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Truncated green fluorescent protein mutants and their expression in *Aplysia* neurons

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ABSTRACT: We determined in detail the primary structure requirements for green fluorescence of the jellyfish green fluorescent protein (GFP) and of its improved mutants (S65T and I167T). We performed a deletion mapping in combination with fluorescence-activated cell sorting (FACS) and spectrofluorometry. This showed that deletion of up to nine amino acids at the C-terminal end of GFP had no deleterious effect on the fluorescent activity of GFP; in fact the deletion increased intensity of fluorescence. Further truncation of up to 11 amino acids resulted in partial impairment in maximal fluorescence. The GFP fluorescence was completely disrupted when more than 12 amino acids were deleted out of the C-terminal. Truncated mutants or their fusion genes with *lacZ* emitted fluorescence when plasmids encoding them were introduced by microinjection into *Aplysia* neurons. © 1998 Elsevier Science Inc.

KEY WORDS: Gene transfer into neurons, Deletion mutagenesis, β-Galactosidase, Fluorescence-activated cell sorting (FACS).

INTRODUCTION

The green fluorescent proteins (GFP) produce stable green fluorescence by absorbing blue light [3,24]. The GFP of the bioluminescent jellyfish *Aequorea victoria* consists of 238 amino acid residues and does not require the presence of other proteins or substrate for fluorescence activity [2,23]. Many neurobiologists became aware of GFP's potential as a tracer for individual neurons during the development and maturation of complex neural circuits as well as a marker for the specific proteins during trafficking within complex dendritic and axonal subcellular structures [2,18,19,21].

A variety of improved GFP mutants have been developed so far to further strengthen their applicability by increasing GFP fluorescence or by modifying fluorescence spectra [4–6,8–10]. A GFP mutant S65T, having its 65th amino acid serine mutated to threonine, has a single excitation peak at 489 nm [9]. It forms fluorophore [3] approximately four times faster than the wild type does [9]. Another mutant, I167T, having its 167th residue isoleucine mutated to threonine, was found with alteration in the ratio of the two excitation peaks and more fluorescent at 475 nm excitation than at 395 nm [10].

Recently, the three-dimensional structures of both wild type GFP and S65T were revealed to show a β-barrel or β-can in which the fluorophore is wrapped around with 11 β-strands [22,25]. The structural requirement of the GFP backbone for fluorescence was also studied by deletion mapping to define a minimal domain necessary for fluorescence of the wild type GFP [7]. The investigators reported that GFP required 2–232 amino acid residues for green fluorescence. However, fluorescence properties of deletion mutants were not extensively compared with those of wild type. In our study, we generated several truncated GFPs using not only wild type but improved mutants (S65T and I167T) in order to extensively map the minimal structure of GFP without disrupting GFP function. We also analyzed the effects of these deletion mutations on spectral properties and intensity of green fluorescence of GFP by spectrofluorometry and fluorescence-activated cell sorting (FACS) analysis. Finally, in order to assess the feasibility of these truncated GFP mutants as gene expression markers in the nervous system, we expressed them by gene transfer into giant neurons of the marine snail *Aplysia*.

MATERIALS AND METHODS

DNA Construction of GFP Mutants in the E. coli Expression Vector pET3a

The coding region of *A. victoria* GFP was amplified from pNEXδ-GFP [11] by polymerase chain reaction (PCR) with the primers gfp1 and gfp2 (Table 1). The PCR product was cleaved with NdeI and BamHI and introduced into NdeI/BamHI-linearized pET3a vector (Novagen, Madison, WI, USA), which drives GFP expression in *E. coli* under the control of T7 promoter. Various deletion mutants of GFP were generated by using restriction enzyme digestion within the coding region, exonuclease digestion using ExoIII/Mung bean kit (Stratagene, La Jolla, CA, USA), and PCR with designed recombinant primers. All these variants of GFP were constructed and subcloned into *E. coli* expression vector pET3a (Fig. 1). All the variants of GFP were derived from pET3a-GFP as follows and their sequences and orientations were checked by DNA sequencing using T7 sequenase system (USB, Cleveland, OH, USA).

Construction of pET3a-GFPΔC4, −GFPΔC8, and −GFPΔC12. A double-stranded oligonucleotide containing three stop codons (TGACTAGTTGA) was inserted right after KpnI site of pNEXδ-GFP. A PvuII fragment (0.11 kb) containing these multiple stop codons was inserted at the BamHI site of pET3a-GFP after pET3a-GFP was digested with BamHI and treated with Mung bean

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TABLE 1

OLIGONUCLEOTIDE PRIMERS USED IN POLYMERASE CHAIN REACTIONS

Name	Sequence
gfp1	5′GGGAATTCCATATGAGTAAAGGAGAAGA3′
gfp2	5′GACACCAGACCAACTGG3′
gfp3	5′CGGGATCCAAGCTTATGAGTAAAGGAGAAGA3′
gfp4	5′CGGGATCCTTTGTATAGTTCATCCA3′
gfp5	5′CCCAAGCTTTTAAATCCCAGCAGCTGTTA3′
gfp6	5′CCCAAGCTTAATCCCAGCAGCTGTTA3′
gfpΔC8	5′CGGGATCCTTATGTAATCCCAGCAGCTG3′
gfpΔC9	5′CGGGATCCTTAAATCCCAGCAGCTGTTA3′
gfpΔC10	5′CGGGATCCTTACCCAGCAGCTGTTACAA3′
gfpΔC11	5′CGGGATCCTTAAGCAGCTGTTACAAACT3′
gfpΔC14	5′CGGGATCCTTATACAAACTCAAGAAGGA3′
gfpΔC22	5′CGGGATCCTTAGTCTCTCTTTTCGTTGG3′
s65t-s	5′ACTTTCACTTATGGTGTTCAATGC3′
s65t-a	5′GAACACCATAAGTGAAAGTAGTGACAAGTG3′
i167t-s	5′AACTTCAAAACTAGACACAACATTGAAG3′
i167t-a	5′TGTGTCTAGTTTTGAAGTTAACTTTG3′
rsv-s	5′AGTGCCTAGCTCGATAC3′
lac-s	5′GCTCTAGAGATTCACTGGCCGTCGT3′
lac1-a	5′GGAATTCTTTTTGACACCAGACCA3′
lac2-a	5′CGGGATCCTTATTTTTGACACCAGA3′

Underlined are EcoRI site (gfp1), BamHI site (gfp3, gfp4), HindIII site (gfp5, gfp6), BamHI site and stop codon (gfpΔC8-gfpΔC22), point mutations (s65t, i167t), XbaI (lac-s), EcoRI site (lac1-a), and BamHI (lac2-a), respectively.

nuclease. The resulting plasmid was digested with BamHI/KpnI, treated with exonuclease III, and subsequently with Mung bean nuclease to generate a group of serially digested GFP according to manufacturer's instruction manual (Stratagene).

Construction of pET3a-GFPΔC8, −GFPΔC9, −GFPΔC10, −GFPΔC11, −GFPΔC14, and −GFPΔC22. Coding sequences of deletion mutants was individually generated by PCR from pET3a-GFP with T7 promoter primer (Novagen) and antisense primers (Table 1) gfpΔC8, gfpΔC9, gfpΔC10, gfpΔC11, gfpΔC14, and gfpΔC22, respectively. The PCR products were digested with NcoI and BamHI and substituted for NcoI/BamHI fragment of pET3a-GFP.

Construction of pET3a-S65T, −S65TΔC9, −S65TΔC10, −S65TΔC11, and −S65TΔC12. Mutation of Ser at residue 65 to Thr (TCT → ACT) was done by two-step recombinant PCR. A 0.29-kb fragment was amplified from pET3a-GFP with T7 promoter primer (Novagen, Madison, WI, USA) and an antisense primer s65t-a (Table 1). A 0.60 kb fragment was amplified from pET3a-GFP with a sense primer s65t-a (Table 1) and T7 terminator primer (Novagen). These two overlapping fragments were mixed and recombinant PCR was done with T7 promoter primer and T7 terminator primer to generate a 0.87 kb fragment. This fragment was cut with NdeI and substituted for the 0.23 kb NdeI fragments from pET3a-GFP, −GFPΔC9, −GFPΔC10, −GFPΔC11, and −GFPΔC12 to create pET3a-S65T, −S65TΔC9, −S65TΔC10, −S65TΔC11, and −S65TΔC12, respectively.

Construction of pET3a-I167T, pET3a-I167TΔC9, −I167TΔC10, and −I167TΔC11. Mutation of Ile at residue 167 to Thr (ATT → ACT) was done by the recombinant PCR using a sense primer i167t-s and antisense primer i167t-a (Table 1) in a similar way by which pET3a-S65T was generated. A recombinant PCR product was cut with NcoI and BamHI and substituted for the 0.56 kb NcoI/BamHI fragment from wild-type GFP to create pET3a-I167T. I167TΔC9, I167TΔC10, and I167TΔC11 were amplified by PCR from pET3a-I167T with T7 promoter primer and their antisense primers gfpΔC9, gfpΔC10, and gfpΔC11, respectively. The PCR products were cleaved with NcoI/BamHI and substituted for the same fragment of pET3a-GFP.

Plasmid construction of pET3a-GFPΔN7, −GFPΔN17, −GFPΔN23, −GFPΔN42, −GFPΔN77, −GFPΔC160, −GFPΔ(77–151), and −GFPΔ(49–57) were similarly done as described earlier (methods not shown).

Qualitative Determination of GFP Fluorescence from E. coli *Expressing GFP Variants*

Each GFP variant was transformed into *E. coli* strain BL21(DE3)pLysS and grown on the Luria-Bertani agar plate containing 60 μg/ml ampicillin, 34 μg/ml chloramphenicol, and 0.4 mM isopropyl thio-β-D-galactoside (IPTG). After 12–16 h in culture, the fluorescence of transformed *E. coli* colonies on the plate was qualitatively assessed by exposing the plate to long-wavelength (366 nm) excitation from a portable ultraviolet (UV) lamp (Model UVGL-25, UVP Inc., San Gabriel, CA, USA) (Fig. 1).

Quantitative Analysis of GFP Fluorescence Using FACS

A single colony of *E. coli* strain BL21(DE)pLysS transformed with various pET3a-GFPs was grown with shaking at 37°C in LB broth containing 60 μg/ml ampicillin and 34 μg/ml chloramphenicol until OD_{600} reached 0.6−0.9. The IPTG was then added to the broth to a final concentration of 0.4 mM, followed by incubation for an additional 2.5 h. After induction with IPTG, the bacterial culture was diluted with phosphate-buffered saline (PBS) containing 8 mM sodium azide at an 1:20 ratio. Flow cytometry analysis and sorting were performed with FACStar^{plus} (Becton Dickinson, San Jose, CA, USA) by exciting 10^4 cells at 488 nm and relative fluorescence intensity was analyzed by LysisII program (Becton Dickinson).

DNA Construction of His-Tagged GFP Mutants

Wild type GFP, S65T, and their respective truncated versions were individually inserted into His-Tag expression vectors, pET21a (Novagen) or pRSETa (Invitrogen, San Diego, CA, USA) to tag six histidines into GFP for facilitating the affinity-purification of GFP variants as follows. Wild type GFP, S65T, and I167T were amplified by PCR from their respective pET3a subclones using the primers gfp3 and gfp4 (Table 1); GFPΔC9, S65TΔC9, and I167TΔC9 with the primers gfp3 and gfpΔC9 (Table 1); GFPΔC10, S65TΔC10, and I167TΔC10 with the primers gfp3 and gfpΔC10 (Table 1). Individual PCR products were digested with BamHI and inserted into BamHI site of pET21a and pRSETa, creating pET21a-GFP, −S65T, −I167T, pRSETa-GFPΔC9, −GFPΔC10, −S65TΔC9, −S65TΔC10, −I167TΔC9, and −I167TΔC10, respectively.

β-Galactosidase gene *lacZ* was rescued by PCR from pNEX-lacZ [15] with primers rsv-s and lac1-a (Table 1). The PCR product was cut with BamHI/EcoRI. The coding sequence of truncated S65T was amplified by PCR with primers gfp1 and gfp5 (Table 1) from pET3a-S65T and digested with EcoRI/HindIII. The resulting DNA fragments containing *lacZ* and S65TΔC9 were inserted in order into BamHI/EcoRI and EcoRI/HindIII sites of pRSETa to produce pRSETa-lacZ-S65TΔC9.

Purification of His-Tagged GFP Variants and Spectrofluometric Assay

Induction and expression of His-tagged GFP variants in *E. coli* strain BL21(DE3)pLysS were done as described previously, ex-

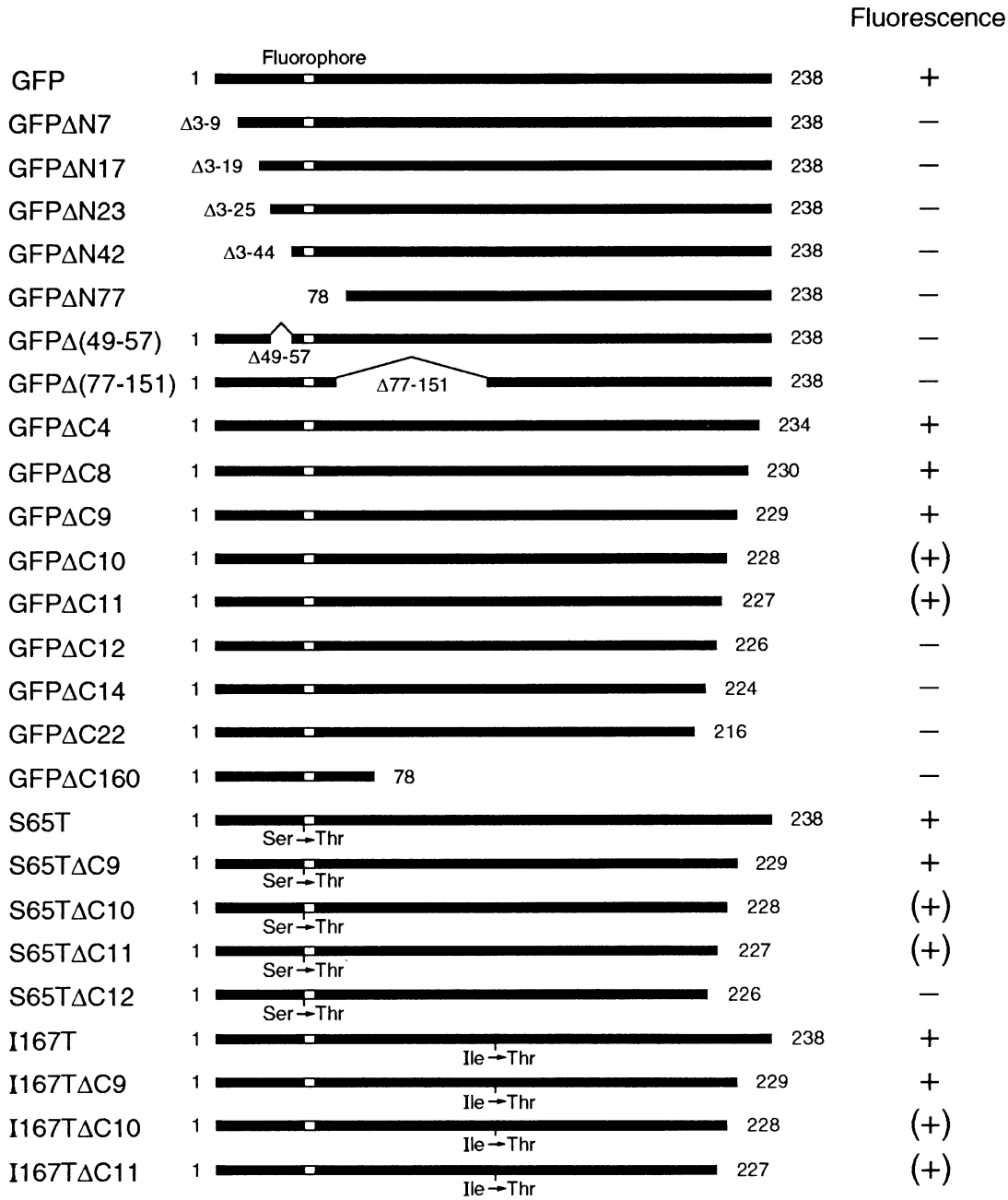

FIG. 1. Schematic representation of various deletion mutants derived from wild type GFP, S65T, and I167T and their fluorescence activity. Fluorescence activity was qualitatively determined by excitation with long-wave ultraviolet (366 nm) of transformed *E. coli* colonies growing on the agar plates. Fluorophore indicates the residues 65–67, Ser-Tyr-Gly.

cept using final concentration of 0.5 mM and 1 mM IPTG for the induction of pRSETa and pET21a subclones, respectively. Purification of His-tagged GFP variants was done using immobilized nickel affinity chromatography as described in the pET System manual (Novagen). The concentration of purified protein was 10 μg/ml in the elution buffer (1 M imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9). The spectral properties of purified GFP variants were obtained using *JASCO* FP-777 spectrofluorometer (Japan Spectroscopic Co., Tokyo, Japan). Excitation spectra were recorded at emission wavelength 510 nm. Emission spectra were recorded at excitation wavelength 395 nm. Fluorescence intensity was obtained by subtracting with the elution buffer blank. Spectra were measured with 10 nm bandwidths. Scan speed was 100 nm/min.

DNA Construction of Truncated GFP Variants in the Aplysia Expression Vector pNEXδ

The NcoI/XbaI fragment of pET3a-GFP was substituted for that of pNEXδ-GFP [11] to create pNEXδ-S65T. GFPΔC9 and S65TΔC9 were amplified by PCR with primers rsv-s and gfpΔC9 (Table 1) from pNEXδ-GFP and −S65T, respectively. The PCR fragments were cut with HindIII/BamHI and inserted into pNEXδ

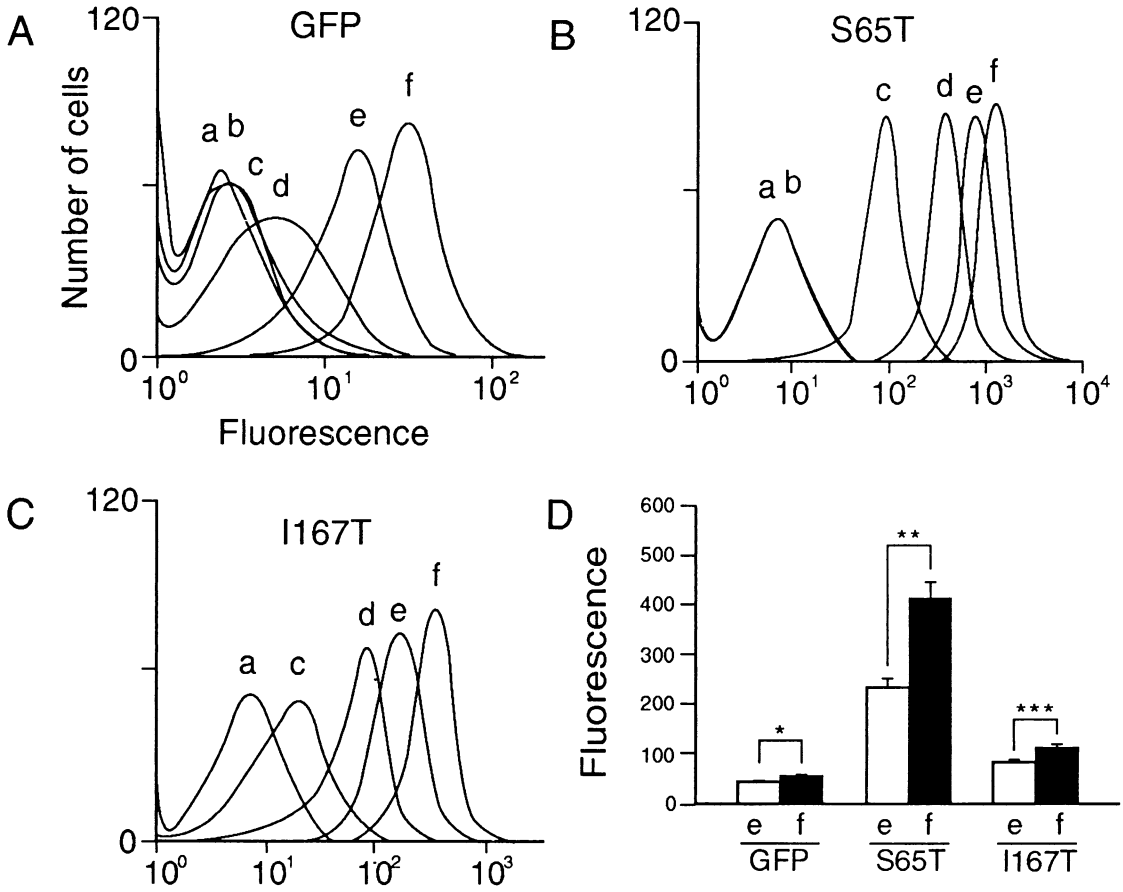

FIG. 2. The FACS analysis of GFP mutants expressed in *E. coli* system. (A–C) show representative FACS profiles for truncated mutants derived from wild-type GFP (A), S65T (B), and I167T (C), respectively: a, uninduced negative control; b, truncation of 12 residues (ΔC12) from the C-terminal; c, truncation of 11 residues (ΔC11); d, truncation of 10 residues (ΔC10); e, no truncation; f, truncation of nine residues (ΔC9); (D) illustrates group data of FACS analyses: e, no truncation; f, truncation of nine residues (ΔC9). Each independent clone of transformed *E. coli* was blindly subjected to FACS analysis. The number (n) of individual clones analyzed in this blind experiment was 34 (GFP), 31 (GFPΔC9), 29 (S65T), 29 (S65TΔC9), 10 (I167T), and 10 (I167TΔC9), respectively. The height of each bar corresponds to the mean fluorescence ±SEM. Truncation of nine amino acids significantly increased fluorescence of GFP, S65T, and I167T: $*p < 0.05$; $**p < 0.00002$; $***p < 0.02$ vs. corresponding untruncation (unpaired Student's t-test, two-tailed).

digested with the same enzymes to generate pNEXδ-GFPΔC9 and −S65TΔC9, respectively. A fusion gene was inserted by the following procedure into pNEXδ, an expression vector for *Aplysia* neurons [13]. β-Galactosidase gene *lacZ* was rescued by PCR from pNEX-lacZ [15] with primers lac-s and lac2-a (Table 1). The PCR product was cut with XbaI/BamHI and inserted into XbaI/BamHI sites of pNEXδ to produce pNEXδ-lacZxb. The coding sequences of truncated GFP and S65T were amplified by PCR with primers rsv-s and gfp6 (Table 1) from pNEXδ-GFP and −S65T, respectively. The PCR products were digested with HindIII. The resulting DNA fragments containing GFPΔC9 and S65TΔC9 were inserted into pNEXδ-lacZxb digested with HindIII to produce pNEXδ-GFPΔC9-lacZ and pNEXδ-S65TΔC9-lacZ, respectively.

Cell Culture of Aplysia Neurons, DNA Microinjection, and Reporter Assays

Aplysia kurodai weighing 50–250 g were purchased from a local supplier in Pusan, Korea and maintained in recirculating sea water tanks at 14°C. The nervous system of *A. kurodai* is very similar to that of *A. californica*, both in anatomy and in electrophysiological properties of identified neurons [17].

All plasmid DNAs used in microinjection were prepared by a standard midiprep procedure using Qiagen-tip 100 (Qiagen, Valencia, CA, USA). Animal dissection, desheathing of ganglia, preparation of cell cultures, and microinjection of DNA plasmids were done as previously described [12,15,17]. Microinjection solution composed of 1 mg/ml pNEXδ DNA construct, 10 mM Tris · Cl (pH 7.3), 100 mM KCl, and 0.1% fast green (Sigma, St. Louis, MO, USA).

The injected neurons were maintained at 18°C for 12–18 h. Green fluorescence from the neurons was detected through a Nikon Diaphot-TMD microscope with a standard fluorescence filter set (B-2A: dichroic mirror, 510 nm; excitation filter, 470–490 nm; barrier filter, 520 nm). β-Galactosidase staining was done with X-gal (GIBCO BRL, Gaithersburg, MD, USA) as previously described [15].

RESULTS

Analysis of GFP Fluorescence from the Various GFP Deletion Mutants

The results are shown in Fig. 1. The GFP fluorescence was not detectable from the GFP mutants from which either N-terminal

TABLE 2

WAVELENGTH PEAKS OF EXCITATION AND EMISSION SPECTRA OF THE AFFINITY PURIFIED GFP VARIANTS

Proteins	Excitation (nm)	Emission (nm)
GFP	399.5 (481.0)	509.3
GFPΔC9	400.8 (479.3)	509.8
GFPΔC10	400.8 (479.3)	509.0
S65T	491.9	512.3
S65TΔC9	492.0	512.0
S65TΔC10	492.0	512.3
I167T	469.8 (401.8)	509.3
I167TΔC9	469.8 (399.8)	507.3
I167TΔC10	470.0 (398.3)	507.0
LacZ-S65TΔC9	492.5	511.0

Numbers in parentheses indicate the properties of minor peaks. Data refer to averages of peak wavelengths of His-tagged GFP proteins obtained from two to four independent experiments.

amino acid residues or the parts of internal sequence of GFP were deleted. These include the mutants that lack seven, 17, 23, 42, or 77 amino acids from the N-terminal, the residues 49–57, and the residues 77–151, respectively. The deletion mutants lacking 12, 14, 22, or 160 amino acids from the C-terminal end did not emit green light, either. In contrast, deletions of four, eight, nine, 10, or 11 amino acid residues from the C-terminal did not disrupt the emission of green fluorescence. However, the appearance of fluorescence from bacterial colonies on agar plates was delayed by more than 24 h after plating in mutants devoid of 10 or 11 amino acids at the C-terminal. Other mutants lacking four, eight, or nine residues developed fluorescence overnight like the wild type. Similar truncations of amino acids at the C-terminal of the improved mutants such as S65T and I167T appeared to have the same consequences as the truncations of GFP.

FACS Analysis of Functional GFP Variants

In order to compare fluorescence intensities between these functional GFP variants in detail, we performed FACS analysis using an equal density of *E. coli* transformed with variants of pET3a-GFP (Fig. 2A–D). The cells were grown and induced under identical conditions. Unexpectedly, the truncation of nine amino acids at the C-terminal (GFPΔC9, S65TΔC9, and I167TΔC9) appeared to increase green fluorescence (Fig. 2A–C). Statistical analyses of blind experiments indicated that by this truncation fluorescence intensity increased 23% more in GFPΔC9 (54.10 ± 3.68: mean ±SEM, $n = 31$), 78% more in S65TΔC9 (413.20 ± 34.13, $n = 29$), and 34% more in I167TΔC9 (110.83 ± 7.93, $n = 10$) than the full-length GFP (44.06 ± 3.11, $n = 34$), S65T (232.61 ± 18.09, $n = 29$), and I167T (82.77 ± 6.96, $n = 10$), respectively (Fig. 2D). These increases in intensity of fluorescence after truncation of nine amino acids from the C-terminal were statistically significant ($p < 0.05$, GFPΔC9 vs. GFP; $p < 0.00002$, S65TΔC9 vs. S65T; $p < 0.02$, I167TΔC9 vs. I167T) (unpaired Student *t*-test, two-tailed).

By contrast, the mutants that lack either 10 (GFPΔC10, S65TΔC10, and I167TΔC10) or 11 (GFPΔC11, S65TΔC11, and I167TΔC11) amino acids at the C-terminal gave rise to much weaker fluorescence than did GFP, S65T, or I167T, respectively (Fig. 2A–C). A further deletion including the 12th amino acid Ala^{227} abolished GFP fluorescence completely (Fig. 2A and B).

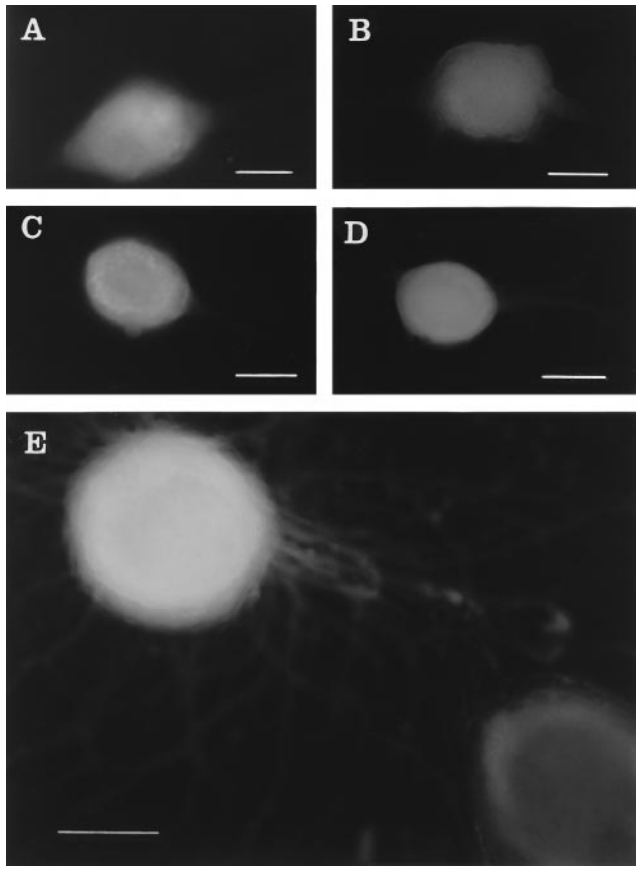

FIG. 3. Expression of fluorescence of GFP derivatives in *Aplysia* neurons: (A) GFP; (B) GFPΔC9; (C) S65T; (D,E) S65TΔC9. Green fluorescence from the neurons was detected through a Nikon Diaphot-TMD microscope with a standard fluorescence filter set (B-2A: dichroic mirror, 510 nm; excitation filter, 470–490 nm; barrier filter, 520 nm). A negative control neuron shown at the bottom right in panel E emits "orange" autofluorescence weakly that is clearly distinguishable from "green" fluorescence of GFP. Scale bar, 100 μm.

Spectral Properties of GFP Variants

We analyzed spectral properties of wild type and S65T and their truncated variants by using purified His-tagged proteins (Table 2). Spectra of GFP, S65T, and I167T were nearly identical to those reported in the literatures [2,9,10]. Both excitation and emission spectra did not seem to be affected by truncation of nine or 10 residues at the C-terminals of wild type or improved mutants of GFP. The spectra of a truncated GFP mutant did not change even after it was fused with *E. coli* β-galactosidase.

Expression of GFP and Its Fusion Gene in Aplysia *Neurons*

Finally, we addressed whether the GFP truncated mutants may still function as a fluorescent marker in the cell either alone or after fusing with other proteins such as β-galactosidase. To do this we used *Aplysia* neurons instead of *E. coli*, which contains a high background level of its own β-galactosidase. In-frame fusion of *lacZ* to truncated GFP or S65T was accomplished. Recombinant fusion genes and deletion variants of GFP were subcloned into pNEXδ [13] to be expressed under the control of a strong promoter/enhancer complex in *Aplysia* neurons. Constructed plasmids

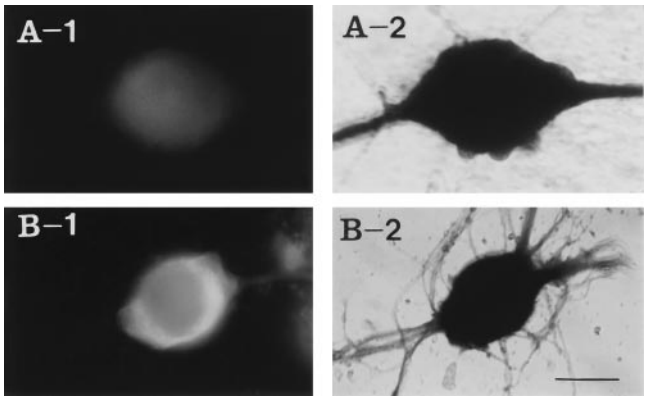

FIG. 4. Expression of fluorescence and β-galactosidase in *Aplysia* neurons of truncated GFP derivatives fused with lacZ: (A) GFPΔC9-lacZ; (B) S65TΔC9-lacZ. The same neurons were observed by fluorescence microscopy before β-galactosidase staining (A-1 and B-1) and light microscopy after staining with X-gal (A-2 and B-2). Scale bar, 100 μm.

(pNEXδ-GFP, $-$GFPΔC9, $-$S65T, $-$S65TΔC9, $-$GFPΔC9-lacZ, and $-$S65TΔC9-lacZ) were microinjected into cultured giant neurons.

We found some (approximately 20% or more) of the injected neurons emitted green light as early as 12 h after microinjection (Fig. 3A–E). Most of the neurons expressing S65T or its derivatives emitted green fluorescence at high level (Fig. 3C,D,E). However, certain proportion (approximately 40%) of the cells expressing wild type GFP or its truncated variants produced rather a weak fluorescence. Expression of GFP and its derivatives could be detected in the neurites as well as in the somata of *Aplysia* neurons (Fig. 3E). Although some of the negative control neurons emitted weak native "orange" autofluorescence (Fig. 3E, bottom right), it was easily distinguished from "green" fluorescence of expressed GFP. Neurons injected with fusion genes expressed both green fluorescence and β-galactosidase activity (Fig. 4A and B). In our experimental condition using fusion proteins expressed in neurons, X-gal staining assay for β-galactosidase activity seemed to be more sensitive than the detection of green fluorescence by fluorescence microscopy.

DISCUSSION

In the present study, we analyzed in detail the primary structure requirements for green fluorescence of GFP and of its improved mutants (Figs. 1 and 2A–D). Our results showed that unexpectedly the deletion of nine amino acids at the C-terminal end of wild type, S65T, and I167T mutant increased intensity of fluorescence. However, further deletion of 10 or 11 residues decreased intensity of fluorescence. Truncation of more than 12 residues at the C-terminal completely abolished green fluorescence of GFP.

The spectrofluometric analysis indicated that enhancement or partial decrease in GFP fluorescence by deletion of amino acids at the C-terminal end is not due to a shift in the wavelength spectra of excitation and emission (Table 2). These differences in fluorescence between mutants did not seem to result from differences in expression levels of GFP variants in *E. coli* because production levels were nearly identical to each other as shown by SDS-PAGE analyses (data not shown).

This data provides a more detailed analysis of C-terminal deletions than the recent result from Dopf and Horiagon [7], which suggested that six amino acids could be deleted from the C-terminal end of His-tagged GFP without disturbing GFP fluorescence. Our data show clearly that further deletion up to nine amino acid is not only permissive in securing the fluorescence property of GFP, but also increase the fluorescence significantly.

The stretch of nine amino acids from Thr^{230} to Lys^{238} residing at the C-terminal end forms a flexible loop [22,25]. The removal of this flexible loop does not seem to have any deleterious effect on the overall structure of GFP. Instead, the structural change by the removal of this loop caused a significant increase in fluorescence intensity. It, however, is not clear if the discrepancies in the degree of green fluorescence between the truncated and untruncated proteins are due to different kinetics in protein folding or fluorophore formation or due to changes in quantum yield. It requires further investigations to understand why this structural change leads to an enhanced GFP fluorescence. The 10th amino acid Ile^{229} from the C-terminal is the last residue of the 11th β-sheet in the cylindrical structure of GFP, presumably involved in protecting the fluorophore from the surrounding water molecule [22,25]. Therefore, a partial impairment in the 11th β-sheet by removing only up to Ile^{229} or Gly^{228} from the C-terminal may lead to partial destruction of GFP fluorescence.

Deletion at the N-terminal or within the internal sequence of GFP completely disrupted GFP fluorescence, although the fluorophore sequence (Ser^{65}-Tyr^{66}-Gly^{67}) still remained intact. This shows that both the N-terminal sequence and the internal sequence of GFP are crucial in forming the fluorophore core or in maintaining the intact structure that enables the fluorophore to function properly. This data confirms the previous result published by Dopf and Horiagon [7] and is consistent with molecular structure data of GFP [25] and S65T [22]. According to the molecular structure, the amino acid 49–57 forms a loop structure linking the third β-sheet to the α-helical fluorophore located at the center of the molecule. Therefore, deletion of this part may shift the position of the central helix to the lower site inside the cavity, thereby eventually disrupting the multiple interactions between the fluorophore and the amino acids projecting inwardly from the cylinder. Three dimensional structure also shows that the parts deleted in the other deletion mutants form either α-helix or β-sheet and that they all correspond to the regions either capping or surrounding the fluorophore. Therefore, the deletion of these parts may result in failure in protecting the fluorophore domain from surrounding water molecules.

Finally we asked if the GFP truncated mutants still function as a fluorescent marker in *Aplysia* neurons. Most *Aplysia* neurons have quite large cell somata from 40–500 μm in diameter, making it relatively easy to introduce complementary DNAs (cDNAs) into them by microinjection. A constitutive expression vector pNEX was developed for *Aplysia* neurons using a Rous sarcoma virus promoter and AP-1 enhancer elements [13,15]. This vector drives high-level constitutive expression in *Aplysia* neurons of any coding sequences subcloned downstream of its enhancer and promoter complex [12,13]. This constitutive expression does not appear to be restricted to specific neurons in the nervous system of *Aplysia* nor to specific culture conditions [11]. Using pNEX and direct microinjection as a tool of gene transfer, it has been possible to study gene regulation in sensory neurons involved in learning and memory [11,13] as well as to examine in identified neurons the function of gene products such as ion channels [15,26], transcription factors [13], synaptotagmin [20], and cell adhesion molecules [1].

Our result indicates that the truncated variants of GFP work as normally as wild type GFP in *Aplysia* neurons and that the truncated GFP fused with *E. coli lacZ* gene retained both GFP fluorescence and β-galactosidase. The GFP expression was detected not only in the somata, but also in the neurites, suggesting that expressed proteins are transported to the distal regions of the neurites from cell body. Spectrofluometric analysis also showed

that the fusion did not change excitation and emission spectra of fluorescence at least in the case of lacZ-S65TΔC9, suggesting that the fusion of lacZ to the GFP mutant did not seem to change the quality of GFP fluorescence at all. Therefore, LacZ-GFP fusion gene cassette can be utilized as a double-check marker of gene expression in many cells including neurons by using two different reporter assay systems.

In summary, we have shown that only nine amino acids at the C-terminal of GFP could be removed without any deleterious effect on green fluorescence. This deletion actually increased green fluorescence. In addition, the truncated mutants were expressed in *Aplysia* neurons either alone or as fused forms with another reporter β-galactosidase. These reporter genes could be particularly useful for studying expressions of ion channels and receptors [14] in identified neurons in the nervous system of *Aplysia* or any other species. These genes could also be used for understanding the molecular mechanisms underlying specific gene expression required for such functions as long-term facilitation, a simple form of learning and memory in *Aplysia* [16].

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