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Research Article

Protein-stabilizing and cell-penetrating properties of α-helix domain of 30Kc19 protein

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The protein-stabilizing and cell-penetrating activities of *Bombyx mori* 30Kc19 α-helix domain (30Kc19α) are investigated. Recently, 30Kc19 protein has been studied extensively as it has both protein-stabilizing and cell-penetrating properties. However, it is unknown which part of 30Kc19 is responsible for those properties. 30Kc19 protein is composed of two distinct domains, an α-helix N-terminal domain (30Kc19α) and aβ-trefoil C-terminal domain (30Kc19β). The authors construct and produce truncated forms of 30Kc19 to demonstrate their biological functions. Interestingly, 30Kc19α is shown to be responsible for both the protein-stabilizing and cell-penetrating properties of the 30Kc19 protein. 30Kc19α shows even higher protein delivery activity than did whole 30Kc19 protein and has low cytotoxicity when added to cell culture medium. Therefore, based on its multifunctional properties, 30Kc19α can be developed as a novel candidate for a therapeutic protein carrier into various cells and tissues.

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1 Introduction

Proteins are essential components of living organisms and participate in various cellular processes in humans by interacting with other molecules. In addition, because of their catalytic properties, proteins are widely used in the biological and chemical industries. However, the low stability and short shelf life of proteins, even in mild environmental conditions, have been pointed out as critical problems for many applications [1]. A considerable amount of literature has focused on protein stabilization. One method developed for the enhancement of enzyme stability is protein immobilization, as it is a simple process that allows for easy reuse [2]. In addition, the encapsulation of proteins in liposomes has been used to protect proteins from external stresses [3]. Buffers have been supplemented with additives such as salts, amino acids, and polyols in order to prevent protein aggregate formation [4]. However, for the in vivo application of therapeutic proteins, it is impossible to maintain protein stability by using such additives. Also, it is difficult to control artificial environments to avoid direct interactions with target proteins [5]. The stabilization of multimeric proteins must be fastidious because dissociated subunits tend to be inactivated [6]. Thus, it is necessary to develop more efficient and economical methods of protein stabilization which can be widely applied in bio-industry.

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Abbreviations: CHO, Chinese hamster ovary; **CPP**, Cell-penetrating protein/peptide; **CTD**, C-terminal domain; **DMEM**, Dulbecco's modified eagle medium; **EPO**, erythropoietin; **FPLC**, fast protein liquid chromatography; **β-gal**, β-galactosidase; **GFP**, green fluorescent protein; **HRP**, horseradish peroxidase; **IPTG**, isopropyl-β-thiogalactopyranoside; **NTD**, N-terminal domain; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **SH**, silkworm hemolymph; **PVDF**, polyvinylidene difluoride; **X-gal**, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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In previous studies, the silkworm hemolymph (SH) of *Bombyx mori* was shown to exhibit anti-apoptotic properties in insect [7–14] and mammalian [15–16] cells. Also, apoptosis was inhibited by 30K gene and recombinant protein from SH [17–21]. Moreover, the productivity of human erythropoietin (EPO) in Chinese hamster ovary (CHO) cells was enhanced by expression and supplementation of 30Kc19 protein, one of the major anti-apoptosis proteins in SH [22–24]. Recently, we found that 30Kc19 protein had cell-penetrating [25] as well as protein-stabilizing [26] properties. Because of these properties, 30Kc19 was adopted and utilized as an intracellular delivery partner of proteins including transcription factors [27] or a major component of protein nanoparticles for drug delivery [28]. Incubation of enzymes with 30Kc19 protein increased their activity mainly as a result of enhanced enzymatic stability conferred by 30Kc19 [26]. For instance, incubation of mitochondrial enzyme complex and sialytransferase with 30Kc19 protein increased these enzymes stability and activity [29]. It is interesting that Pep-c19 which is a cell-penetrating peptide (CPP) of 30Kc19 does not have homology to other previously reported CPPs [30], and the dimerization of this Pep-c19 is the prerequisite for intracellular penetration [31]. Cell-penetrating proteins can be applied to the in vitro and in vivo delivery of various cargo molecules [32]. However, most cell-penetrating proteins originate from viruses and thus are of limited clinical usefulness. In addition, unlike other cell-penetrating proteins, 30Kc19 not only delivers but also stabilizes cargo proteins. In this context, 30Kc19 protein provides unique functions compared to other cell-penetrating proteins, and is suitable as a novel delivery tool of therapeutic molecules for the potential treatment of diseases. Herein, 30Kc19 protein was fractionated into two domains, and each domain was investigated for its cell penetration and stabilization properties. Based on structural studies [33], both the α-helix N-terminal domain (NTD) and the β-trefoil C-terminal domain (CTD) of 30Kc19 protein were constructed and produced in *Escherichia coli*, and their functions were observed and compared to those of whole 30Kc19 protein.

2 Materials and methods

2.1 Plasmid construction

30Kc19 protein has two distinct domains (all-α NTD and all-β CTD) [33]. In order to produce each domain, 30Kc19 alpha-helix (30Kc19α) and 30Kc19 beta-sheet (30Kc19β) were cloned and inserted into the pET-23a plasmid (Novagen, Madison, WI, USA) using *Eco*R1 and *Xho*I sites. Each recombinant protein was designed to contain a T7 tag for immunoassays and a His tag for protein purification.

2.2 Protein expression and purification

The constructed plasmid was transformed into *E. coli* BL21 (Novagen) for recombinant protein production. *E. coli* were cultured in LB broth medium at 37°C in a shaking incubator. One mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was used for induction when the OD_{600} reached 0.6, and cells were further incubated for 4 h. Cell lysates were prepared using ultra-sonication, and the recombinant proteins were purified by fast protein liquid chromatography (FPLC; GE Healthcare, Uppsala, Sweden). Lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, pH 8.0), washing buffer (20 mM Tris-HCl, 0.5 M NaCl, 50 mM imidazole, pH 8.0), and elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 350 mM imidazole, pH 8.0) were used for protein purification. Purified proteins were finally dialyzed against 20 mM Tris-HCl buffer (pH 8.0).

2.3 Immunoblot analysis

To compare the soluble expression levels of 30Kc19, 30Kc19α, and 30Kc19β, cell lysates were analyzed using Western blot. 15 μl of total lysates or soluble fractions were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was carried out against anti-T7 primary antibody (Abcam, Cambridge, UK). Anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Milipore, Bedford, MA, USA) was used as secondary antibody. Each protein was visualized with the G:BOXChemi XL system (Syngene, Cambridge, UK). For the investigation of the cell-penetrating ability of 30Kc19α, HeLa cells were incubated with medium containing 0.5 or 1.0 mg/mL of 30Kc19α protein. 4 h after incubation, cells were collected by centrifugation at 12 000 rpm for 5 min and lysed with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and protease inhibitor cocktail) for 20 min. After centrifugation at 12 000 rpm for 15 min, supernatants were separated by SDS-PAGE and then analyzed by Western blot.

2.4 GFP stability measurement

Recombinant green fluorescent protein (GFP) was expressed in *E. coli* BL21 and purified by FPLC (95% purity). To evaluate the GFP-stabilizing effect of 30Kc19 and 30Kc19α, 15 μM or 400 μg/mL of 30Kc19 and 30Kc19α, respectively, were added to a solution containing GFP. After 24 h of incubation at 37°C, GFP fluorescence intensity was measured with a spectrofluorometer (λ_{ex} = 485 nm/λ_{em} = 535 nm). The experimental data were calculated as relative GFP fluorescence comparing to initial GFP fluorescence. The statistical analysis has been performed using SigmaPlot software. The p values were obtained from t-test in the software (*$p < 0.05$, **$p < 0.01$,

***$p < 0.001$ and ****$p < 0.0001$). To measure time-dependent stability, 400 μg/mL of 30Kc19 and 30Kc19α were added to a solution containing GFP, and fluorescence intensity was measured for up to 60 min. Finally, to explore the concentration-dependent effect of 30Kc19α on GFP stability, 40, 80, 160, and 400 μg/mL of 30Kc19α were added to a solution containing GFP, and then fluorescence intensity was measured for up to 60 min.

2.5 Live cell imaging of 30Kc19α

30Kc19α was labeled with Alexa Flour® 488 (Invitrogen, Carlsbad, CA, USA) in solution and dialyzed against Dulbecco's modified eagle medium without phenol red (DMEM, Invitrogen). HeLa cells were incubated with fluorescence-labeled 30Kc19α protein for 4 or 24 h. Cells were then counter-stained with 2 μg/mL of Hoechst 33342 (Life Technologies, Gaithersburg, MD, USA). Penetration and localization of 30Kc19α protein in live HeLa cells were observed using confocal laser scanning microscopy (Olympus, Lake Success, NY, USA).

2.6 Intracellular GFP delivery analysis

HeLa cells were cultured in 96-well plates (Nunc Lab-Tek, Thermo Fisher Scientific, Rockford, IL, USA) and incubated with 20 μM of GFP-30Kc19 or GFP-30Kc19α protein. GFP-30Kc19 and GFP-30Kc19α were expressed and purified from *E. coli* [30]. Cells were washed three times with PBS and intracellular GFP delivery by 30Kc19 or 30Kc19α was analyzed by measuring GFP fluorescence intensity with a spectrofluorometer. The statistical analysis has been performed using SigmaPlot software. The *p* values were obtained from *t*-test in the software (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$).

2.7 β-galactosidase activity assay

The β-galactosidase (β-gal) gene was introduced into HeLa cells using Lipofectamine® 3000 (Invitrogen) according to the manufacturer's instructions. Two days after the transfection, HeLa cells were incubated with 40 and 80 μg/mL of 30Kc19α for 24 h. β-gal activity was determined using a β-gal assay and X-gal staining. For the β-gal assay, HeLa cells were collected and suspended using 1x RIPA Lysis buffer (Thermo Fisher Scientific). 10 μl of cell lysate was added to one well of a 96-well plate containing 50 μl of 1x cleavage buffer (60 mM $Na_2HPO_4{\cdot}7H_20$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$, pH 7) and 17 μl of 4 mg/mL o-nitrophenol-β-D-galactoside (Sigma-Aldrich). After 30 min of incubation at 37°C, 125 μl of stop buffer (1 M Na_2CO_3) was added and absorbance at 420 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Thermo LabSystems, Milford, MA, USA). The statistical analysis has been performed using SigmaPlot software. The *p* values were obtained from *t*-test in the software (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$). For X-gal staining, cells were fixed in 4% paraformaldehyde for 15 min, then treated with 0.5 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma-Aldrich) for 4 h. The development of blue color was observed using a fluorescence microscope (Olympus IX71, Tokyo, Japan).

2.8 Cytotoxicity test

To investigate the cytotoxicity of 30Kc19α, HeLa cells were seeded on 96-well plates at 70% confluence, and then incubated with medium containing 40 or 80 μg/mL of 30Kc19α for 24 h. A cytotoxicity assay was carried out using a Cell Counting Kit-8 (Dojindo Laboratories, Kumanoto, Japan), and 2 h after incubation with CCK-8 solution, the absorbance at 450 nm was measured using a spectrophotometer.

3 Results

3.1 Plasmid design and expression of 30Kc19, 30Kc19α and 30Kc19β

30Kc19 protein consists of an all α-helix N-terminal domain (NTD) and an all β-trefoil C-terminal domain (CTD) [33]. Recently, we demonstrated that 30Kc19 protein has protein-stabilizing and cell-penetrating properties [25, 26]. Hence, it is intriguing which part of 30Kc19 is responsible for these properties. The 3D structures of 30Kc19, 30Kc19α (Ala1-Asn88) and 30Kc19β (Ala89-Phe239) were predicted using a Swiss-model program [34, 35] (Fig. 1A). From the modeling, we observed that each sequence was well separated into two distinct domains. Hence, 30Kc19, 30Kc19α, and 30Kc19β open reading frames were inserted into the pET-23a expression vector system (Fig. 1B). The vector contained a T7 tag for immunoblot analysis and a His tag for protein purification. Based on a previous work showing that 30Kc19 is highly soluble and enhances the soluble expression of fusion proteins from *E. coli* [27], we considered soluble expression to be a key property of 30Kc19, and therefore checked the expression pattern of 30Kc19α and 30Kc19β. Western blot results showed that the soluble expression level of 30Kc19α was as high as that of 30Kc19 (Fig. 1C). In contrast, the expression level of soluble 30Kc19β was extremely low. Therefore, 30Kc19α was chosen and the soluble form of 30Kc19α was purified for further investigation of protein-stabilizing and cell-penetrating properties as the expression patterns of 30Kc19α and 30Kc19 were similar (Fig. 1D).

3.2 In vitro protein-stabilizing property of 30Kc19α

To investigate the stabilizing ability of 30Kc19α, the green fluorescent protein (GFP) activity was monitored

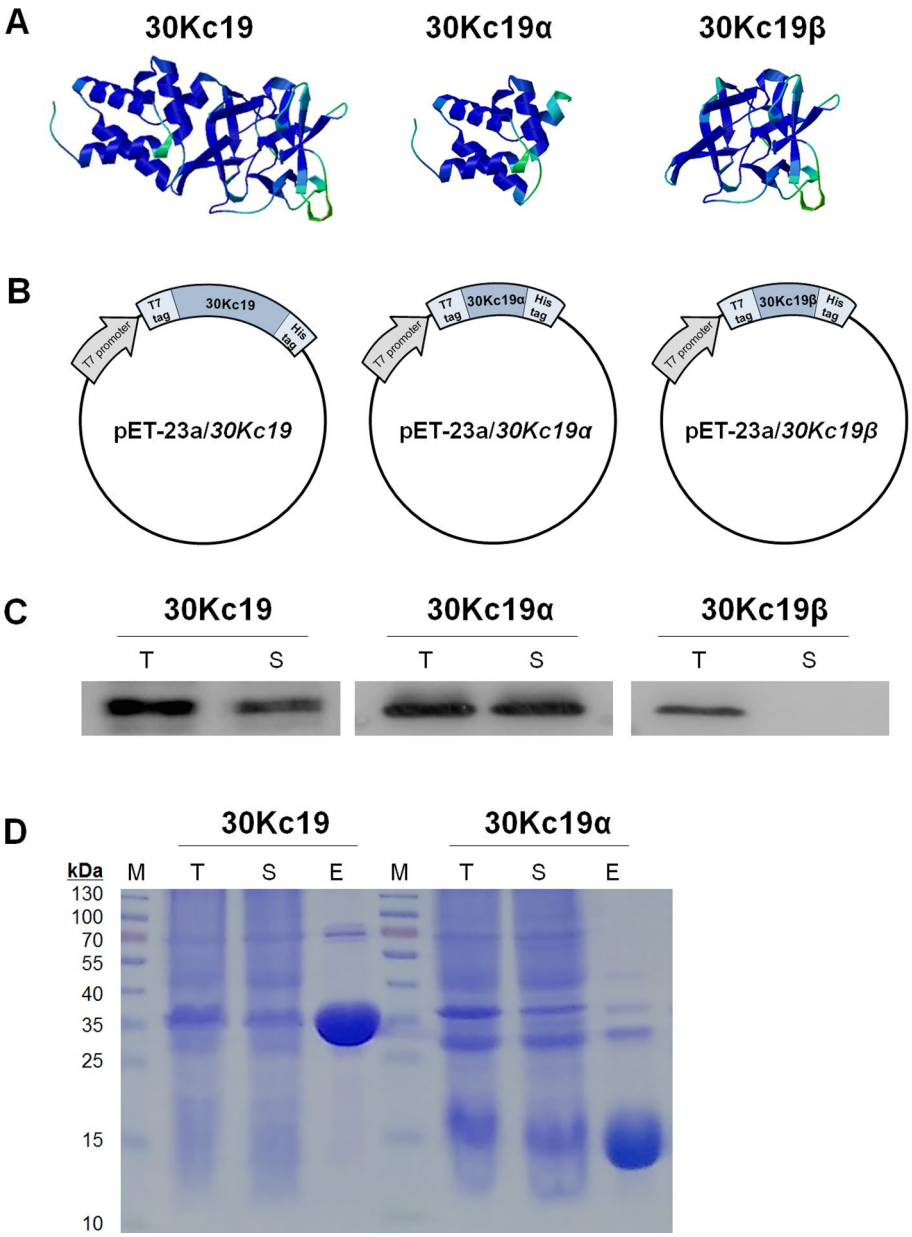

Figure 1. Gene construction and protein expression. (**A**) The structure of 30Kc19 protein and the expected structures of 30Kc19α and 30Kc19β as given by a Swiss-model program. (**B**) Construction of 30Kc19, 30Kc19α, and 30Kc19β in the *E. coli* expression pET-23a vector. (**C**) Western blot analysis of soluble protein expression. (**D**) SDS-PAGE analysis of purified soluble 30Kc19 and 30Kc19α proteins. T, total lysate; S, soluble fraction; E, eluted fraction.

over time [36]. A decrease in GFP fluorescence indicates that the GFP becomes unstable and inactivated in the solution. In the absence of 30Kc19 in solution, GFP fluorescence intensity decreased more than 50% within 24 h at 37°C (Fig. 2A). However, when the same molar concentration of 30Kc19 or 30Kc19α (15 μM) was added to the solution for 24 h, the decrease in GFP fluorescence was smaller than that of none-added control. When the same mass concentration (400 μg/mL) of 30Kc19 and 30Kc19α was added to GFP solution and incubated for 24 h, the fluorescence was higher than the fluorescence of none-added control (Fig. 2B). There were no significant differences between 30Kc19 and 30Kc19α when they were added to solution in the same molar and weight concentration.

To observe the time-dependent stabilizing effect of 30Kc19 and 30Kc19α, GFP fluorescence was measured every 10 min for 60 min (Fig. 2C). GFP fluorescence gradually decreased to 36% after 60 min incubation in the absence of 30Kc19 and 30Kc19α proteins. In contrast, GFP fluorescence decreased to 72 and 74%, respectively, in the first 10 min, with 30Kc19 and 30Kc19α, and then relative fluorescence slightly decreased to 68 and 73%, respectively, after 60 min incubation. The concentration-dependent effect of 30Kc19α on GFP stabilization was analyzed by adding 40, 80, 160, or 400 μg/mL of 30Kc19α into GFP solution. Even with the low concentration of 30Kc19α in solution, the decrease in GFP fluorescence was smaller than that of none-added control. And, relative

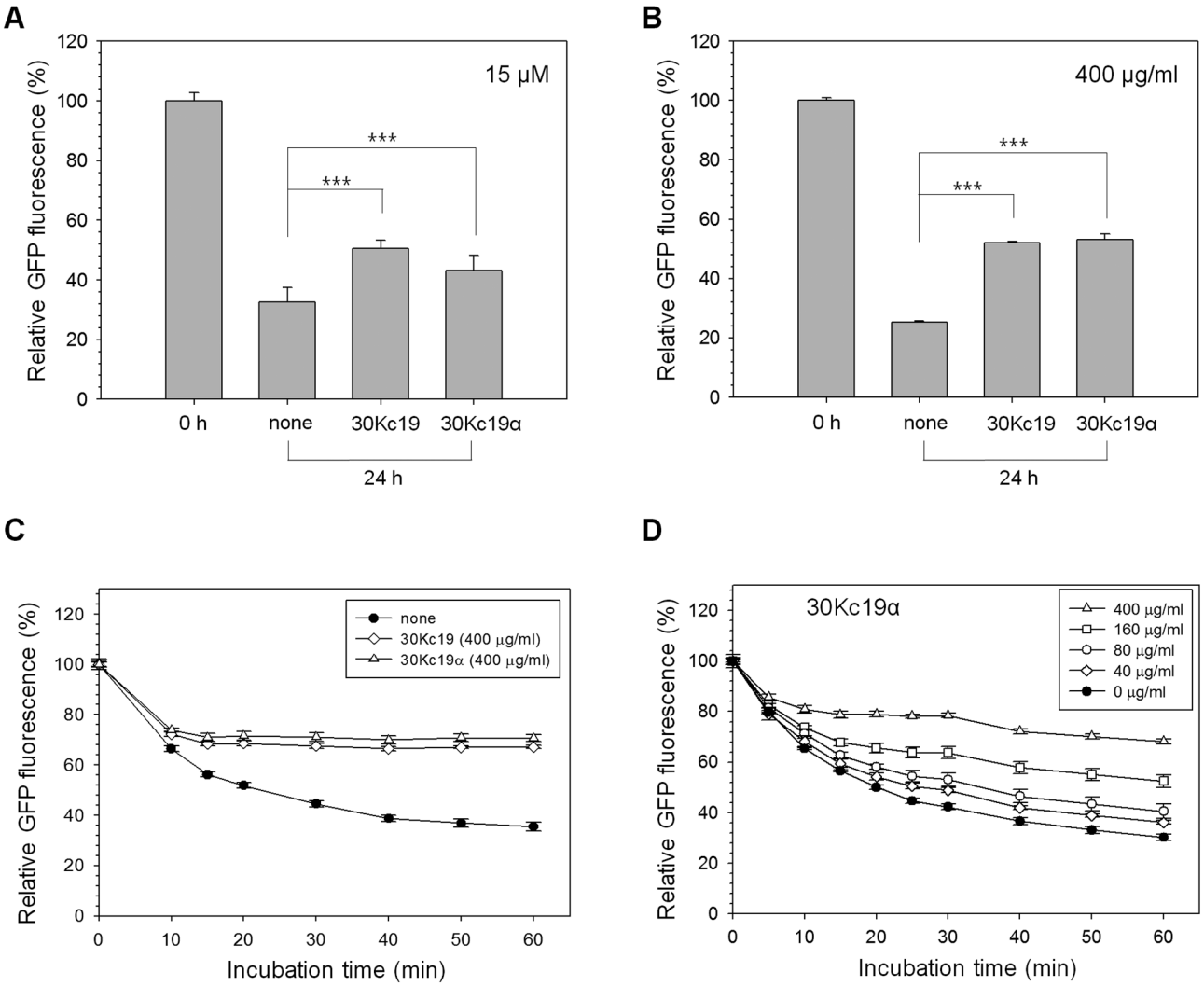

Figure 2. Stabilization of GFP by 30Kc19α. The activity of GFP was measured with a spectrofluorometer. (**A**) 15 μM of 30Kc19 and 30Kc19α were added to a GFP-containing solution for 24 h ($n = 3$). (**B**) 400 μg/mL of 30Kc19 and 30Kc19α were added to a GFP-containing solution for 24 h. GFP fluorescence before protein addition (0 h) was 100%. ***$p < 0.001$, compared with the untreated (control) group ($n = 3$). (**C**) After 400 μg/mL of 30Kc19 and 30Kc19α were added to a GFP-containing solution, GFP fluorescence was measured every 10 min for 60 min ($n = 3$). (**D**) Stabilization of GFP by 30Kc19α in a dose-dependent manner over 60 min ($n = 3$).

GFP fluorescence increased in accordance with the increase of 30Kc19α concentration (Fig. 2D). Thus, it is clear that 30Kc19α significantly increased GFP stability in a concentration-dependent manner.

3.3 Intracellular penetration and protein delivery properties of 30Kc19α

Previously, our group reported that 30Kc19 has a cell-penetrating property regardless of cell type [25] and encompasses a CPP, which was identified as a short peptide with 13 amino acids (Val45-Cys57) [30]. Thus, we predicted that 30Kc19α may be capable of cell penetration as this CPP is located within the 30Kc19α domain. To explore the cell-penetrating property of 30Kc19α, HeLa cells were incubated with 0.5 or 1.0 mg/mL of 30Kc19α for 4 h. Cells were lysed and cell lysates were analyzed to search for 30Kc19α inside the cells (Fig. 3A). Western blot results revealed that the intracellular amount of 30Kc19α increased as the concentration of 30Kc19α in the culture medium increased, which indicates that 30Kc19α penetrated cells. Intracellular penetration of 30Kc19α was also analyzed using confocal microscopy (Fig. 3B). 30Kc19α was labeled with Alexa Fluor® 488 and 0.4 mg/mL of 30Kc19α was added to the culture medium. Live cells were observed after 4 h incubation, and a significant amount of fluorescence was detectable, especially in the cytosol.

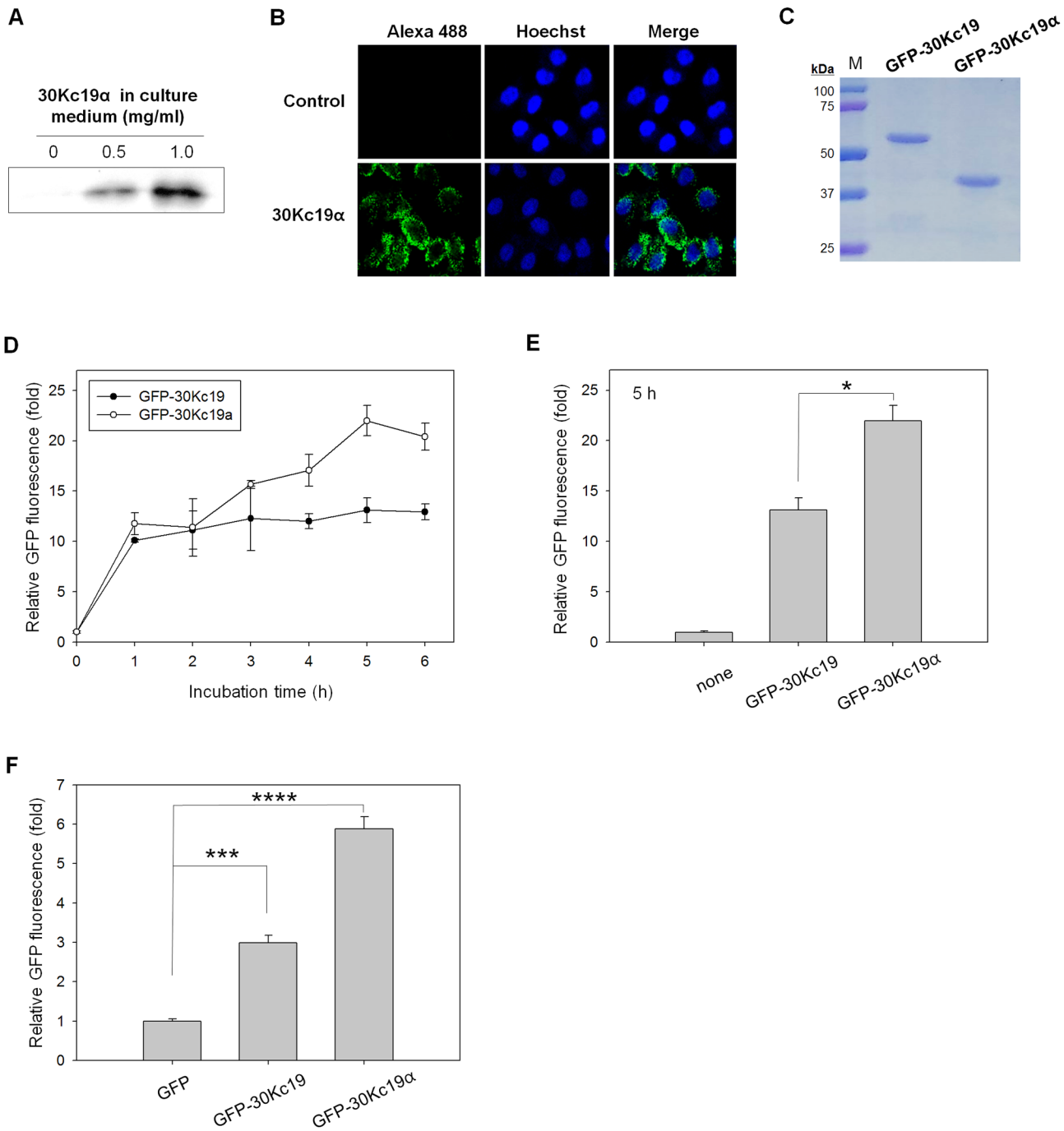

Figure 3. Cell penetration and protein delivery of 30Kc19α. (**A**) HeLa cells were treated with 0.5 or 1.0 mg/mL of 30Kc19α for 4 h. Cell lysates were analyzed by Western blot. (**B**) 30Kc19α was labeled with Alexa Fluor 488 (green) for live cell imaging. HeLa cells were incubated with 0.4 mg/mL of labeled 30Kc19α for 4 h. Nuclei were stained by Hoechst (blue). (**C**) GFP-30Kc19 and GFP-30Kc19α were expressed and purified from *E. coli*. SDS-PAGE showed the purified proteins. (**D**) Time-dependent fluorescence intensity when HeLa cells were incubated with 20 μM of GFP-30Kc19 or GFP-30Kc19α for 6 h. GFP fluorescence in cells was measured with a spectrofluorometer ($n = 3$). (**E**) The graph of intracellular fluorescence of proteins at 5 h, as compared to none-added control, $^{*}p < 0.05$ ($n = 3$). (**F**) Intracellular fluorescence of proteins after treatment with trypsin-EDTA, as compared to GFP protein, $^{***}p < 0.001$, $^{****}p < 0.0001$ ($n = 3$).

In order to investigate the protein delivery ability of 30Kc19α, GFP-conjugated 30Kc19 or 30Kc19α were expressed and produced as recombinant proteins from *E. coli*. The sizes of purified GFP-30Kc19 and GFP-30Kc19α were verified (57 and 40 kDa each) by SDS-PAGE (Fig. 3C). Then, cells were incubated with 20 μM of GFP-30Kc19 or GFP-30Kc19α in the culture medium for 6 h and fluorescence intensity was measured. As shown in Fig. 3D,

intracellular GFP fluorescence drastically increased in a time-dependent manner. It is interesting that the intracellular protein delivery efficiency of GFP-30Kc19α was higher than that of GFP-30Kc19 at 5 h (Fig. 3E). There were 13.1- and 22.0-fold increases in relative fluorescence intensity when GFP was delivered by 30Kc19 and 30Kc19α, respectively, as compared to none-added control, which is a basal level of fluorescence in cells. It means that 30Kc19α is 1.4 times higher than 30Kc19 in the cell-penetrating ability. In our previous work, Pep-c19 which is a CPP of 30Kc19 was 1.6 times higher than 30Kc19 in the cell-penetrating ability [30]. This indicates that the penetrating property of 30Kc19α is similar or a little lower than that of Pep-c19. However, no stabilizing activity on the Pep-c19 was detected at the concentration tested.

To quantify the intracellular GFP fluorescence without the interference of membrane-bound GFP-conjugated proteins, cells were treated with trypsin-EDTA after 5 h incubation with GFP-conjugated proteins. Then, after further 6 h incubation, intracellular GFP fluorescence was measured (Fig. 3F). Compared with GFP control without any fusion protein, the relative fluorescence intensity increased 3.0- and 5.9-fold in GFP-30Kc19 and GFP-30Kc19α, respectively. The higher increase in GFP-30Kc19α is probably due to the reduced size as compared with GFP-30Kc19. However, although the size of Pep-c19 is smaller than that of 30Kc19 or 30Kc19α, the fluorescence intensity of GFP-Pep-c19 was even lower than GFP-30Kc19 and GFP-30Kc19α after the 6 h incubation (data not shown). This is considered to be due to that the peptide did not contribute to the stability of GFP protein at the tested concentration. Overall, we have shown that 30Kc19α not only has a cell-penetrating property, but also has the ability to deliver cargo protein into cells. The stabilizing effect of 30Kc19α will be described in the following section.

3.4 Intracellular protein-stabilizing property of 30Kc19α

We further tested if 30Kc19α has protein-stabilizing activity inside cells. After the introduction of the β-galactosidase (β-gal) gene to HeLa cells using Lipofectamin®, cells were reseeded onto 96-well plates. Then, cells were incubated for 24 h in the absence or presence of 30Kc19α, and cell lysates were collected in order to measure intracellular β-gal activity and cell viability (Fig. 4). Intracellular β-gal activities from cells incubated with different concentration of 30Kc19α were normalized to the β-gal activity of control cells incubated without 30Kc19α. As shown in Fig. 4A, the maximum relative β-gal activity was obtained at 80 μg/mL of 30Kc19α. Intracellular β-gal activity was also visualized by X-gal staining (Fig. 4B). CCK-8 assay was carried out for cell viability tests. Cell viability was maintained at a constant level higher than 95% until 120 μg/mL of 30Kc19α (Fig. 4C). However, when the cells were treated with 200 μg/mL of 30Kc19α, the viability was decreased to 85%. Thus, the optimal concentration of 30Kc19α for the maximum functional activity without cell cytotoxicity was 80 μg/mL.

4 Discussion

In this study, *Bombyx mori* 30Kc19 was truncated into two distinct domains (30Kc19α and 30Kc19β) and it was shown that the protein-stabilizing and cell-penetrating properties of 30Kc19 are attributable to 30Kc19α. To observe the biological properties of each domain, we first asked whether 30Kc19α can be expressed as a soluble form in an *E. coli* expression system. Soluble expression is very important in practice, as recombinant proteins produced from *E. coli* tend to aggregate and form inclusion bodies. Although these aggregates could be converted back to a soluble form by refolding processes, there is no guarantee that such reconstructed proteins will have the same bioactivity as the original proteins [37], and there will be loss during the refolding process. Several proteins have been reported as soluble fusion partners, including maltose-binding protein (MBP) and N utilization substance A (NusA), which are highly soluble and are capable of enhancing the solubility of fusion partners [38]. 30Kc19 is also a highly soluble protein and has been shown to enhance the solubility of fusion transcription factors produced in *E. coli* [27].

After expressing 30Kc19α and 30Kc19β, we found that 30Kc19α was responsible for the solubility of the whole 30Kc19 protein (Fig. 1C), and thus was subjected to further protein-stabilizing tests. When the same concentration of both proteins was added to culture media with GFP, we observed that 30Kc19α exhibited strong protein-stabilizing activity, which was comparable to that of 30Kc19 (Fig. 2). In a previous study, our group found that 30Kc19 increased the stability of various enzymes with effectiveness similar to that of bovine serum albumin (BSA) [26]. BSA has been widely used in numerous biochemical applications to increase protein stability during biological reactions. The 30Kc19 and BSA may stabilize proteins by a crowding effect. Molecular crowding is a surrounding effect rather than a specific interaction with other molecules [39] and some proteins have been reported to have this property [40]. Although the stabilizing effect is not a specific property of 30Kc19 or 30Kc19α, these proteins also have the cell penetration property. In this study, we found that 30Kc19α has a stabilizing-effect on both GFP and β-gal in a concentration-dependent manner (Fig. 2.4). Some CPPs have a multifunctional property. For example, hunter-killer peptides (HKPs) have cell-penetrating and cationic antibacterial properties [41]. However, the multifunctional property with cell-penetrating and cargo-

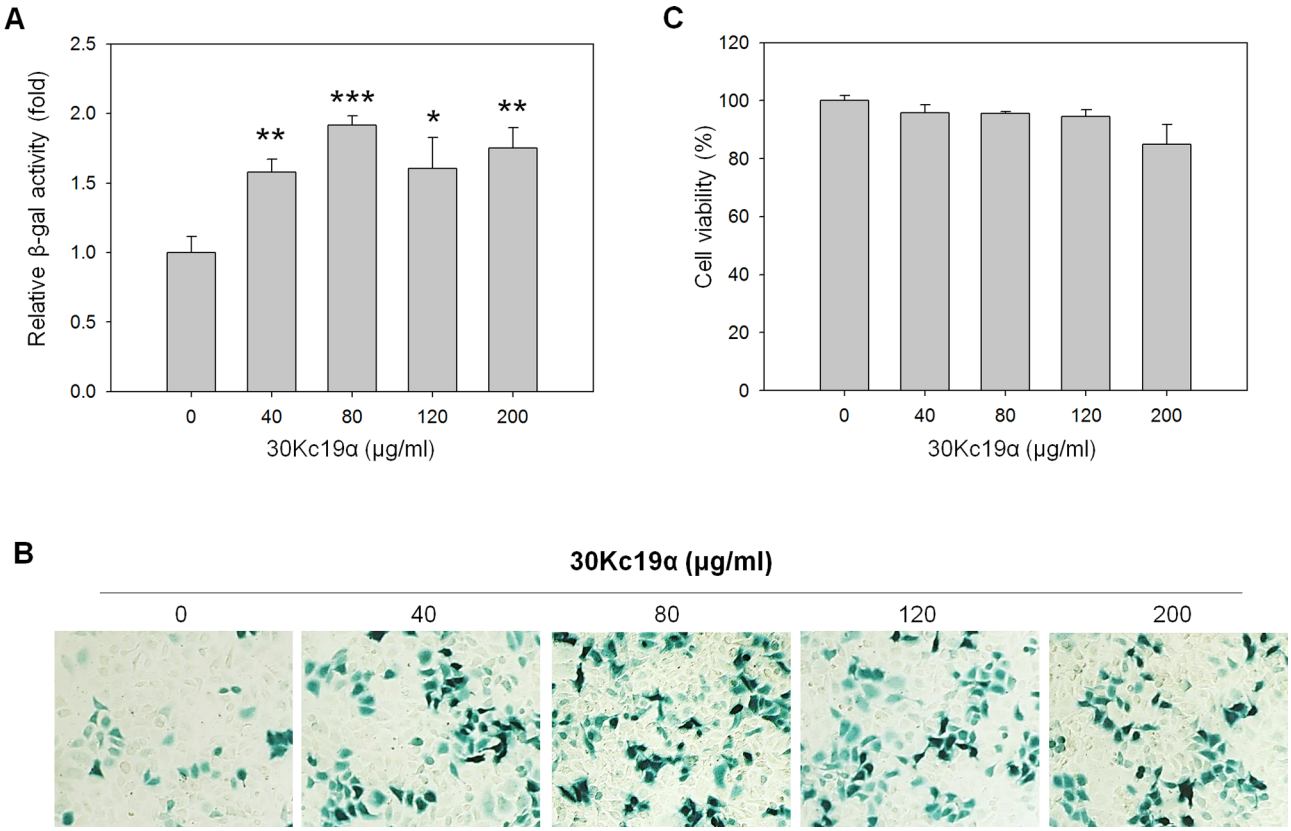

Figure 4. Intracellular protein stabilization by 30Kc19α. (**A**) β-gal-transfected HeLa cells were incubated with 40 or 80 μg/mL of 30Kc19α for 24 h. β-gal activity was measured with an ELISA reader. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with the control group ($n = 3$). (**B**) β-gal activity of HeLa cells as assessed by X-gal staining. (**C**) Cell viability assay for 30Kc19α as assessed by a CCK-8 assay ($n = 3$).

stabilizing effects is a unique property of 30Kc19 and 30Kc19α. The benefit of 30Kc19α over other proteins including BSA is that 30Kc19α simultaneously delivers and stabilizes cargo proteins. Tat is one of the well-known CPPs which have the cargo-delivery activity. Compared with GFP control without the fusion protein or CPP, the relative fluorescence intensity increased 5.9-fold in GFP-30Kc19α in 5 h incubation, while it increased 2.4-fold in Tat-GFP during the same incubation time [42]. Further experiments will be carried out to explore the in vivo application of 30Kc19α without hampering the cell viability as a potential drug carrier.

The biological role of 30Kc19β, the β-trefoil C-terminal domain, is still unknown. However, 30Kc19α was proven to be the domain responsible for protein-stabilizing and cell-penetrating activities, and 30Kc19β can be excluded. Also, the reduced size of 30Kc19α as compared to untruncated 30Kc19 can be of great practical advantage.

In conclusion, 30Kc19α has protein-stabilizing activity as well as cell-penetrating ability, and so 30Kc19α is a novel candidate for a therapeutic protein drug carrier.

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The authors declare no financial or commercial conflict of interest.

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Cover illustration

This special issue, in collaboration with the Asian Federation of Biotechnology and edited by Professors Hyung Joon Cha, Noriho Kamiya and S. Vikineswary Sabaratnam, covers the most advanced biotech research from Asian Congress of Biotechnology 2015. This issue includes articles on drug delivery, enzyme engineering, cellular therapy, biosensors, etc. The 30Kc19 protein derived from the silkworm hemolymph consists of two domains, which are 30Kc19α (blue) and 30Kc19β (red). The cover image shows that 30Kc19α has multifunctional properties, which are cell penetration, protein stabilization, and cargo delivery. The Image is provided by Jina Ryu, Hyoju Kim, Hee Ho Park, Hong Jai Lee, Ju Hyun Park, Won Jong Rhee and Tai Hyun Park authors of "Protein-stabilizing and cell-penetrating properties of α-helix domain of 30Kc19 protein" (http://dx.doi.org/10.1002/biot.201600040).

Biotechnology Journal – list of articles published in the November 2016 issue.

Research Article

Enzymatically prepared redox-responsive hydrogels as potent matrices for hepatocellular carcinoma cell spheroid formation

Kousuke Moriyama, Shono Naito, Rie Wakabayashi, Masahiro Goto and Noriho Kamiya

http://dx.doi.org/10.1002/biot.201600087

Research Article

Theoretical calculations on the feasibility of microalgal biofuels: Utilization of marine resources could help realizing the potential of microalgae

Hanwool Park, Choul-Gyun Lee

http://dx.doi.org/10.1002/biot.201600041