-KZsp-GL and pcDNA3-GLsp-GL were also constructed and used as control vectors. Expression vectors for pcDNA3-nanoLuc and pcDNA3-GLsp-nanoLuc were prepared by the same procedures (Fig. 1A). All plasmids contained the identical nucleotide sequence from the CMV promoter to the Kozak consensus sequence at the initial methionine codon. In pcDNA3-GLsp-dnKAZ, the secreted nanoKAZ lacked the extra 7 amino acid residues (K-L-G-T-T-M-V) at the amino terminus on comparison with pcDNA3-GLsp-nanoKAZ [7] (Fig. 1A). For expression in *Escherichia coli* cells, the *Eco*RI/*Xba*I fragment of *nanoKAZ* was ligated to the *Eco*RI/*Xba*I site of pCold II (Takara Bio, Kyoto, Japan) [13] and pCold-ZZ-P-X [14] to produce pCold-nanoKAZ and pCold-ZZ-P-nanoKAZ [7], respectively (Fig. 1B, Supplementary Fig. S1). Furthermore, the truncated nanoKAZ at the amino- or carboxyl-terminal regions was expressed as follows. *Eco*RI/*Xba*I fragments of *nanoKAZ* obtained by PCR using the synthetic primers (Supplementary Table S1) were inserted into the *Eco*RI/*Xba*I site of pcDNA3, pcDNA3-GLsp [7], pCold II [13], and pCold-ZZ-P-X vectors [14] (Fig. 1C).

2.2. Expression of nanoKAZ in mammalian and E. coli cells

To express nanoKAZ in mammalian cells, a Chinese hamster ovary-K1 (CHO-K1) cell line was cultured in Ham's F-12 medium (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Biowest, France), 100 units/mL of penicillin G, and 0.1 mg/mL of streptomycin (Wako Pure Chemicals) at 37 °C in a humidified atmosphere of 5% CO_2. For transient expression, cells (1×10^5 cells in 2 mL) in a 6-well plate (Nunc) were transfected using the transfection reagent of FuGENE (3 μL) (Promega, Madison, WI) with 1 μg of the expression vector and 0.1 μg of pGL4.13 [Luc2/sv40] (Promega) as an internal control [8]. To establish a stable cell line expressing nanoKAZ in the culture medium, cells transfected with pcDNA3-GLsp-nanoKAZ [7] or pcDNA3-nanoKAZ (this study) were selected in 800 μg/mL of G418 sulfate (Calbiochem, CA), and stable transformants of

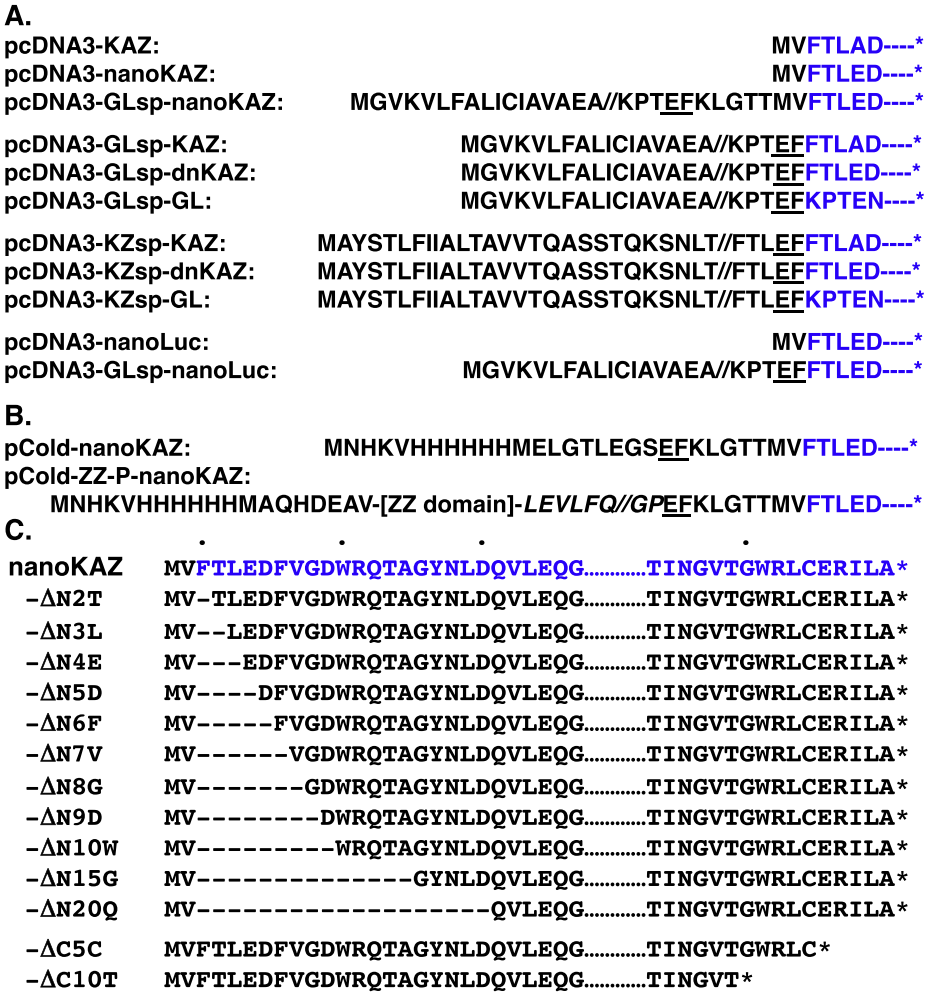

Fig. 1. Expression of the catalytic 19 kDa protein (KAZ) and mutated 19 kDa protein (nanoKAZ) of *Oplophorus* luciferase in mammalian cells and *E. coli* cells. Amino acid sequences shown in blue indicate the coding regions of KAZ, nanoKAZ, nanoLuc, and GLase. The underlined EF sequence indicates the restriction site of *Eco*RI. "//" represents the cleavage sites of the amino-terminal signal peptide and PreScission protease (the recognition site *LEVLFQGP* is italicized). "*" indicates the carboxyl terminus of the protein. (A) Amino-terminal sequences of KAZ and nanoKAZ expressed in mammalian cells in the presence or absence of the amino-terminal signal peptides of *Gaussia* luciferase (GLsp) and *Oplophorus* luciferase (KZsp). (B) Amino-terminal sequences of nanoKAZ expressed in *E. coli* cells using pCold-nanoKAZ and pCold-ZZ-P-nanoKAZ. (C) Deletion of amino and carboxyl regions of nanoKAZ. Dashes (–) indicate deleted amino acid residues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CHO-K1/pcDNA3-GLsp-nanoKAZ/clone No. B10 and CHO-K1/pcDNA3-nanoKAZ/clone No. B11 were isolated. For luminescence assay, the culture medium and cell extracts were used. To prepare cell extracts, cells were washed 3 times with 3 mL of PBS (D-PBS (–), Wako Pure Chemicals), suspended in 1 mL of PBS, and disrupted by sonication using a Branson (Danbury, CT) model 250 sonifier for 3 s. For Western blot analysis, the culture medium of CHO-K1 cells stably expressing nanoKAZ was replaced with a serum-free medium (MCDB201, Sigma, St. Louis, MO). After incubation for 72 h, the medium was concentrated using an Amicon Ultra centrifugal filter unit (MWCO 10,000, Millipore Co., Billerica, MA) and was used for the experiments.

The procedures for protein expression in *E. coli* cells were the same as previously described [8]. Briefly, the host strain BL21 possessing the expression vector was grown in 10 mL of Luria–Bertani broth containing ampicillin (50 μg/mL) at 37 °C for 3 h. After addition of IPTG to the culture medium at a final concentration of 0.1 mM, bacterial cells were further incubated at 15 °C for 20 h. One milliliter of cultured cells was collected, suspended in 0.5 mL of 30 mM Tris–HCl (pH 7.6)–10 mM EDTA, disrupted by sonication for 5 s, and used as "cell extracts" of *E. coli* cells.

2.3. Determination of luminescence activity

Luminescence activity was determined using an Atto (Tokyo, Japan) AB2200 luminometer (Ver.2.07, rev4.21) with a 0.23% neutral density filter, as previously described [7,8]. The reaction mixture (100 μL) contained coelenterazine (1 μg/μL in ethanol, JNC Co., Tokyo, Japan) in 30 mM Tris–HCl (pH 7.6)–10 mM EDTA, and the luminescence reaction was started by adding 1–5 μL of the enzyme solution to the reaction mixture. Luminescence intensity was recorded in 0.1 s intervals for 60 s.

2.4. Protein analysis

SDS–PAGE analysis was performed under reducing conditions using a 12% or 16% separation gel (TEFCO, Tokyo, Japan), and the gel was stained with a colloidal CBB staining kit (TEFCO). Protein concentration was determined with a commercially available kit (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard (Pierce, Rockford, IL). Western blot analysis was performed using anti-*Oplophorus* luciferase serum (×1000 dilution), as previously described [2].

2.5. Amino acid sequence analysis

The protein bands separated by SDS–PAGE were transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), stained with 0.1% Ponceau S (TCI, Tokyo, Japan) in 5% acetic acid, and then were subjected to amino acid sequence analysis on an Applied Biosystems (Foster City, CA) model Procise 492HT gas-phase protein sequencer.

2.6. Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was measured on an AutoFLEX III (Bruker Daltonics) in the positive linear mode using sinapic acid as a matrix, as previously described [15].

2.7. Bioluminescence imaging

The methods of video-rate bioluminescence imaging were essentially the same as previously described [16–18]. CHO-K1 cells stably expressing nanoKAZ (1×10^4 cells in 2 mL) were cultured on a 35-mm glass-bottom dish (Iwaki Glass, Cat. No. 3910-35, 27ϕ). After incubation for 48 h, cells were washed 3 times with Hanks' balanced sodium solution (HBSS, Wako Pure Chemicals), and the luminescence reaction was started by addition of *h*-coelenterazine (3 μg/mL, JNC Corporation, Tokyo, Japan) in HBSS. Luminescence signals were detected at 37 °C using a model IX81-ZDC microscope (Olympus Co., Tokyo, Japan) equipped with a back-thinned EM-CCD camera (model C9100-14; 1024 × 1024 pixels, pixel size 13 μm; Hamamatsu Photonics, K.K., Hamamatsu, Japan) in a dark box. A high numerical aperture (NA) objective lens of UPLSAPO 60× O (NA1.35, Olympus) was used, and exposure time was 500 ms or 5 s. Data of luminescence signals were collected on a computer hard disk using AQUACOSMOS software version 2.6 (Hamamatsu Photonics, K.K.) with an acquisition mode of 1 × 1 binning, fast scanning, EM gain level = 255, and photon-counting level = 1.

3. Results and discussion

3.1. Amino-terminal signal peptide of KAZ acts as a functional signal peptide for secretion in mammalian cells

Secretory proteins in mammalian cells contain an amino-terminal signal peptide for secretion. Previously, we tried to express KAZ in mammalian cells using a putative amino-terminal signal peptide of wild-type KAZ (KZsp) [2] and the functional signal peptide of *Gaussia* luciferase (GLsp) [8]. However, significant secretion of KAZ into the culture medium was not observed using KZsp and GLsp [2,8]. To investigate whether KZsp is a functional secretory peptide in mammalian cells, we constructed the expression vectors of pcDNA3-KZsp-KAZ, pcDNA3-KZsp-dnKAZ, and pcDNA3-KZsp-GL. As a control vector for secretion of nanoKAZ and GLase, we used pcDNA3-GLsp-dnKAZ and pcDNA3-GLsp-GL, respectively (Fig. 1A, Supplementary Fig. S1). The expression vectors of pcDNA3-KAZ and pcDNA3-nanoKAZ, which lacked the amino-terminal signal peptide for secretion, were also constructed (Supplementary Fig. S1). These plasmids were transiently expressed in CHO-K1 cells, and the luminescence activities of the culture medium and cell extracts were determined. The luminescence activities in the culture medium using pcDNA3-GLsp-nanoKAZ [7] and pcDNA3-GLsp-dnKAZ were almost the same, indicating that the extra 7 amino acid residues (K-L-G-T-T-M-V) at the amino terminus did not affect the secretion of nanoKAZ (unpublished results). As summarized in Table 1, wild-type KAZ was not secreted into the culture medium when KZsp and GLsp were used, as previously described [2,8]. In contrast, nanoKAZ possessing KZsp and GLsp were efficiently secreted into the culture medium. These results indicated that KZsp is a functional signal peptide for the secretion of the extracellular proteins from mammalian cells. The ability of KZsp for GLase secretion was

Table 1
Evaluation of the amino-terminal signal peptide of the 19 kDa protein (KZsp) for secretion in CHO-K1 cells after transfection for 23 h.

Expression vector (pcDNA3-)	Signal peptide for secretion	Relative luminescence activity (I_{max})	
		Culture medium	Cell extracts
Blank	–	0.006	0.008
KAZ	–	0.006	0.004
KZsp-KAZ	+	0.005	0.002
GLsp-KAZ	+	0.005	0.006
nanoKAZ	–	0.54	3.65
KZsp-dnKAZ	+	1.0 [a]	0.35
GLsp-dnKAZ	+	5.0	0.96
KZsp-GL	+	60.8	25.6
GLsp-GL	+	129.4	25.0

[a] 3.6×10^5 rlu/well.

Table 2
Transient expression of the mutated 19 kDa protein of *Oplophorus* luciferase (nanoKAZ) in the presence and absence of the amino-terminal signal peptide for secretion in CHO-K1 cells after transfection for 23 h and 46 h.

Expression vector (pcDNA3-)	Signal peptide for secretion	Relative luminescence activity (I_{max})			
		23 h		46 h	
		Culture medium	Cell extracts	Culture medium	Cell extracts
nanoKAZ	–	0.57	1.92	8.80	2.59
nanoLuc	–	0.31	1.19	7.10	1.79
GLsp-dnKAZ	+	1.0 [a]	0.10	4.21	0.11
GLsp-nanoLuc	+	0.75	0.10	3.92	0.07
GLsp-KAZ	+	0.008	0.029	0.003	0.050
Blank	–	0.004	0.004	0.001	0.002

[a] 5.6×10^5 rlu/well.

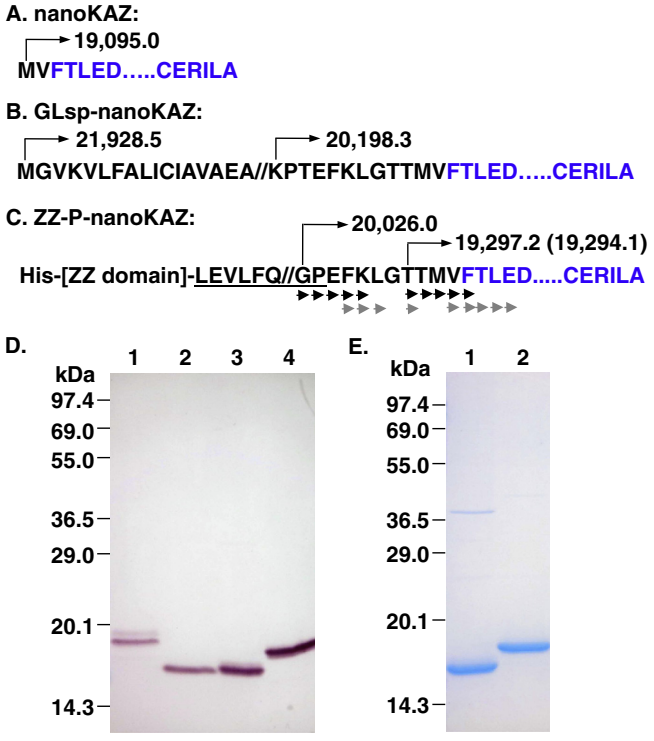

Fig. 2. Identification of nanoKAZ secreted from CHO-K1 cells in the absence of the signal peptide sequence by mass spectrometry and amino-terminal sequence analysis. (A) Predicted average mass value (19,095.0) of secreted nanoKAZ using pcDNA3-nanoKAZ in CHO-K1 cells. (B) Predicted average mass value (20,198.3) of secreted nanoKAZ using pcDNA3-GLsp-nanoKAZ in CHO-K1 cells. (C) Amino-terminal regions of purified ZZ-P-nanoKAZ after digestion with PreScission protease. Black and gray arrowheads show amino acid residues of major and minor sequences, respectively, identified by amino-terminal sequence analysis. The predicted average mass values of cleaved nanoKAZ and truncated nanoKAZ are 20,026.0 and 19,297.2, respectively. The *m*/*z* value of 19,294.1 in parenthesis was determined by MALDI-TOF-MS. (D) Western blot analysis of nanoKAZ secreted into the culture medium from CHO-K1 cells using anti-*Oplophorus* luciferase serum. Lane 1, concentrated serum-free culture medium from 200 μL of CHO-K1/pcDNA3-GLsp-nanoKAZ/clone No. B10 cells (1.8×10^5 rlu); lane 2, concentrated serum-free culture medium from 200 μL of CHO-K1/pcDNA3-nanoKAZ/clone No. B11 cells (2.8×10^5 rlu); lane 3, truncated nanoKAZ from purified nanoKAZ (320 ng protein); lane 4, purified nanoKAZ (320 ng protein) from *E. coli* cells. (E) SDS–PAGE analysis of truncated form of nanoKAZ obtained by treatment of PreScission protease with purified ZZ-P-nanoKAZ. Proteins were analyzed using a 16% gel under reducing conditions. Lane 1, truncated nanoKAZ (1.6 μg) obtained from purified nanoKAZ; lane 2, purified nanoKAZ (1.6 μg).

approximately half of that of GLsp based on luminescence activity in the culture medium (Table 1).

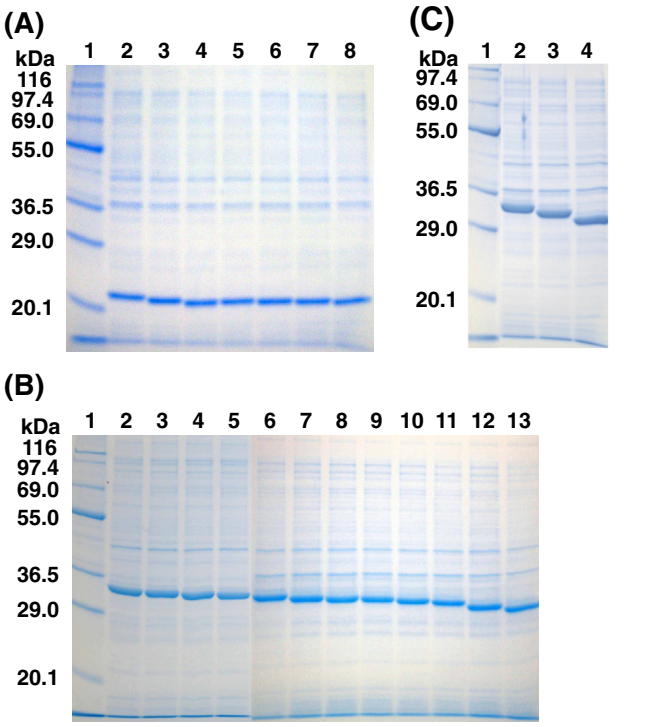

Fig. 3. SDS–PAGE analyses of the amino- and carboxyl-terminal truncated forms of nanoKAZ expressed in *E. coli* cells using pCold II and pCold-ZZ-P-X vectors. Cell extracts of *E. coli* cells (5 μL) as described in Section 2 were run on a 12% gel under reducing conditions. (A) Expression of amino-terminal truncated forms of nanoKAZ using pCold II vector. Lane 1, protein size marker (TEFCO); lane 2, nanoKAZ; lane 3, –ΔN5D; lane 4, –ΔN6F; lane 5, –ΔN7V; lane 6, –ΔN8G; lane 7, –ΔN9D; lane 8, –ΔN10W. (B) Expression of amino-terminal truncated forms of nanoKAZ using pCold-ZZ-P-X vector. Lane 1, protein size marker (TEFCO); lane 2, ZZ-P-nanoKAZ; lane 3, –ΔN2T; lane 4, –ΔN3L; lane 5, –ΔN4E; lane 6, –ΔN5D; lane 7, –ΔN6F; lane 8, –ΔN7V; lane 9, –ΔN8G; lane 10, –ΔN9D; lane 11, –ΔN10W; lane 12, –ΔN15G; lane 13, –ΔN20Q. (C) Expression of carboxyl-terminal truncated nanoKAZ using pCold-ZZ-P-X vector. Lane 1, protein size marker (TEFCO); lane 2, ZZ-P-nanoKAZ; lane 3, –ΔC5C; lane 4, –ΔC10T.

3.2. Secretory expression of nanoKAZ from mammalian cells in the absence of the amino-terminal signal peptide

During our study of transient expression using pcDNA3-nanoKAZ, we detected luminescence activity in the culture medium of CHO-K1 cells after transfection for 23 h (Table 1). Further, after transfection for 46 h, luminescence activity in the culture medium was more than 10-fold higher than that observed at 23 h, and luminescence activity using pcDNA3-nanoKAZ was approximately 2-fold higher than that using pcDNA3-GLsp-dnKAZ (Table 2). Similar results were obtained using pcDNA3-nanoLuc, which contains *nanoLuc* having 72% nucleotide sequence homology to *nanoKAZ* with an identical amino acid sequence [6,7] (Table 2). These results suggested that nanoKAZ lacking the amino-terminal signal peptide was expressed in the cytoplasm and then secreted into the culture medium. Furthermore, similar secretion of nanoKAZ into the culture medium was also observed in HeLa and COS-1 cells using pcDNA3-nanoKAZ (unpublished results). Thus, secretion of nanoKAZ lacking the amino-terminal signal peptide might occur generally in mammalian cells.

3.3. Determination of molecular size of secreted nanoKAZ in the culture medium

To identify the secreted nanoKAZ in the culture medium, we established stable transformants of CHO-K1 cells using

Table 3
Luminescence activity of nanoKAZ with deletions at the amino-terminal region in *E. coli* and CHO-K1 cells.

N-terminal truncated protein	Relative luminescence activity (I_{max}, %)					
	E. coli BL21 cells		CHO-K1 cells[a]			
	pCold-nanoKAZ	pCold-ZZ-P-nanoKAZ	pcDNA3-GLsp-nanoKAZ		pcDNA3-nanoKAZ	
	Cell extracts	Cell extracts	Culture medium	Cell extracts	Culture medium	Cell extracts
nanoKAZ	100[b]	100[c]	100[d]	4.8	100[e]	29.4
−ΔN2T	(−)	93.4	97.0	3.4	93.4	23.3
−ΔN3L	(−)	82.0	87.7	4.4	50.6	16.2
−ΔN4E	(−)	74.0	60.5	2.9	4.6	9.7
−ΔN5D	87.0	73.6	56.2	1.7	0.1	1.5
−ΔN6F	40.0	59.8	0.5	0.3	0.001	0.06
−ΔN7V	1.6	5.8	0	0.1	0	0.007
−ΔN8G	3.9	10.1	0.001	0.2	>0.0001	0.01
−ΔN9D	0.01	0.3	0	>0.0001	0	0
−ΔN10W	0.03	1.6	0	0.002	0	0
−ΔN15G	(−)	0	0	0	0	0
−ΔN20Q	(−)	0	0	0	0	0

(−): not determined.
[a] After transfection for 44 h.
[b] 1.1×10^5 rlu/μL of cell extracts.
[c] 1.7×10^5 rlu/μL of cell extracts.
[d] 6.0×10^6 rlu/well.
[e] 7.4×10^6 rlu/well.

Table 4
Luminescence activity of nanoKAZ with deletions at the carboxyl terminal region in *E. coli* and CHO-K1 cells.

N-terminal truncated protein	Relative luminescence activity (I_{max}, %)					
	E. coli BL21 cells		CHO-K1 cells[a]			
	pCold-nanoKAZ	pCold-ZZ-P-nanoKAZ	pcDNA3-GLsp-nanoKAZ		pcDNA3-nanoKAZ	
	Cell extracts	Cell extracts	Culture medium	Cell extracts	Culture medium	Cell extracts
nanoKAZ	(−)	100[b]	100[c]	5.4	100[d]	29.4
−ΔC5C	(−)	6.1	0.01	0.31	0.08	0.28
−ΔC10T	(−)	0	0	0	0	0

(−): not determined.
[a] After transfection for 44 h.
[b] 2.0×10^5 rlu/μL of cell extracts.
[c] 5.0×10^6 rlu/well.
[d] 7.3×10^6 rlu/well.

pcDNA3-nanoKAZ and pcDNA3-GLsp-nanoKAZ. The secreted proteins in the culture medium were detected by Western blot analysis using anti-*Oplophorus* luciferase [2] (Fig. 2). On SDS–PAGE analysis, a truncated form of recombinant nanoKAZ was used as a protein size marker. Previously, we described the purification of recombinant nanoKAZ from ZZ-P-nanoKAZ by treatment with PreScission protease [7]. When nanoKAZ digested with PreScission protease was stored at 4 °C for 6 months without further purification, we found that nanoKAZ with a smaller size was formed (Fig. 2E). By amino-terminal amino acid sequence analysis and mass spectrometry, we concluded that the smaller-sized nanoKAZ was a truncated form of nanoKAZ (T-T-M-V-F-T-L-E-D-, underline sequenced; black arrowheads in Fig. 2C), having an m/z value of 19,294.1. Also, minor amino acid sequences in the purified nanoKAZ and the truncated nanoKAZ were detected and estimated to be less than approximately 10% of the proteins in terms of the initial yield of the first reaction cycle of amino acid sequence analysis (gray arrowheads in Fig. 2C). Western blot analysis shown in Fig. 2D indicated that the molecular size of the secreted nanoKAZ in the absence of the signal peptide (lane 2) was close to that of the truncated form of nanoKAZ (lane 3). This result suggested that the protein secreted when using pcDNA3-nanoKAZ might have the amino-terminal sequence of M-V-F-T-L-E-D- with 171 amino acid residues (Fig. 2A), and nanoKAZ lacking the amino-terminal signal peptide may be secreted from mammalian cells without further protein processing of nanoKAZ.

3.4. Effect of amino- and carboxyl-terminal truncation on secretory ability and luminescence activity of nanoKAZ in CHO-K1 cells

To investigate the relationship between the secretory ability and luminescence activity of nanoKAZ in CHO-K1 cells, we expressed the truncated forms of nanoKAZ at the amino- and carboxyl-terminal regions in *E. coli* and CHO-K1 cells (Fig. 1C). The protein expression levels of various truncated forms of nanoKAZ in *E. coli* cells were almost the same as those of nanoKAZ using pCold II and pCold-ZZ-P-X vectors (Fig. 3). As summarized in Tables 3 and 4, truncation of 10 amino acid residues at the amino- and carboxyl-terminal regions caused significant loss of luminescence activity and secretory ability in CHO-K1 cells. These results suggest that both terminal regions of nanoKAZ might be structurally necessary to maintain the catalytic activity and secretion of nanoKAZ.

3.5. Unconventional secretion of nanoKAZ in mammalian cells

To clarify whether the secretion of nanoKAZ involves the ER/Golgi-dependent pathway, brefeldin A, an inhibitor of ER/Golgi-dependent secretion [19,20], was added to the culture medium of CHO-K1 cells. Secretion of nanoKAZ using pcDNA3-GLsp-nanoKAZ was inhibited by over 95%, but not using pcDNA3-nanoKAZ (Fig. 4A). The export of unconventional secretory proteins lacking a typical amino-terminal signal peptide is not affected by brefeldin A [11,12,19,20]. Thus, nanoKAZ lacking the amino-terminal signal

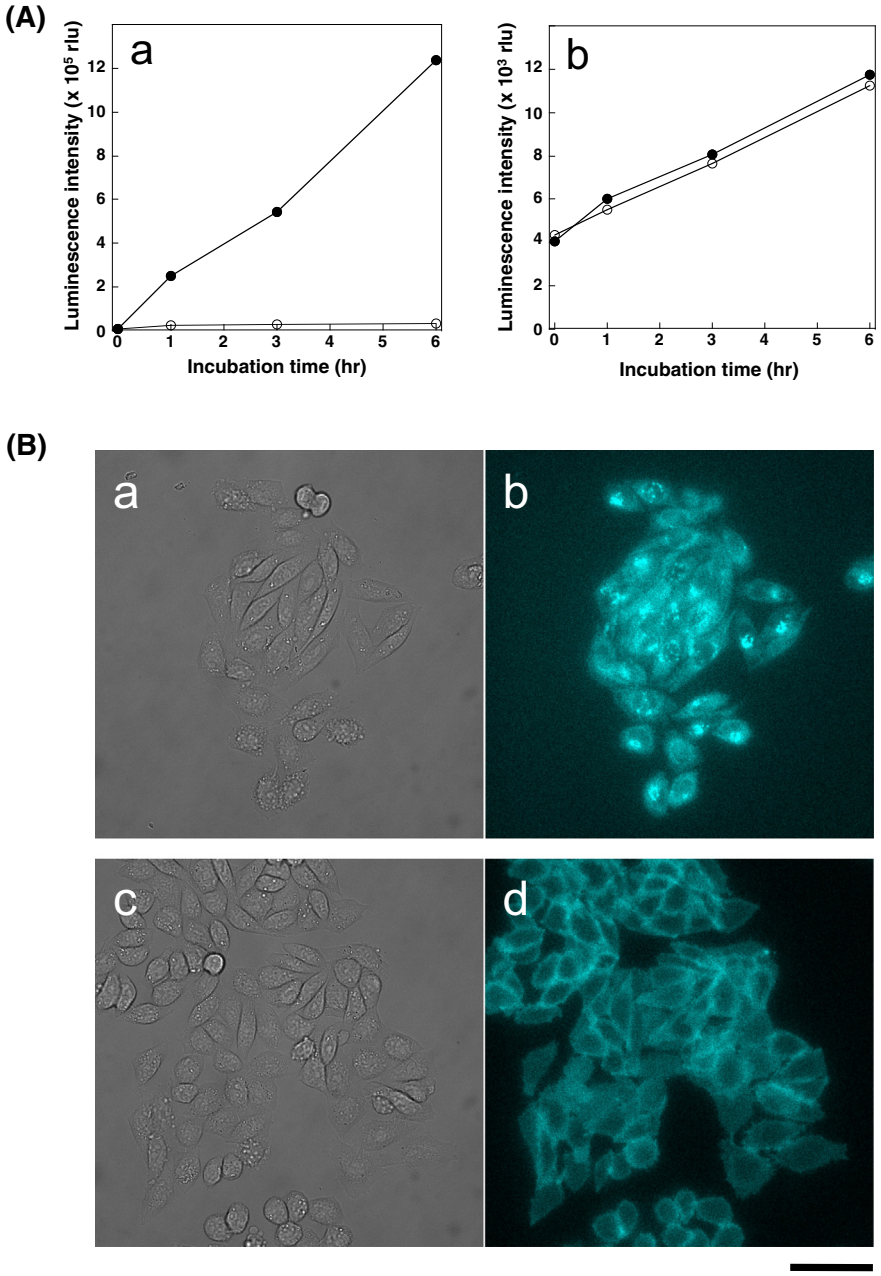

Fig. 4. Secretion of nanoKAZ into the culture medium in the presence and absence of the amino-terminal signal peptide in CHO-K1 cells. (A) Effects of brefeldin A on nanoKAZ secretion from CHO-K1 cells. Stably expressed nanoKAZ cells using pcDNA3-GLsp-nanoKAZ (a) and pcDNA3-nanoKAZ (b). Open circle (○) and closed circle (●) indicate the presence and absence of brefeldin A, respectively, in the culture medium. CHO-K1 cells (2×10^5 cells in a 6-well plate) stably expressing nanoKAZ were cultured for 48 h in 3 mL of Ham's F-12 medium supplemented with 10% FBS and washed twice with pre-warmed medium. Then, brefeldin A (5 mg/mL in methanol, Wako Pure Chemicals) was added to the culture medium at a final concentration of 5 μg/mL, and luminescence activity in the medium (5 μL) was determined. (B) Bioluminescence imaging of nanoKAZ stably expressed in CHO-K1 cells in the presence and absence of the amino-terminal signal peptide for secretion. After immersing cells in *h*-CTZ solution for 7 min, the luminescence image was obtained with an exposure time of 5 s. (a) Bright-field image of CHO-K1/pcDNA3-GLsp-nanoKAZ/clone No. B10 cells. (b) Pseudo-colored bioluminescence image on the bright-field image of (a). (c) Bright-field image of CHO-K1/pcDNA3-nanoKAZ/clone No. B11 cells. (d) Pseudo-colored bioluminescence image on the bright-field image of (c). Scale bar, 50 μm.

peptide was secreted through the ER/Golgi-independent pathway, as described previously for the unconventional secretion of proteins [11,12].

To confirm the distribution of nanoKAZ in CHO-K1 cells, nanoKAZ was visualized by video-rate bioluminescence imaging using *h*-coelenterazine as a substrate. From the luminescence images in Fig. 4B, the distribution of luminescence signals using pcDNA3-GLsp-nanoKAZ (Fig. 4B-b) was different from that obtained using pcDNA3-nanoKAZ (Fig. 4B-d). Notably, strong luminescence signals were observed on the cell membrane using pcDNA3-nanoKAZ, suggesting that nanoKAZ expressed in the cytoplasm accumulated at the cell membrane and was secreted into the culture medium (Supplementary Fig. S2).

In conclusion, nanoKAZ is an artificial protein that can be secreted not only through the ER/Golgi-dependent pathway but also through the ER/Golgi-independent pathway. However, the mechanism whereby nanoKAZ lacking the amino-terminal signal peptide can be secreted into the culture medium is unclear and

the question of why wild-type KAZ possessing the amino-terminal signal peptide cannot be secreted into the culture medium remains unsolved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.140.

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