ACCELERATED COMMUNICATION

Development of a reporter peptide that catalytically produces a fluorescent signal through α-complementation

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Received 13 January 2015; Accepted 23 February 2015
DOI: 10.1002/pro.2667
Published online 4 March 2015 proteinscience.org

Abstract: In α-complementation, inactive N-terminal (α-domain) and C-terminal (ω-domain) fragments of β-galactosidase associate to reconstitute the active protein. To date, the effect of α-domain size on α-complementation activity has not been systematically investigated. In this study, we compared the complementation activities of α-domains of various sizes using an *in vitro* system. We found that the complementation activities are similar for α-domains comprising between 45 and 229 N-terminal residues but are significantly decreased for those containing less than 37 residues. However, these smaller α-domains (15 and 25 residues) exhibited sufficient α-complementation activity for application as reporters.

Keywords: α-complementation; β-galactosidase; reporter gene; cell-free translation system; reporter peptide

Introduction

β-Galactosidase is widely used as a reporter protein both *in vivo*[1–3] and *in vitro,*[4–6] and its structure has been investigated in detail.[7–9] This enzyme can be separated into two fragments: an N-terminal fragment of 45–200 residues known as the α-domain, and a C-terminal fragment that lacks residues 11–41, known as the ω-domain. These two domains are inactive when separated but can combine to regenerate active β-galactosidase in a process known as α-complementation.[10–14] Through α-complementation, a small gene encoding the α-domain can be used as a reporter for gene expression if the ω-domain protein is supplemented in advance.

Owing to its small size, the α-domain has been utilized for various applications. For example, the α-domain is included in various plasmid vectors for blue-white screening[15] because the insertion of this small domain negligibly affects the plasmid size, which affects the growth rate of host bacteria.[16,17] The α-domain is also a useful reporter for gene

Additional Supporting Information may be found in the online version of this article.

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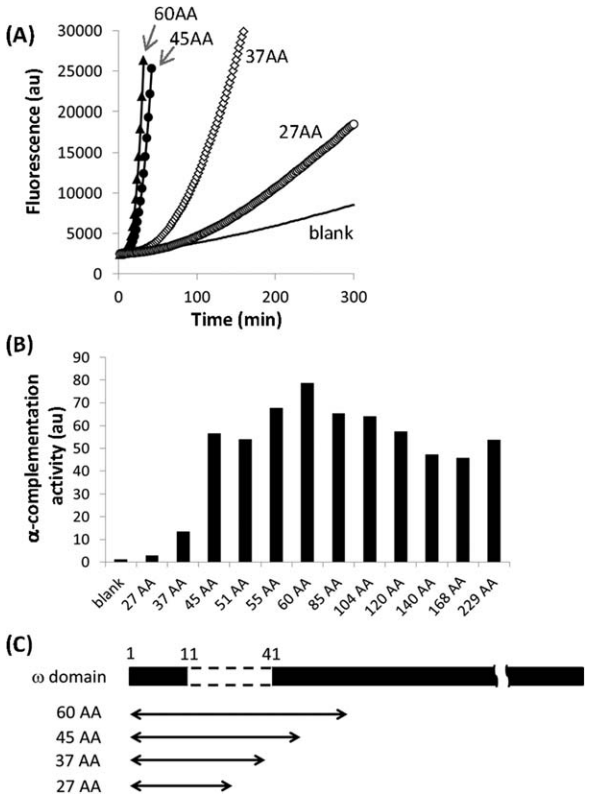

Figure 1. α-Complementation activities of various α-domains. (A) Representative fluorescence kinetics of α-complementation by DNAs encoding different α-domains. The background fluorescence level in the absence of DNA is also shown (blank). (B) α-Complementation activities of DNAs encoding α-domains of various sizes. As an index of the α-complementation activity, the maximum slopes of the fluorescence increases were normalized to the blank and plotted. The numbers before "AA" indicate the number of N-terminal β-galactosidase residues. (C) Relationship between the absent region of the ω-domain and the coding region of each α-domain.

expression in certain *in vitro* systems: we have previously demonstrated that using the α-domain rather than full-length β-galactosidase significantly improves the efficiency of translation-coupled RNA replication[18] because replication efficiency is heavily dependent on RNA size.

Previous studies have used N-terminal sequences of various lengths as the α-domain.[18–22] However, the relationship between α-domain size and α-complementation activity has not been widely explored, and thus, the minimal α-domain that retains the ability to function as a reporter remains unknown. In this study, we compared the α-complementation activities of α-domains of different sizes *in vitro* and developed a smaller reporter peptide than previously reported, which may be useful for various applications.

To assay the α-complementation activity of α-domains of various sizes, we utilized a reconstituted transcription/translation system in *Escherichia coli* (the PURE SYSTEM[23]). As the original system exhibits high β-galactosidase activity because of contamination, we used a customized system consisting of highly purified components that exhibit negligible β-galactosidase activity.[24] We amplified DNA fragments encoding α-domains comprising between 27 and 229 N-terminal residues from β-galactosidase using PCR and incubated these fragments with the transcription/translation system containing the purified ω-domain protein and a fluorescent substrate, 5-chloromethylfluorescein di-β-D-galactopyranoside (CM-FDG). The fluorescence produced by β-galactosidase activity was measured every minute. A representative example of the raw data is shown in Figure 1(A). As an index of α-complementation activity, we determined the maximum rate of the increase in the fluorescence emission for each α-domain [Fig. 1(B)]. The α-domains comprising between 45 and 229 residues (45–229AA) exhibited similar levels of activity, whereas α-domains containing less than 45 residues (27AA and 37AA) exhibited drastically reduced activities. These reductions may be reasonably expected because these smaller α-domains do not perfectly complement the sequence that is absent (residues 11–41) in the ω-domain [Fig. 1(C)]. Unexpectedly, 27AA and 37AA exhibited significantly higher α-complementation activities than the control without DNA (blank). This result suggests that α-complementation by much smaller α-domains may still be possible.

To examine whether the α-domain can be further reduced in length, we amplified DNA fragments encoding shorter N-terminal regions of β-galactosidase [10, 16, 17, 21, 23, and 27AA; Fig. 2(A)] using PCR and determined their α-complementation activities as described above [Fig. 2(B)]. The observed α-complementation activities did not correlate with size, although certain α-domains (27AA, 17AA, and 16AA) exhibited significantly higher activities than the blank. We further deleted the first 2 N-terminal residues (Met and Thr), which are reportedly dispensable for α-complementation,[25] to obtain 25AAd2, 21AAd2, 19AAd2, 15AAd2, 14AAd2, and 8AAd2 [Fig. 2(A)], all of which exhibited increased α-complementation activity relative to the blank [Fig. 2(B)].

These experiments were all performed using DNA fragments encoding α-domains. To confirm that the translated proteins possess α-complementation activity, we chemically synthesized the 25AAd2, 15AAd2, and 8AAd2 peptides and determined their α-complementation activities using the same method as described above, with the exception that the peptides were added to the reaction mixture instead of DNA. The 25AAd2 peptide amplified fluorescence in a concentration-dependent manner [Fig. 2(C)], confirming that the 25AAd2 peptide possesses α-complementation activity. Addition of the 8AAd2 peptide did not result in increased

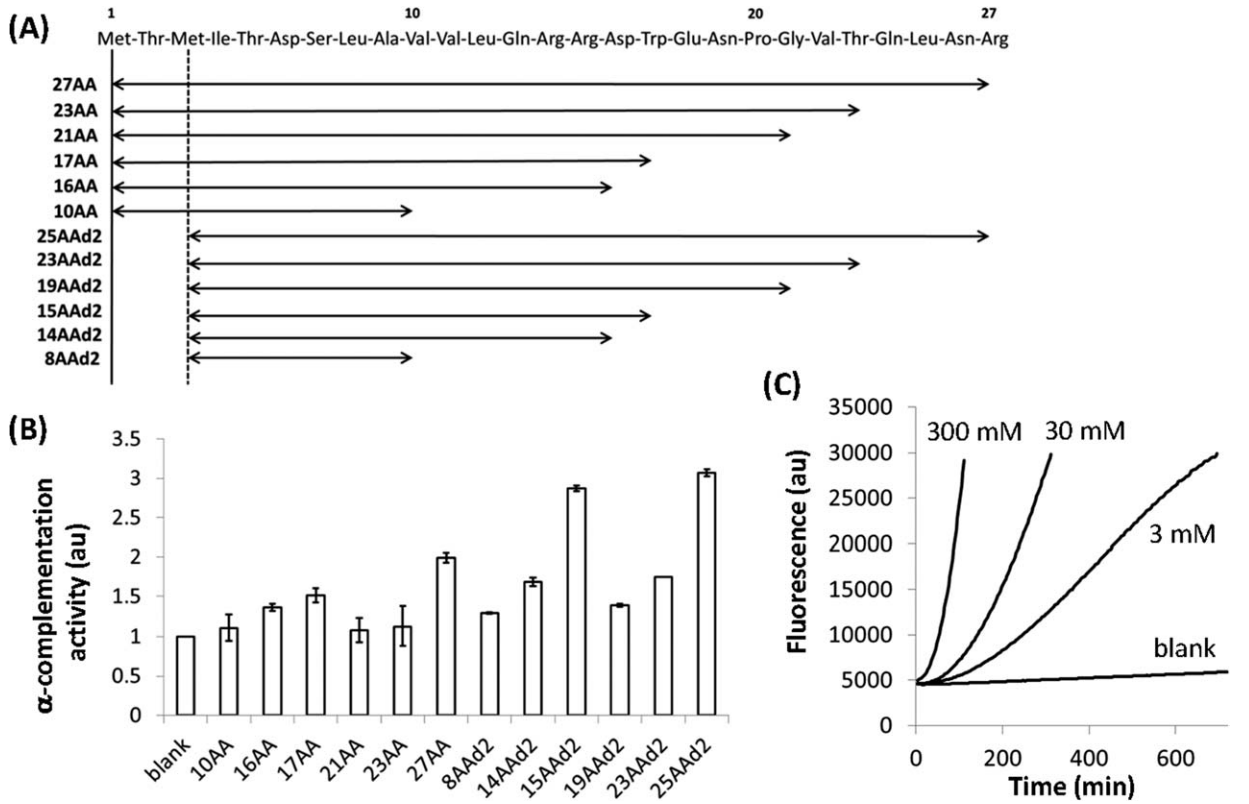

Figure 2. α-Complementation activities of small α-domains. (A) Amino acid sequences of each α-domain. (B) α-Complementation activity of each α-domain normalized to the fluorescence of the blank. The activities were determined as described in Figure 1. (C) Fluorescence kinetics for α-complementation by the chemically synthesized 25AAd2 peptide.

fluorescence, suggesting that the α-complementation activity of 8AAd2 was too weak to be detected using this method. The 15AAd2 peptide was insoluble in water and thus could not be assayed.

Next, we evaluated the ability of the 25AAd2 and 15AAd2 α-domain fragments to serve as reporter genes by performing gene expression experiments in lipid bilayer membranes (liposomes), which are widely used as microreactors. We encapsulated DNA fragments (5 nM) encoding 15AAd2 or 25AAd2 into liposomes together with the transcription/translation system, the ω-domain protein, and the fluorescent substrate. We measured the fluorescence intensity of each liposome using flow cytometry following a 20-h incubation at 37°C (Fig. 3). For both DNA fragments, most of the liposomes exhibited higher fluorescence intensities than the blank, irrespective of the liposome volume. This result demonstrates that 15AAd2 and 25AAd2 are sufficiently active for use as reporter genes in liposomes.

In this study, we investigated the α-complementation activities of variously sized α-domains. We found that α-domains composed of 45–229 residues exhibited similar α-complementation

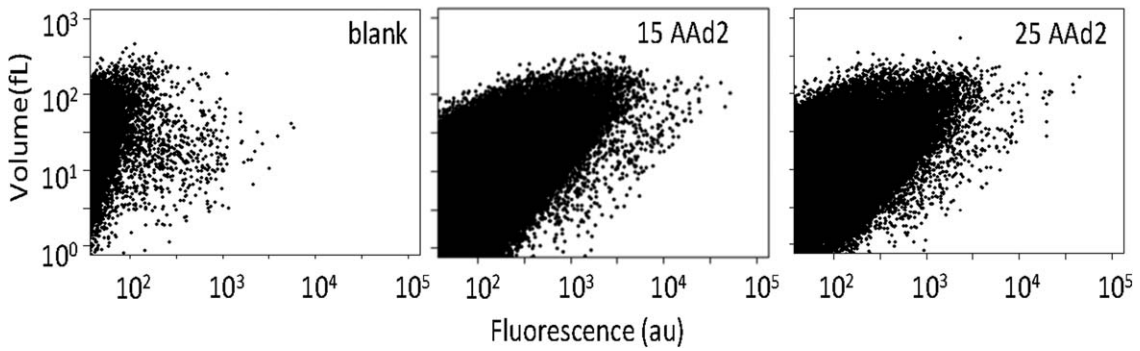

Figure 3. Expression of the small α-domains in liposomes. DNA fragments encoding 15AAd2 and 25AAd2 were encapsulated into liposomes together with the α-complementation assay mix. The fluorescence of each liposome was measured using flow cytometry.

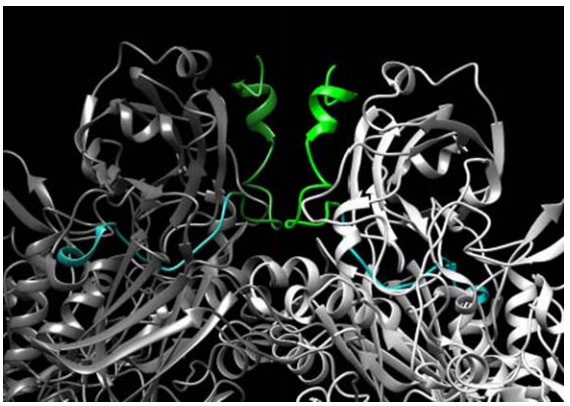

Figure 4. Mapping the region complemented by 25AAd2 in the β-galactosidase tetramer. A portion of the interface structure of the β-galactosidase tetramer[9] is shown. The ω-domain protein lacks the colored regions (green and cyan). The region complemented by 25AAd2 (residues 3–25, green) and the absent region (residues 26–41, cyan) are marked. The lighter and darker colors represent the same domain in different monomers. This image was rendered using UCSF Chimera.[30]

activities but that smaller α-domains, i.e., 37AA and 27AA, exhibited 4-fold and 20-fold decreased activity, respectively, compared with that of 45AA. This result is largely consistent with previous observations based on the protein structure of the α-domain; residues 13–23 directly contribute to the subunit-subunit interface, whereas residues 29–33 serve as "anchors" to connect the interface to each subunit.[7] In this study, the α-domains that were smaller than 37AA lacked these anchor regions, and thus, the observed decreases in α-complementation activity could be expected. However, two results in this study are inconsistent with those from previous studies. The first result is that the activity of 37AA was 4-fold lower than that of 45AA, although 37AA contains the entire "anchor" sequence. This result suggests that residues 38–45 may also serve important roles in anchoring the α-domain. The second result is that the α-domains that lack the entire 'anchor' sequence (i.e., smaller than 37AA) still exhibited modest but significant α-complementation activity, which may be explained by their structures (Fig. 4). The 25AAd2 α-domain (green) primarily consists of the subunit–subunit interface and does not contain the anchor region (cyan), although it still associates with the ω-domain (gray) via the other side of the interface. This weak association with the ω-domain likely accounts for the weaker but persistent α-complementation activity of the small α-domains.

Unexpectedly, we also found that small α-domains (25AAd2 and 15AAd2) retained a certain level of α-complementation activity and could be used as reporter genes. These small reporters can potentially be used for novel applications. For example, protein size is a critical parameter for *in vitro* translation using nonbiological amino acids[26,27] or tRNAs,[28] as well as prebiological ribozymes,[29] which only permit the translation of small peptides. These smaller reporter peptides may be useful for monitoring translation activity that cannot be detected using conventional reporters.

Materials and Methods

The ω-protein was purchased from Clontech (Mountain View, CA, USA), as was the EA reagent of the ProLabel Detection Kit II. The DNA fragments encoding various α-domains were PCR amplified from the pET-lacZ plasmid using the primers shown in Supporting Information Table S1. The pET-lacZ plasmid was constructed as follows, and the partial sequence around lacZ is shown in the Supporting Information: A DNA fragment containing the lacZ gene was amplified from the plasmid encoding Rep(+)Gal(-) RNA[6] and inserted into pET-21a (Novagen). The T7 tag and histidine tag fused to the lacZ gene were then removed. To prepare the α-domains lacking the two N-terminal residues, the plasmid pET-lacZd2 was constructed by PCR using the pET-lacZ template and the primers GAAGGAG ATATACATATGATTACGGATTCACTGGCCG and AT GTATATCTCCTTCTTAAAGTTAAACAAAATTATTTC TAGAGGGG, followed by self-ligation using the InFusion cloning kit (Takara, Japan). The chemically synthesized α-domains were purchased from Gene Design (Japan).

The α-complementation assay was performed as described previously.[18] The α-complementation reaction contained the customized transcription/translation system, 1 n*M* α-domain DNA, 50 μ*M* CM-FDG (Life Technologies), and 2 μL of EA reagent (ω-domain protein) in a 20-μL reaction volume. Reactions were incubated at 37°C for 3 h, and fluorescence was measured every minute. For α-complementation assays using the chemically synthesized peptides, the peptides were added instead of DNA. For the liposome experiments, the reactions additionally contained a red fluorescent compound (1.9 μ*M* transferrin Alexa 647) as a volume marker, and the DNA concentration was increased to 5 n*M*. The mixture was incubated at 37°C for 20 h and subjected to flow cytometry. The composition and preparation of the customized transcription/translation system was described previously.[24]

Acknowledgments

We thank R. Otsuki and T. Sakamoto for technical assistance. We also thank Dr. Y. Shimizu (RIKEN) for helpful comments on this study.

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