EXCLI Journal 2006;5:164-178 – ISSN 1611-2156
Received: 5. September 2006, accepted: 12. October 2006, published: 16. October 2006

Original article:

INSIGHTS INTO THE GENETIC RE-ENGINEERING OF CHIMERIC ANTIBODY-BINDING GREEN FLUORESCENT PROTEINS FOR IMMUNOLOGICAL TAGGERS

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ABSTRACT

Genetic re-manipulation of chimeric antibody-binding green fluorescent proteins was successfully conducted to create versatile tools for immunological diagnosis. Four chimeric GFPs carrying one and two-consecutive sequences of the Fc-binding motif (Z-domain), derivative of IgG-binding B domain of Staphylococcal protein A (SpA), at the C-terminus were constructed. The chimeric Ab-binding GFPs possessed dual characteristics of both IgG-binding and activity of fluorescent emission. The chimeric proteins were purified to homogeneity using an IgG-Sepharose column. Additionally, a hexahistidine was fused to the N-terminal of the GFPZ and GFPZZ to allow a high protein recovery obtained from immobilized metal (Ni^{2+}) affinity chromatography (Ni-NTA), and for protein immobilization to the sensor surface. Results obtained from the Surface Plasmon Resonance (SPR) revealed a high binding affinity (K_A) to immobilized human immunoglobulin up to 6.7 and 81.1 (10^7/M) for the GFPZ and GFPZZ, respectively. This affinity constant was raised up to 2-5 times higher when the chimeric GFPs harboring hexahistidine residues were captured on the sensor chip via metal coordination. The strong binding affinity to IgG of the chimeric GFPs was clinically applied to detect the antinuclear antibody. A strong intensity of fluorescence, higher than that of the classical fluorescein isothiocyanate (FITC) conjugated system, was significantly detected. Moreover, the proteins with double repeats of Fc-binding motif (GFPZZ and H_6GFPZZ) obviously demonstrated a more intense fluorescent signal than those of the single Z domain, which corresponded to the result from SPR. All these findings support a high potential for applying such chimeric Ab-binding GFPs for clinical applications.

Keywords: Chimeric Antibody-binding GFP, Surface Plasmon Resonance, Fluorescent antinuclear antibody analysis, C-terminus GFP fusion

INTRODUCTION

Green fluorescent protein (GFP) is an autofluorescent protein originally isolated from the Pacific Northwest jellyfish, *Aequorea victoria* (Shimomura, 2006). It is a 27 kDa protein that emits fluorescent light (λmax = 508 nm) by excitation at 395 nm. Neither substrate nor cofactor is required for the fluorescent emission. This emission is attributed to the presence of an internal chromophore, formed by cyclization and oxidation of residues Ser65, Tyr66, and Gly67 (Cody et al., 1993). Fusion of the GFP to various kinds of cellular protein has been applied as a reporter for gene expression

(Cha et al., 2002; Kain et al., 1995), as a marker to study cell lineage during development (Raich et al., 1999; Zernicka-Goetz et al., 1997), and as a tag to localize proteins in living cells (Feilmeier et al., 2000; Phillips, 2001).

GFP is a relatively small monomeric protein which possesses a unique conformation of β-barrel structure in which the emission chromophore resides (Phillips, 1997). Such a compact structure makes GFP highly stable in various hazardous conditions and resistant to high concentration of denaturing agents (Ward et al., 1982). Improper folding of the β-barrel structure leads to a decrease of GFP stability and a diminishing of fluorescent activity.

Although, researchers have claimed that fluorescence of the full-length GFP is not significantly affected by fusing of the partner protein to either its N- or C-terminus (Margolin, 2000; Yang et al., 1996), there are studies showing that fusion of some proteins to GFP results in a decrease in its fluorescence, especially when the protein was fused to the N-terminus. In our hands, N-terminal fusion not only influences the fluorescent activity in certain cases but also affects localization of the fusion protein (Prachayasittikul et al., 2004; Prachaya-sittikul et al., 2001). Aoki et al. (1996) and Arai et al. (1998) have found that purified protein A-GFP and protein G-EGFP contain portions of protein without fluorescent activity. The fluorescent and non-fluorescent forms of such proteins have migrated to different locations in SDS-PAGE analysis. Sacchetti et al. (1999) have reported that fusion of Myc-tag to the N-terminus of GFP diminishes folding efficiency of GFP, whereas fusion of Sb-tag to the C-terminus of GFP results in 3-fold enhanced folding. This phenomenon can be explained by the fact that the folding of protein is generally started immediately after their N-terminus is translated. Therefore, fusion of the other proteins to the N-terminus may cause irregularly folding.

It has been found that the fluorescence of GFP decreases when it has been expressed under a *pelB* signal sequence (Casey et al., 2000; Lei et al., 1987). Similar evidence has also been found in the chimeric protein composed of GFP fused to the C-terminus of maltose-binding protein (MBP) (Feilmeier et al., 2000). Fluorescence has not been detected when the chimeric protein is translated in conjunction with the MBP signal sequence. In contrast, when the signal sequence is deleted, the fluorescent activity is regained. In certain circumstances, the N-terminal fusion brings about low protein expression (Griep et al., 1999; Morino et al., 2001).

In our previous study (Prachayasittikul et al., 2005), we constructed the chimeric Fc-binding GFP, where the double repeat of Z-domain, a synthetic binding domain derived from protein A (Fexby et al., 2004), was genetically fused to the N-terminus of GFPuv (Crameri et al., 1996). However, a decrease in fluorescent activity and a low yield of the chimeric protein was revealed. Such effects are plausibly encountered via the presence of a signal peptide and the fusing location at the N-terminus of GFPuv. Therefore, in this study, the signal peptide is omitted and we have constructed the Ab-binding GFPs by genetically fusing one or two consecutive Z-domains to the C-terminus. Furthermore, a hexahistidine tag has been added to allow an alternative purification using immobilized metal affinity chromatography (IMAC) and for metal immobilization on the sensor surface of Surface Plasmon Resonance (SPR). The feasibility of using these chimeric proteins as powerful tools for immunological diagnosis has also been explored.

MATERIALS AND METHODS

Plasmids and Bacterial Strains

Plasmids pEZZ18 and pTrc99A, both obtained from GE Healthcare (Uppsala, Sweden), were used for the chimeric genes construction. The plasmid pTGFPuv previously described elsewhere (Fexby et al., 2004) was used as a source of the gene encoding green fluorescent protein. *E. coli* strain TG1 (*sup*E, *hsd*Δ5, *thi*Δ(lac-proAB),

F'[*tra*D36 *pro*AB$^+$ *lac*I^q lacZΔM15] was used for cloning and protein expression.

Enzymes and Chemicals

Restriction endonucleases were purchased from MBI Fermentas (Vilnius, Lithuania). T4 DNA ligase and the "Expand High Fidelity PCR system" were obtained from New England Biolabs (Beverly, MA, USA) and Roche (Mannheim, Germany), respectively. All other chemicals were of analytical grade and commercially available.

Construction of chimeric genes encoding chimeric Ab-binding GFPs

A series of plasmids harboring chimeric genes encoding Fc-binding green fluorescent proteins was constructed. All cloning procedures were performed according to the standard protocol described by Maniatis et al. (1989). The experiment was initiated by construction of a chimeric gene encoding the green fluorescent protein carrying a hexahistidine at the N-terminal. Briefly, a complementary mixture of 0.1 μM of H6_1 (5′CATGGGTCATCACCATCACCATCAC GGTACCATCGATG-3′) and H6_2 (5′-GATCCATCGATGGTACCGTGATGGTA TGGTGATGACC-3′), synthetic oligo-nucleotides obtained from MWG Biotech (Ebersburg, FRG), was hybridized at 90C for 15 min and slowly cooled down to room temperature. The hybridized DNA fragment containing an *Nco*I site at the 5′-end and a *Bam*HI site at the 3′-end was further inserted in between the *Nco*I and *Bam*HI sites of pTrc99A. Since there was an additional *Kpn*I site following the hexahistidine sequence, the *gfpuv* gene obtained from pTGFPuv was subsequently inserted in between the *Kpn*I and *Pst*I sites, resulting in plasmid pTH$_6$GFPuv.

The fragments of gene encoding Fc-binding domain (Z or ZZ) were obtained by PCR amplification using the plasmid pEZZ18 as template together with the following sense (prim5ZZ_for: 5′-AAAAGAGCTCGTAGACAACAAATTCA ACAAAGAAC-3′) and antisense primers (prim3ZZ_rev: 5′-AAAACTGCAGTCATTT CGGCGCCTGAGCATC-3′) (MWG Bio-tech). Parts of both primers are identical to the Z encoded gene, prim5ZZ includes a 5′ overhang containing a *Sac*I restriction site (underlined) and prim3ZZ includes a 5′ overhang containing a *Pst*I site (underlined) as well as a TCA stop codon (reverse complementary sequence of TGA). Fragments obtained from amplification were then separated by agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen, Basels, Switzerland). The DNA fragments of Z and ZZ encoded genes were digested with *Sac*I and *Pst*I and individually cloned into the 3′-end of hexahistidine-tagged GFP encoded gene in the plasmid pTH$_6$GFPuv, resulting in plasmids pTH$_6$GFPZ and pTH$_6$GFPZZ, respectively. The genes encoding GFPZ and GFPZZ were then cleaved from pTH$_6$GFPZ and pTH$_6$GFPZZ by *Kpn*I and *Pst*I digestion and inserted into a new pTrc99A plasmid vector, resulting in plasmids pTGFPZ and pTGFPZZ, respectively. The cloning strategy of the different GFP constructs is presented in Figure 1. The newly constructed plasmids were checked for the in-frame fusion by DNA sequencing using BigDye terminators v3.0 from Applied Biosystems (Warrington, UK), according to the supplier's recom-mendations.

Expression and purification of chimeric Ab-binding GFPs

Crude protein extracts preparation

E. coli cells carrying various kinds of chimeric gene were grown in 400 ml of LB medium (Tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L) supplemented with ampicillin (100 mg/L). The culture was incubated at 37C under shaking (150 rpm) for 2 hrs. Protein expression was then induced upon addition of IPTG to a final concentration of 1 mM and the cells were further grown at 30C until the late exponential phase (16-18 hrs). Cells were harvested by spinning at 7,000 *g* for 5 min, re-suspended in an appropriate working buffer (TST; 50 mM Tris-HOAc, 150 mM NaCl and 0.05 % Tween 20, pH 7.6 or IMAC

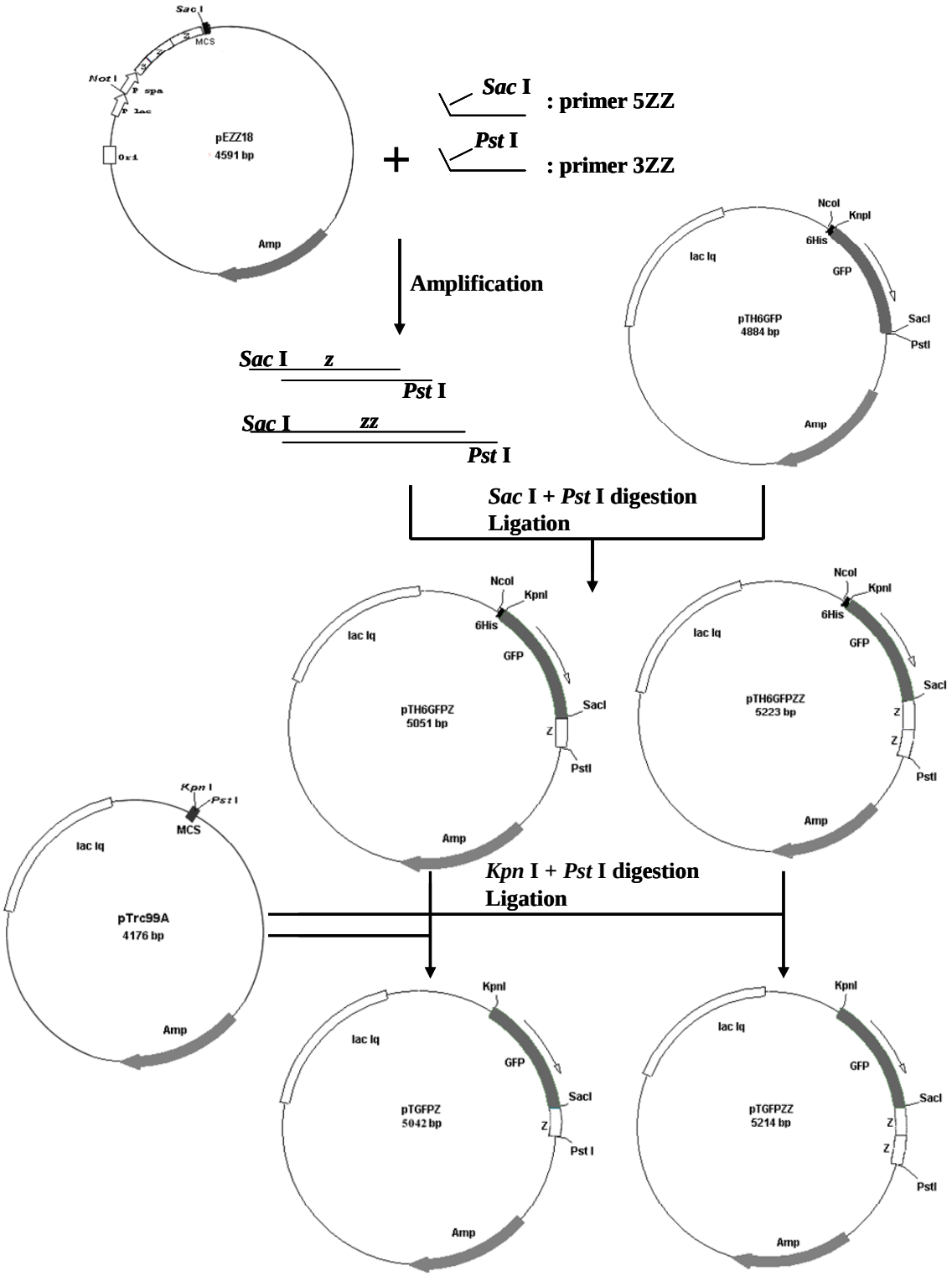

Figure 1: Diagrammatic representation of gene encoding antibody-binding GFP constructs.

buffer; 0.05 M Na-Phosphate, 0.5 M NaCl, pH 7.6), and disrupted by sonic disintegration. Cell debris was removed by centrifugation at 17,000 *g* for 15 min. Bulk proteins were partially eliminated by heating of the crude extracts at 70C for 15 min and the denatured proteins were subsequently removed by centrifugation at 17,000 *g* for 15 min. The clear supernatants were collected for further purification.

Purification of chimeric protein via IgG-Sepharose affinity chromatography

The supernatant, dissolved in TST buffer, was applied onto 2 ml of IgG-Sepharose 6 Fast Flow gel (GE Healthcare) pre-packed in a glass Econo-column (1 cm × 10 cm) from BioRad Laboratories (Sundbyberg, Sweden). Unbound protein was washed out with 20 ml TST buffer followed by 4 ml of 5 mM ammonium acetate, pH 5.0. The chimeric Ab-binding GFPs were then eluted using 0.3 M acetic acid, pH 3.4 and the collected fractions were immediately neutralized with an equal volume of 0.5 M Tris-HOAc, pH 10.0. The green fluorescent fractions were pooled and stored at 4C until further analysis.

Purification of chimeric protein via Immobilized Metal Affinity Chromatography (IMAC)

The supernatants of H_6GFPZ and H_6GFPZZ dissolved in IMAC buffer obtained after heat treating step were further applied onto 2 ml of NTA-agarose gel (Qiagen) immobilized with nickel ions (Ni^{2+}-NTA) pre-packed in a C10 column (1 cm ×10 cm) using an AC10 adaptor (GE Healthcare). The column was washed using IMAC buffer containing 12 mM imidazole. Elution of chimeric GFPs was performed upon addition of 80 mM imidazole dissolved in the same buffer. Monitoring of protein elution profile was done using a UV detector at 280 nm and the different fractions were collected in accordance with the absorbance.

The recovered samples from both purification methods were finally dialyzed using Spectra/Por tubings (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) soaked in 0.01 M HEPES, 0.15 M NaCl, pH 7.6. Protein concentrations were measured according to the Bradford's method (Bradford, 1976) using "BioRad Protein Assay".

Characterization of chimeric Ab-binding GFPs

Molecular size estimation and fluorescent determination using SDS-PAGE

Protein separation was performed on 12% polyacrylamide gels in a discontinuous buffer system, as described by Laemmli (1970). Samples were mixed with loading buffer and applied directly to the gel with or without heat denaturation. Molecular size estimation and fluorescent determination of the chimeric Ab-binding GFPs was performed on the SDS-PAGE with and without heat denaturation, respectively.

Investigation of native state of chimeric Ab-binding GFPs by gel filtration

The gel filtration column was prepared using Sephacryl S-200 superfine (GE Healthcare). Gel was resuspended in running buffer composed of 10 mM Tris-HCl, 0.15 M NaCl, pH 8.0 and packed at 1 ml/min into a C16 glass column (1.6 cm × 70 cm) with adaptors, generating a bed height of 48.5 cm. All samples were run at a flow rate of 0.1 ml/min, monitored with a UV detector (280 nm) and collected into 2 ml fractions. Blue-dextran was used to determine the void volume. Lactate dehydrogenase (LDH), bovine serum albumin (BSA) (Sigma), luciferase and GFPuv were used to calibrate the column. Each protein construct was individually applied to the gel filtration column to determine the molecular mass in the native state and to detect the feasibility of dimer formation.

Analysis of fluorescent property of chimeric Ab-binding GFPs

To investigate the fluorescence properties of cells expressing GFPuv and of the purified proteins, the fluorescence intensities of cell suspension and purified protein extracts were measured. *E. coli* harboring plasmids pTGFPZ, pTGFPZZ, pTH_6GFPZ and pTH_6GFPZZ were grown in 100 ml flask containing 50 ml of LB broth supplemented with 100 µg/ml ampicillin and IPTG was then added to a final concentration of 1 mM at 2 hrs after inoculation. The cultures were further incubated at 30C for 18 hrs. Cells were harvested and resuspended in Tris-HCl, pH 8.0 to the same density (OD_{600} = 1). The measurements were performed in 4.0 ml cuvettes containing 3.0 ml of cell suspensions. Fluorescence intensities at 508 nm were measured upon excitation at 400 nm using a Fluorimeter system from Photon Technology International (West Sussex, UK).

Fluorescence intensity of the purified chimeric Ab-binding GFPs was also measured in a similar manner. Specific fluorescence activity was further calculated as the fluorescence intensity per milligram protein.

Biospecific interaction analysis of chimeric Ab-binding GFPs

Binding affinity to immobilized IgG molecules on sensor surface

To characterize the IgG binding affinity of all four constructs, the interaction between the chimeric Ab-binding GFPs and human IgG was investigated in real time using the BIACORE 3000 system (Biacore AB, Uppsala, Sweden). Human purified IgG (DakoCytomation, Glostrup, Denmark) was utilized as an immobilized ligand. Immobilization was performed using the amine-coupling procedure as described elsewhere (Johnsson et al., 1991). Briefly, a carboxylated dextran layer of the Biacore CM5 sensor chip was activated by N-hydroxysuccinimide (NHS) and N-ethyl-N′-(3-diethylaminopropyl)-carbodiimide (EDC). The IgG solution used for immobilization was prepared in 0.1 M sodium acetate, pH 5.6 to a concentration of 100 nM. A background response of approximately 1800 response units (RU), caused by the immobilized molecule, was reached before the surface was deactivated using 1 M ethanolamine-hydrochloride solution. A flow cell, activated and deactivated in the same way, but without IgG immobilization, was used as reference. The chimeric Ab-binding GFPs were diluted in HBS-P buffer (0.01 M HEPES, 0.15 M NaCl, 0.005% Surfactant P20, pH 7.4) to final concentrations of 200, 100, 50, 25, and 12.5 nM. One hundred and fifty microlitres of each diluted sample was injected (30 µL/min) over the immobilized IgG surface. The association and dissociation rates of GFPZ, GFPZZ, H_6GFPZ and H_6GFPZZ to the IgG ligand were monitored and the results were displayed as sensograms. The data were subsequently evaluated using the BIAevaluation 3.2 software (Biacore). The association rate constant (k_a), dissociation rate constant (k_d) and affinity constant (K_A) were calculated using a 1:1 Langmuir adsorption model.

Binding affinity to free IgG molecules in solution

To further determine the binding affinity of the chimeric Ab-binding GFPs to free IgG molecules, the chimeric H_6GFPZ and H_6GFPZZ were immobilized onto the NTA sensor chip via a metal-binding interaction. In detail, nickel ions (500 µM in running buffer; 0.01 M HEPES, 50 µM EDTA, 0.15 M NaCl, pH 7.4) were firstly immobilized onto the chip by 1 minute injection at a flow rate of 10 µl/min. The H_6GFPZ or H_6GFPZZ obtained from IgG-Sepharose purification was further immobilized to a level of approximately 400 RU. Samples of human IgG prepared at 5 different concentrations (275, 137.5, 69, 34.5 and 17 nM) were prepared in running buffer and injected at a flow rate of 30 µl/min. Between each sample injection, the chip was regenerated by 30 µL of regeneration buffer (0.01 M HEPES, 0.15 M NaCl, 0.35 M EDTA, 0.005% surfactant P20, pH 8.3) at a flow rate of 10 µl/min. The BIAevaluation 3.2 software was used to evaluate the data and the kinetics constants were calculated using a 1:1 Langmuir model.

Evaluation of potential usage of chimeric Ab-binding GFPs on fluorescent antinuclear antibody (FANA) analysis

To evaluate the potential usage of applying chimeric Ab-binding GFPs for immunological diagnosis, fluorescent antinuclear antibody (FANA) assay was chosen. Cryosections of rat liver were prepared onto microscope glass slides and kept at -20C until used. Immediately prior to the FANA test, the slides were removed from the freezer, allowed to warm up to room temperature, and air dried. Thirty microlitres of serum from patient were applied onto the slides. After 30 min incubation at room temperature in a moist chamber, the slides were washed three times with PBS and allowed to dry at room temperature. Subsequently, 20 µl of the chimeric proteins (1 µM in PBS) was applied to the liver

section and incubated at room temperature in a moist chamber for an additional 30 min. After being washed three times with PBS and air dried at room temperature, the slides were covered with glycerin and a cover slip. The results were observed under a fluorescence microscope using an FITC-filter set. The negative control was included by performing the test in the same manner using the normal serum. The test was also performed using an FITC-conjugated antibody (DakoCytomation, Glostrup, Denmark) for comparison.

RESULTS AND DISCUSSION

In our previous work, the chimeric Fc-binding green fluorescent protein (ZZGFPuv) was constructed by gene fusion at the N-terminus of GFP (Prachayasittikul et al., 2005). However, the low protein expression and loss of fluorescence in the secreted portion are a major concern. The N-terminal extension has been found to decrease the fluorescence activity in the fusion of some individual proteins to the GFP. This probably is caused by the improper folding or defolding of the protein (Prachayasittikul et al., 2004; Prachayasittikul et al., 2001). Moreover, the presence of *SpA* signal sequence has been found to reduce gene expression and in some circumstances the chimeric protein was ineffectively secreted by *sec* pathway during translocation process (Feilmeier et al., 2000). Meanwhile, it has been reported by Sacchetti et al. (1999) that fusion at the C-terminus provides a better folding of GFP, especially when the tag protein possesses a stable helix conformation. These have motivated us to re-engineer the chimeric Fc-binding GFPs in order to obtain a higher yield of fully function chimeric proteins.

Genetic re-engineering of chimeric Ab-binding green fluorescent proteins

In the present study, a series of chimeric genes encoding chimeric green fluorescent proteins carrying one and two-consecutive Fc-binding motifs (GFPZ, GFPZZ) at the C-terminus have successfully been constructed. In addition, the H_6GFPZ and H_6GFPZZ, which are identical to those of GFPZ and GFPZZ and contain an additional hexahistidine tag at the N-terminus of GFP, have also been constructed, to assist protein purification as well as for immobilization via metal-chelation. Strong cellular fluorescence could be detected upon exposure of *E. coli* expressing chimeric Ab-binding GFPs to UV illumination as represented in Figure 2. This finding infers that extension of the peptide on the C-termini does not have any significant disturbance on the GFP's fluorescence.

Levels of protein expression on different variants of chimeric Ab-binding GFPs in *E. coli* were also determined. Both the fluorescence activities at the cellular level and the specific fluorescence of purified protein obtained from IgG-Sepharose purification were measured (Figure 3). Total cellular fluorescence of the cells expressing H_6GFPZ and H_6GFPZZ were approximately 2.5 times lower than those of the others. Meanwhile, a non-significant difference of specific fluorescence activity among the purified proteins was observed. Our findings lend support to the notion that disparity of fluorescent emission of cell suspensions might be attributable to the variation of protein expression level. This was supported by findings that the amounts of purified protein obtained from 1 litre of culture were at 14.1, 18.2, 4.7 and 4.9 mg for cells expressing GFPZ, GFPZZ, H_6GFPZ and H_6GFPZZ, respectively. Thus, the presence of a hexahistidine tag appears to be detrimental to chimeric protein expression. However, this limitation can be overcome by supplementation of free histidine residue to the growth medium (Lilius et al., 1991).

Our results revealed that the specific fluorescence of purified GFPZ, GFPZZ, H_6GFPZ and H_6GFPZZ were approximately 1.13, 1.91, 0.85 and 0.82 times as compared to that of the native GFPuv. More importantly, the chimeric GFPZZ exhibited 3.8 times higher specific fluorescence than that of the fluorescent form of ZZGFP (36 kDa). Such findings are an indication that fusion of Z-domains to the C-terminus of

GFP can overcome the loss of fluorescent activity due to the incomplete or improper folding of chimeric GFP derived from N-terminus fusion.

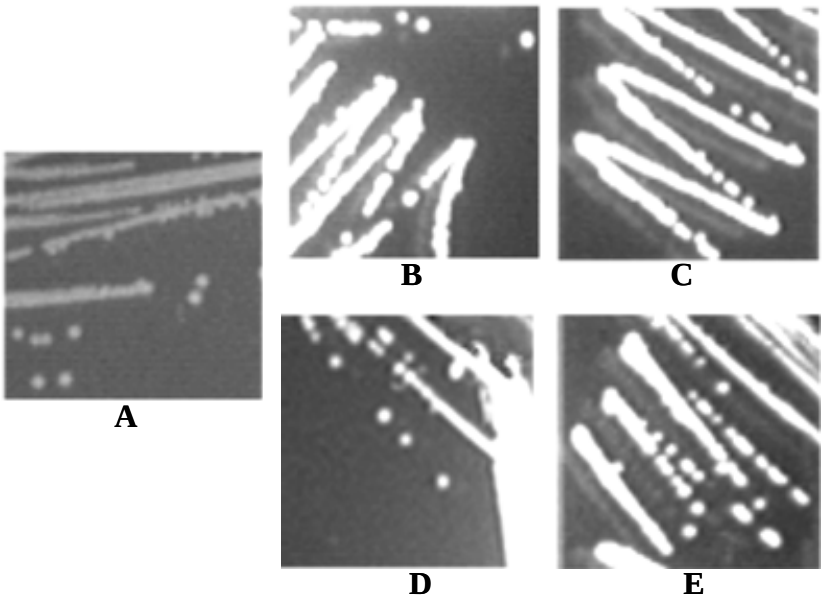

Figure 2: Colony of *E. coli* TG1 carrying plasmid pTrc99A (A), pTGFPZ (B), pTGFPZZ (C), pTH_6GFPZ (D) and pTH_6GFPZZ (E), observed under UV illumination.

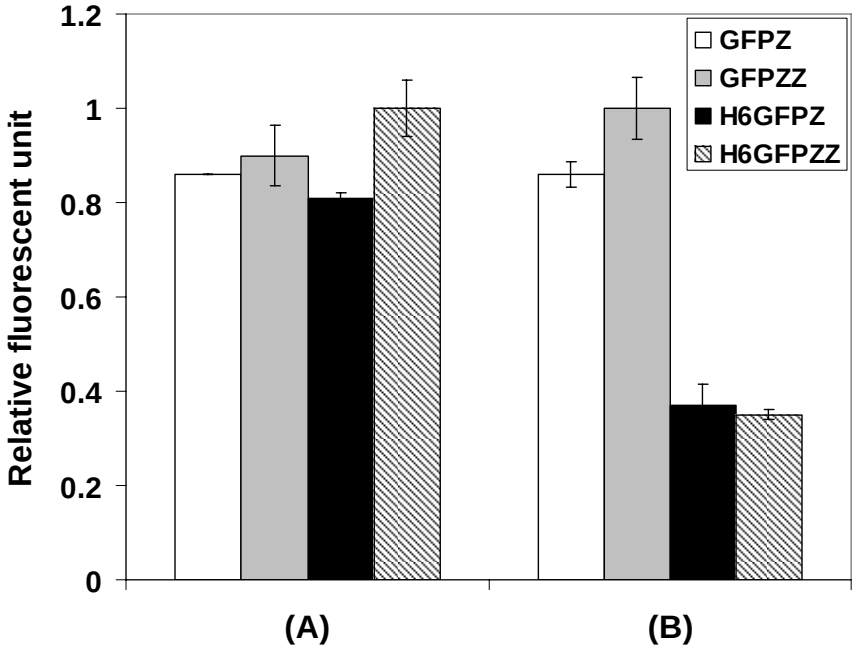

Figure 3: Specific fluorescence activity of each chimeric protein (A) and fluorescence level of *E. coli* cells expressing chimeric antibody-binding green fluorescent proteins (GFPZ and GFPZZ denoted as chimeric GFPs carrying one and two consecutive Z-domains at C-terminal, respectively; H_6GFPZ and H_6GFPZZ denoted as chimeric GFPZ and GFPZZ harboring an additional hexahistidine at their N-terminal) (B) were determined. The results were normalized by dividing by the highest value of each category. The values represent the means calculated from three independent experiments.

To further examine the molecular weight of chimeric Ab-binding GFPs and the tendency to form dimer (Phillips, 1997), all the four constructs were individually subjected to gel filtration. A standard curve for molecular weight determination was created using lactate dehydrogenases, BSA, luciferase and GFPuv. Estimation of the molecular masses of the chimeric proteins was calculated from their elution volumes as represented in Table 1. The molecular masses of protein in the native state were 30.9, 41.7, 33.9 and 40.7 kDa for the GFPZ, GFPZZ, H_6GFPZ and H_6GFPZZ, respectively. These values were in good agreement with the calculated values, proving that monomeric proteins were all obtained.

Purification efficiency of chimeric proteins using IgG Sepharose column vs. immobilized metal ion affinity chromatography (IMAC)

All chimeric Ab-binding GFPs possessed dual characteristics of both IgG-binding and fluorescence properties. They could all be purified to homogeniety via IgG-Sepharose column while retaining the high fluorescence intensity after purification, as seen in Figure 4. However, elution of the chimeric protein from the IgG column requires very low pH, which may lead to the disturbance of protein function. Therefore, the IMAC was chosen as an alternative method due to its mild-elution conditions. The IMAC is also low cost and applicable for multiple reusage.

Further experiments were then conducted on the efficiency of protein purification via IgG Sepharose and IMAC columns charged with nickel ions. Results obtained from the purification of H_6GFPZ and H_6GFPZZ using both methods are illustrated in Figure 5 and Table 2. As seen in Figure 5, the proteins were successfully purified to homogeneity via the two approaches, in which the majority of protein bands were represented at 34.3 and 40.9 kDa, for the chimeric H_6GFPZ (top panel) and H_6GFPZZ (low panel), respectively. It seemed that the purity of the chimeric GFPs purified from Ni-NTA was slightly lower. However, the IMAC purification has been proven to be beneficial, as the recovered yields (ca. 69%) were higher than that of IgG sepharose purification (ca. 56%) (Table 2).

It is noteworthy that a marked decrease of fluorescence was found when the protein was solubilized in TST buffer. This is most probably due to the presence of Tween, which may induce the exposure of hydrophobic residues buried inside the protein molecule and cause destabilization of GFP by favoring aggregation.

In a separate work, we have compared the influencing effects which occur during the cell lysis processes either via sonication or detergent-containing lysing buffer. When the extracts were further heat-treated, a greater loss of GFP was observed in the presence of detergent (data not shown). This suggests that the buffering system influences the heat durability of the GFP. The thermostability of GFPuv at pH 7-8 is more intense in phosphate buffer than in the Tris-HCl (Penna et al., 2004).

Table 1 The molecular mass of chimeric Fc-binding GFPs determined by gel filtration and the theoretical molecular mass calculated from amino acid sequences.

Chimeric proteins	Molecular mass (kDa) determined by gel filtration	Theoretical molecular mass (kDa)
GFPZ	30.9	33.1
GFPZZ	41.7	39.8
H_6GFPZ	33.9	34.3
H_6GFPZZ	40.7	40.9

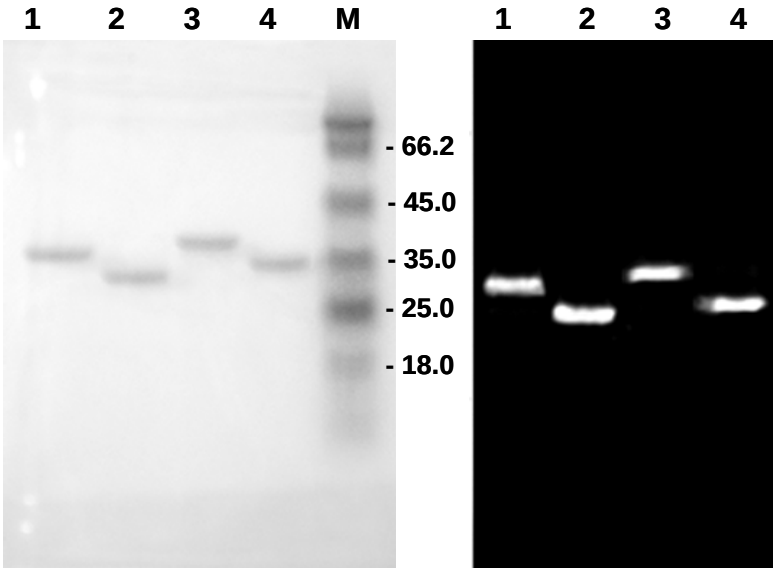

Figure 4: SDS-PAGE of chimeric proteins purified using IgG immobilized affinity chromatography. Approximately 3 μg of protein were applied to electrophoresis gel with (left) or without (right) heat denaturation. The gel on the left side was stained with Coomassie brilliant blue whereas on the right the gel was exposed to UV irradiation. Lane 1, 2, 3, and 4 represented chimeric GFPZZ, GFPZ, H_6GFPZZ and H_6GFPZ, respectively, M indicated a protein molecular weight marker (MBI fermentas) presented in kDa.

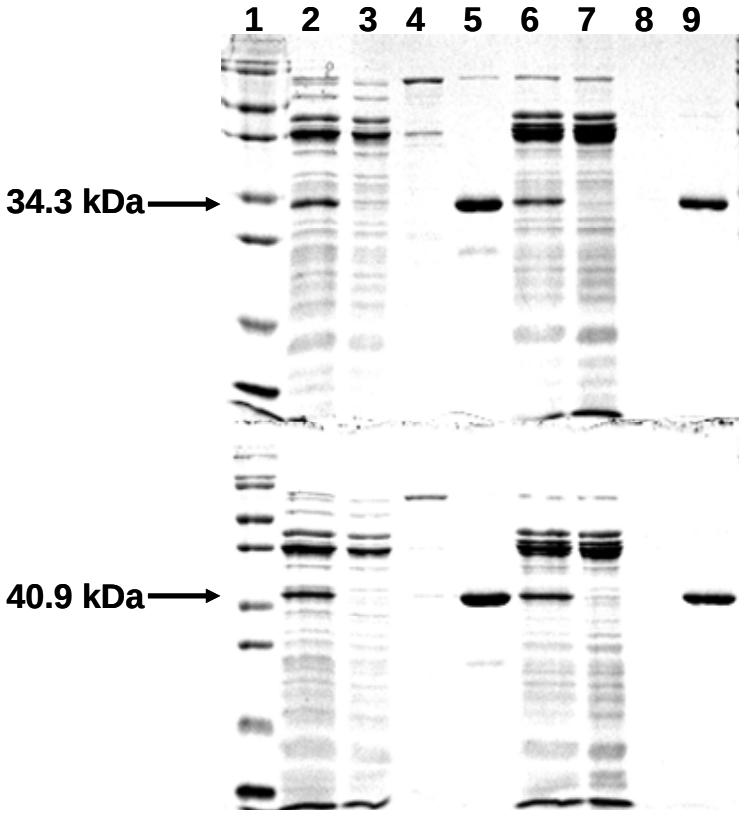

Figure 5: SDS-PAGE demonstrating purification of H_6GFPZ (upper gel) and H_6GFPZZ (lower gel). Lane 2-5 were samples from IMAC and lane 6-9 were from IgG sepharose purification. lane 1: molecular weight marker, lanes 2 and 6: heat treated crude extract, lanes 3 and 7: unbound protein, lane 4: 12 mM imidazole wash, lane 8: NH_4Ac (pH 5.0) wash, lanes 5 and 9: eluate fraction.

Table 2 Purification efficiency of chimeric Fc-binding GFPs via IgG Sepharose and IMAC.

Protein	Purification step	Total protein (mg)	Total fluorescence (count/sec)x10^6	Specific fluorescence (count/sec/mg)x10^6	%recovery	Purification fold
GFPZ	crude extract	193	697	3.6	100	1
	Heat treatment	38.4	607	15.8	86	4.4
	IgG sepharose	2.8	488	174.2	70	48.3
GFPZZ	crude extract	226	859	3.8	100	1
	Heat treatment	45	765	17	89	4.5
	IgG sepharose	3.5	638	182.3	74	47.9
H_6GFPZ	crude extract	82.6	244	3	100	1
	heat treatment	18.3	218	11.9	89	4
	IMAC (Ni-NTA)	0.92	167	181.2	68	60.4
	crude extract	83.4	248	3	100	1
	heat treatment	16.6	200	12	81	4
	IgG sepharose	0.75	138	182.6	56	60.9
H_6GFPZZ	crude extract	84.3	273	3.2	100	1
	heat treatment	17.6	230	13.1	84	4.1
	IMAC (Ni-NTA)	1.15	188	163.6	69	51.1
	crude extract	84.7	294	3.5	100	1
	heat treatment	16.4	225	13.7	77	3.9
	IgG sepharose	1.00	164	163.6	56	46.7

Binding analysis of chimeric Ab-binding GFPs towards IgG

In order to determine the kinetic parameters of the binding interaction between the chimeric Ab-binding GFPs and IgG, a binding analysis using surface plasmon resonance was performed. Experiments were carried out to include the binding affinity to immobilized IgG on the sensor surface and to free IgG molecules in the solution. Human IgG (hIgG) was immobilized onto the sensor surface by amino coupling. The chimeric GFPs were then individually injected and their association and dissociation constants were analyzed. For the binding test of free IgG, the H_6GFPZ and H_6GFPZZ were applied as ligands and were non-covalently captured on the Ni-NTA sensor chip. The binding interaction was analyzed upon injection of the free IgG molecules into the system. For controls, both the GFPuv and the histidine tagged GFPuv variant (His_6GFP) were tested against hIgG. None of the two proteins exhibited any affinity towards hIgG (data not shown), indicating the necessity of Z domain for high specificity achievements.

As shown in Table 3, the affinity constants (K_A) obtained from amino coupling of the chimeric GFPZ, GFPZZ, H_6GFPZ and H_6GFPZZ were 6.7, 81.1, 2.4 and 60.3, respectively. Our findings revealed that the chimeric GFP harboring two-repetitive Z-domains exhibited ten times higher affinity towards IgG than a single Z-domain chimera. These values are consistent with those reported in the literature for the Z and ZZ-domains (K_A = 1.0-10.0 and 10-100, respectively) (Fexby et al., 2004; Gulich et al., 2000; Tashiro and Montelione, 1995). One main reason for the higher affinity constant of ZZ is owing to the lower dissociation rate, presumably caused by a weak interaction between different molecules

Table 3 Kinetics binding constants of chimeric Fc-binding GFPs for human IgG

	k_a* (10^5/Ms)	k_d* (10^{-3}/s)	K_A* (10^7/M)
IgG-immobilization			
Z**	0.5-2.0	2.0-10.0	1.0-10.0
ZZ**	1.0-5.0	0.2-1.0	10-100
GFPZ	1.1	1.64	6.7
GFPZZ	1.9	0.23	81.1
H_6GFPZ	0.5	2.00	2.4
H_6GFPZZ	1.4	0.23	60.3
Metal-immobilization			
H_6GFPZ	0.2	0.16	11.4
H_6GFPZZ	0.4	0.03	125.0

*k_a is the association rate constant. k_d is the dissociation rate constant. K_A is the affinity constant described as the ratio between k_a and k_d.
**The kinetic parameters of Z and ZZ were obtained from previous studies (Fexby et al., 2004; Gulich et al., 2000; Tashiro and Montelione, 1995).

of Z domain or by the second site cooperation on the Fc molecule (Jendeberg et al., 1995; Jendeberg et al., 1996). In addition, it should be noted herein that the hexahistidine tagged constructs exhibited a small decrease in affinity constant. However, this affinity is still satisfactory for further applications in immunoassays.

Results obtained from the NTA chip had a much higher affinity than those obtained from amine coupling experiments. This high affinity resulted from approximately 13 and 8 times lower dissociation rates (K_d), in conjunction with 2.5 and 3.5 times lower association rates (K_a), for the H_6GFPZ and H_6GFPZZ, respectively. The difference in K_a could be explained by the discrepancy in binding orientation. Plausible explanations can be drawn from i) the decrease of K_a might be due in part to the high accessibility of small molecules (H_6GFPZ or H_6GFPZZ; ~30-40 kDa) to bind to IgG molecules (~150 kDa); ii) the difficulty of IgG to bind to the chimeric H_6GFPZ or H_6GFPZZ pointed toward the chip surface, since the N- and C-terminal of GFP are located on the same side of β-can (Phillips, 1997). Therefore, the occupancy upon binding to immobilized metal ions is needed to be taken into account. For the lower K_d obtained from the IgG-immobilized experiment, one plausible reason could be the fact that IgG might lose part of its binding ability during the amino coupling (Johnsson et al., 1995). However, non-specific binding to the immobilized metal surface is negligible since no binding was observed without pre-capturing of GFP molecules (data not shown).

Application of chimeric Ab-binding GFPs on fluorescent antinuclear antibody analysis (FANA)

To suit an ultimate goal of this study, the feasibility of applying the chimeric Ab-binding GFPs as a powerful tool for detection of antinuclear antibodies (ANAs), was evaluated. In this study, the chimeric GFPZ, GFPZZ, H_6GFPZ and H_6GFPZZ, were individually applied in FANA instead of FITC-conjugated anti-human antibodies. Results indicated that all chimeric Ab-binding GFPs could be used as efficiently as the FITC-conjugate to detect antinuclear antibodies (Figure 6). However, a divergence

in fluorescence intensity could be observed among different kinds of chimeric GFPs. The proteins with double repeats of Fc-binding motif (GFPZZ and H_6GFPZZ) demonstrated obviously more intense and clearer signals than those harboring a single Z domain. More importantly, it should be noted that the chimeric Ab-binding GFPs provided a stronger fluorescence intensity than the FITC-conjugated antibodies. A negative serum of ANAs was applied as a control experiment.

In conclusion, our findings strongly support the high potential of using such chimeric Ab-binding GFPs as versatile reagents for immunological diagnosis. Moreover, the following listed notions supportively make our chimeric GFPs become more attractive and ideal for clinical applications: i) the fluorescent signal is relatively stable and resistant to photobleaching as compared with the FITC-conjugation system, ii) no special chemical or substrate is required for the reaction, iii) the sensitivity is sufficient to analyze a low level of antibody, iv) the fluorescence intensity and the IgG-binding capability are extremely stable for long periods of storage, at least 2 yrs at -20C, or even exposure to repetitive freezing and thawing and v) the process is simple and can further be developed as a rapid assay in the future.

Acknowledgements: Y.S. is a postgraduate student supported by the collaborative Ph.D. fellowship from the Royal Thai Government under the supervision of V.P. This project was partially supported by a governmental grant under Mahidol University (annual 2546-2550 B.E.).

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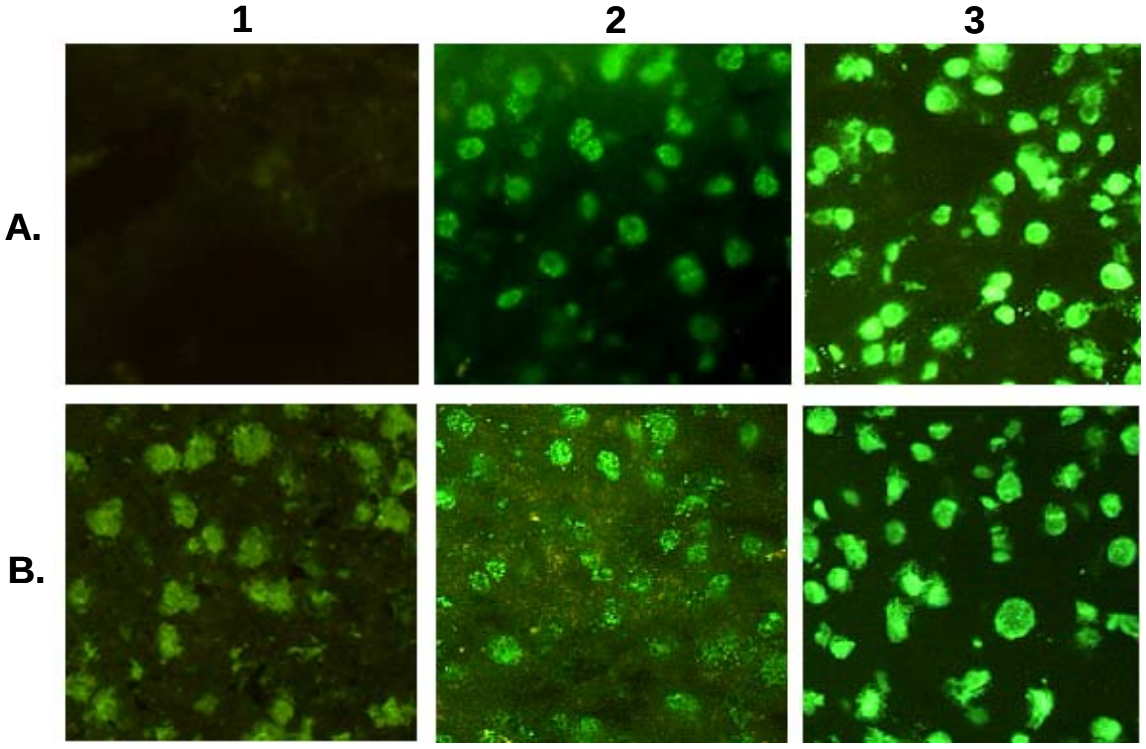

Figure 6: Microscopic images showing result of anti-nuclear antibody test by IFA. Negative serum (1A) was used, GFPZ (2A), GFPZZ (3A), FITC-labelled anti-human antibody (1B), H_6GFPZ (2B) and H_6GFPZZ (3B) were used as detection probes.

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