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Rapamycin-induced oligomer formation system of FRB–FKBP fusion proteins

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Most proteins form larger protein complexes and perform multiple functions in the cell. Thus, artificial regulation of protein complex formation controls the cellular functions that involve protein complexes. Although several artificial dimerization systems have already been used for numerous applications in biomedical research, cellular protein complexes form not only simple dimers but also larger oligomers. In this study, we showed that fusion proteins comprising the induced heterodimer formation proteins FRB and FKBP formed various oligomers upon addition of rapamycin. By adjusting the configuration of fusion proteins, we succeeded in generating an inducible tetramer formation system. Proteins of interest also formed tetramers by fusing to the inducible tetramer formation system, which exhibits its utility in a broad range of biological applications.

[**Key words:** FRB; FKBP; Rapamycin; Oligomer; Chemically-induced oligomerization; Protein engineering]

Many proteins do not perform their function alone in the monomeric state, but work as oligomers by forming larger protein complexes with other proteins (1,2). If we can artificially control the association and dissociation of protein complexes, the cellular functions involving protein complexes can be regulated. To date, several regulation methods for protein dimer formation using external stimuli have been developed (3–5). These dimerization methods are used as research tools to investigate the roles of protein–protein interaction and to manipulate various cellular functions.

One of the most useful dimerization systems is the FRB–FKBP–rapamycin heterodimer formation system (3,6). Rapamycin, an antifungal antibiotic macrolide, simultaneously binds to the 12-kDa FK506 binding protein (FKBP) and the FKBP–rapamycin binding (FRB) domain of the mammalian target of rapamycin (mTOR) and mediates their tight heterodimer formation ($K_d \approx 2.5$ nM) (Fig. 1A) (7,8). By the conditional dimerization of proteins of interest fused to FKBP or FRB on the addition of rapamycin, various cellular functions, such as gene expression and protein translocation, were controlled artificially (9–12). Recently, more convenient light-induced dimerization systems have been reported by several groups and made great contributions to emerging fields such as optogenetics (4,5,13).

In contrast to the induced dimerization systems, cellular protein complexes form not only simple dimers but also larger oligomers. In fact, the average oligomeric state of cellular proteins is tetrameric (1). Although oligomeric proteins and their oligomerization contribute to important cellular functions, their detailed functional mechanisms remain largely enigmatic. In addition, abnormal protein assembly causes many diseases (14,15). Thus, the construction of an artificial control system for oligomer formation is expected to attract great interest for basic biological research and clinical applications. Although a couple of oligomer formation systems have been developed, their biomedical applications have been very limited due to their incompatibility in cellular use. (3,16,17).

In this study, we hypothesized that a fusion protein containing FRB and FKBP domains works as an oligomeric building block and forms oligomer by interacting among the fused building blocks on addition of rapamycin (Fig. 1B). Using various biophysical techniques, we found that addition of rapamycin induced oligomerization of fusion proteins consisting of FRB and FKBP and that oligomers with biologically relevant size could be generated by adjusting the configuration of the fusion proteins. Our induced oligomerization system would be a unique and useful tool for regulating many cellular functions involving oligomeric proteins.

MATERIALS AND METHODS

Proteins design Proteins were derived from several different domains: FK506 binding protein (FKBP) (18), FKBP–rapamycin binding (FRB) domain in FRAP (residues 2021–2113) (8), 27th Ig (I27) domain of the giant muscle protein titin (19), and aminopeptidase N (pepN) (20). FRB and FKBP were fused in six different combinations in different orders, with or without two linkers (Fig. 1C). FRB and FKBP domains were connected to each other in frame, either directly or through a six-residue linker (Gly-Gly-Leu-Glu-Gly-Gly) or I27 domains. Fusion proteins consisted of an N-terminal Strep tag followed by a factor Xa cleavage site. This was followed by FRB–FKBP fusion proteins or pepN-fused FR–FK proteins, followed by a C-terminal hexahistidine (His_6) tag. Their coding sequences were cloned into the plasmid pET3a (Novagen) for *Escherichia coli* expression and purification. All genes were constructed using standard molecular biology techniques and verified by DNA sequencing.

Protein expression and purification Fusion proteins coded in the plasmid pET3a were expressed from the T7 promoter in *E. coli* strain Rosetta2 (DE3) pLysS (Novagen). Transformed bacteria were grown at 37°C, and expression was induced

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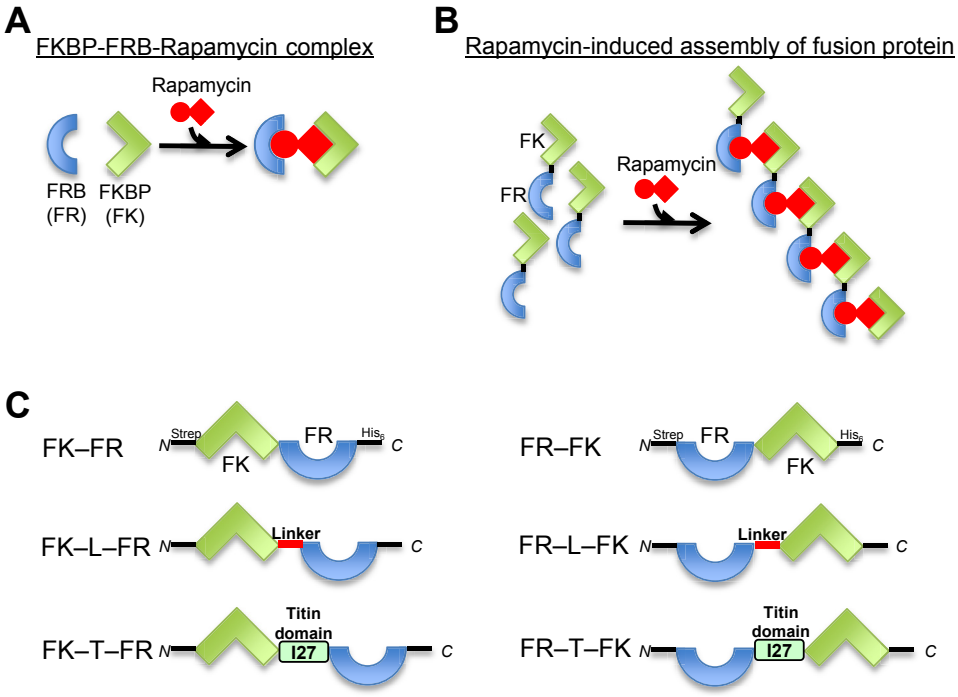

FIG. 1. Design of the oligomer-forming protein using an FRB–FKBP–rapamycin system (A) Schematic representation of induced heterodimer formation of FRB (FR) and FKBP (FK) by rapamycin. (B) Schematic design of an oligomer-forming fusion protein consisting of FR and FK. Binding of rapamycin induces polymerization of the fusion proteins. (C) Schematic representation of constructed fusion proteins with different orders of FR and FK, with or without six-residue amino acid linkers (L) or titin immunoglobulin I27 domain linkers (T).

with 0.2 mM isopropyl β-D-1-thiogalactopyranoside for 12–16 h at 22°C after the culture reached an optical density of 0.6 at 600 nm. Proteins were purified using a Proteus IMAC midi kit (AbD Serotec) equilibrated in PBS with 10 mM imidazole and 5 mM 2-mercaptoethanol. Proteins were eluted from the resin with PBS and 150 mM imidazole and 5 mM 2-mercaptoethanol. The eluted fractions were bound to a 1 mL Strep-Tactin gel (IBA Life Science) and proteins were eluted from the resin with 100 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 2.5 mM desthiobiotin. Finally, the eluted proteins were applied to a HiPrep 16/60 Sephacryl S-300 HR column equilibrated with PBS and were eluted with PBS. Fractions containing monomeric fusion proteins were analyzed by SDS-PAGE and stored at −80°C in 15% glycerol. Protein concentration was spectrophotometrically determined using an extinction coefficient calculated by ProtParam in ExPASy (http://www.expasy.org).

Size exclusion chromatography Oligomer formation was studied by gel filtration chromatography using a Superdex-200 10/300 column (GE Healthcare). Fusion proteins were incubated in the presence or absence of 200 μM rapamycin (LC laboratories) for 10 min in PBS supplemented with 5 mM 2-mercaptoethanol and 0.1% ethanol. Next, 100 μL of fusion protein filtered through a 0.22-μm pore-size filter was loaded onto a Superdex-200 column equilibrated with PBS, 5 mM 2-mercaptoethanol, and 0.1% ethanol with or without 5 μM rapamycin and eluted in the same buffer at 0.5 mL/min, and the relative peak sizes were quantitated by absorbance at 280 nm. For pepN-fused FR–FK, the buffer was supplemented with 0.05% Tween 20. To check the reversibility of tetramer formation of FR–FK, FR–FK tetramer was first collected from the gel filtration fractions in the presence of rapamycin and then incubated with 50 μM FK506 in PBS supplemented with 5 mM 2-mercaptoethanol and 5% ethanol. The FR–FK incubated with FK506 was loaded onto the gel filtration column equilibrated with PBS, 5 mM 2-mercaptoethanol, and 0.1% ethanol with 5 μM FK506 and eluted in the same buffer at 0.5 mL/min. The column was calibrated with the following proteins (Sigma–Aldrich): apoferritin (440 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa). Blue dextran (Sigma–Aldrich) was used to define the void volume of the column.

Static light scattering measurements Molecular weights were determined by static light scattering (SLS) using a Zetasizer Nano S ZEN1600 (Malvern Instruments) at 25°C. Fusion proteins were incubated in PBS with 5 mM β-mercaptoethanol, in the presence or absence of 200 μM rapamycin for 10 min and filtered through a 0.22-μm pore-size filter. Four to six different sample concentrations in the range 0.25–3.2 mg/mL were prepared and measured. Scattering intensities for SLS analysis were derived from the average count rate of the samples and were calibrated against toluene, using the Rayleigh ratio value of 1.35×10^{-5} cm^{-1}, as quoted by the manufacturer. A refractive index increment of 0.185 mL/g was assumed. Each sample was measured in a square glass cuvette. First, it was equilibrated for 2 min at 25°C and then the time scale of the scattered intensity was measured. Molecular weight was given by the Rayleigh equation:

$$\frac{KC}{R_\theta} = \frac{1}{M} + 2A_2C \quad (1)$$

K, optical constant; R_θ, Rayleigh ratio of the scattered to incident light intensity; M, weight-averaged molecular weight; A_2, second virial coefficient; C, is the sample concentration. A Debye plot, a linear fit of KC/R_θ against C, was generated from the data, with the intercept equal to the inverse molecular weight.

Dynamic light scattering measurements Sizes of fusion proteins were determined by dynamic light scattering (DLS) using a Zetasizer Nano S ZEN1600 (Malvern Instruments) at 25°C. The same conditions as for gel-filtration experiments were used (10 μM protein in PBS, 5 mM 2-mercaptoethanol, and 0.1% ethanol with various concentrations of rapamycin). Using an air-driven ultracentrifuge (Air-fuge, Beckman Coulter), each sample was ultracentrifuged to avoid high backscattering. Each sample was measured in single-use UV-transparent disposable cuvettes (Sarstedt). It was first equilibrated for 2 min at 25°C and the time scales of the scattered light intensity fluctuations were measured. Every sample was measured three times, each with 16–48 runs, and autocorrelation was analyzed with Zetasizer Software (Malvern Instruments). The apparent Z-average hydrodynamic diameter was obtained by cumulant analysis by fitting a single exponential to the correlation function.

Crosslinking Fifty microliters of sample measured in the DLS experiment was mixed with 2.5-μl, freshly prepared glutaraldehyde (10% stock solution). The proteins were crosslinked for 5 min at room temperature and the reaction was terminated by the addition of 10 μl Tris–HCl stock solution (1 M, pH 8.0). The crosslinked proteins were analyzed by 5–20% SDS-PAGE.

RESULTS

The design of an oligomer-forming protein by fusion of inducible dimer-forming proteins To construct artificial oligomerization system, we designed a series of fusion proteins that contain FRB (FR) and FKBP (FK), which form a heterodimer in the presence of rapamycin, as building blocks (Fig. 1C). In principle, structural configurations of the building block define the structure and polymerization degree of the produced oligomer (17,21). Considering this, the FR and FK domains were connected with different orders of FK and FR using three kinds of linkers; no