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Cholera toxin B subunit—Five-stranded α-helical coiled-coil fusion protein: "Five-to-five" molecular chimera displays robust physicochemical stability

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ABSTRACT

To create a physicochemically stable cholera toxin (CT) B subunit (CTB), it was fused to the five-stranded α-helical coiled-coil domain of cartilage oligomeric matrix protein (COMP). The chimeric fusion protein (CTB–COMP) was expressed in *Pichia pastoris*, predominantly as a pentamer, and retained its affinity for the monosialoganglioside GM1, a natural receptor of CT. The fusion protein displayed thermostability, tolerating the boiling temperature of water for 10 min, whereas unfused CTB readily dissociated to its monomers and lost its affinity for GM1. The fusion protein also displayed resistance to strong acid at pHs as low as 0.1, and to the protein denaturant sodium dodecyl sulfate at concentrations up to 10%. Intranasal administration of the fusion protein to mice induced anti-B subunit serum IgG, even after the protein was boiled, whereas unfused CTB showed no thermostable mucosal immunogenicity. This study demonstrates that CTB fused to a pentameric α-helical coiled coil has a novel physicochemical phenotype, which may provide important insight into the molecular design of enterotoxin-B-subunit-based vaccines and vaccine delivery molecules.

1. Introduction

Cholera toxin (CT) B subunit (CTB) is used as a component of killed oral cholera and enterotoxigenic *Escherichia coli* vaccines [1,2]. It has also been intensively investigated as a mucosal delivery molecule for chemically or genetically conjugated antigens to induce protective immunity against infections and autoimmune diseases [3–7]. Crystallographic studies and mutational analyses of CTB have identified the amino acid residues involved in the B–B intersubunit interactions, which are mediated by numerous hydrogen bonds and ionic and hydrophobic interactions [8,9]. The pentameric structure and monosialoganglioside GM1 (GM1) affinity of CTB are essential for its mucosal immunogenicity and its mucosal antigen delivery function. Any physicochemical stress that disrupts its pentameric configuration reduces its receptor affinity and concomitantly abrogates its unique biological functions [7,10–13].

Molecular perturbation agents, including high temperatures, extreme pHs, and protein denaturants, exemplify such physicochemical stresses. However, vaccine antigens or delivery molecules are expected to have physicochemically stable phenotypes, not only because such phenotypes confer molecular stability during their manufacture and storage [14,15], but also because they allow their efficient delivery as intact molecules to sites of immune response, such as draining lymph nodes.

We recently created a mutant CTB pentamer with a robustly thermostable phenotype by introducing *de novo* intersubunit disulfide bonds between adjacent B subunits [16]. With site-directed mutagenesis, we replaced Thr1 and Thr92 with cysteine residues. Thr1 and Thr92 are spatially located in close proximity, as evident in the X-ray crystal structure of the CTB pentamer [8], so we inferred that the substituted residues would form disulfide bonds. The restriction of molecular movement by five intersubunit disulfide crosslinks imposed strong physical constraints, preventing the pentamer from dissociating to monomers, even under harsh physicochemical conditions. In this study, we used a different approach, placing a 'molecular bundle' at the C-terminal end of the B subunit, predicting that it would strongly tie the constituent subunits together, preventing their escape from the pentameric unit. We also envisaged that a certain level of molecular freedom might

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be required, so we introduced an intervening 'molecular leash' between the two protein moieties.

Cartilage oligomeric matrix protein (COMP), a thrombospondin-like pentameric glycoprotein found in cartilages and tendons [17–19], contains an N-terminal multimerization domain [17,20] which is a five-stranded α-helical coiled-coil motif spanning amino acid residues 20–83 [20–22]. Coiled coils share a common heptated repeat $(a–g)_n$, in which the amino acids at positions a and d are usually hydrophobic residues, and the other positions are often occupied by hydrophilic residues [23]. The side chains of amino acids a and d are generally involved in hydrophobic interactions between the helices, and are the main stabilizing forces in coiled coils [23]. The coiled coil of COMP, when expressed alone, displays robust physicochemical stability [20–22,24].

In this study, we exploited the physicochemical stability of the COMP coiled coil and its pentameric configuration, and used it as a molecular bundle to confer a physicochemically stable molecular phenotype on the enterotoxin B subunit, which is otherwise heat labile. We designed the chimeric CTB–COMP fusion protein based on our inference that the pentamer would gain physicochemical stability if its C-terminal ends were tightly bundled by a molecule such as a coiled coil with the same valence as that of CTB. We analyzed the physicochemical stability and heat-resistant mucosal immunogenicity of the chimeric fusion protein.

2. Materials and methods

2.1. Molecular design of the chimeric CTB–COMP fusion protein

The full-length CTB gene (GenBank: U25679) was PCR amplified from plasmid pB [5] with primers: sense, 5′-GCGCCAATTG-GCCACCATGATTAAATTAAAATTTGGTGTT-3′ and antisense: 5′-GGCAATTGTTAATGATGGTGATGGTGATGGAATTCATGGTGATGGT-GATGATGTCCAGGTCCTGGACCATTTGCCATACTAATTGCGG-3′, containing the *Mun*I and *Mun*I/*Eco*RI recognition sequences, respectively (underlined). The amplified fragment was digested with *Mun*I and inserted at the *Eco*RI site of plasmid pPIC3.5K (Life Technologies, Carlsbad, CA, USA) to generate the plasmid pB-spacer encoding the fusion protein CTB-$(GP)_2GH_6$–(*Eco*RI)–H_6 (first subcloning in Fig. 1), in which a new *Eco*RI site became available for further subcloning.

The nucleotide sequence for $(G_4S)_3$–$COMP_{Gly26–Gly80}$ was then PCR amplified from a plasmid containing the synthetic $COMP_{Gly26–Gly80}$ sequence [25] with primers: sense, 5′-GCGCAATTGGGCGGTGGCGGTAGCGGCGGT-3′ and antisense, 5′-GCGGAATTCGCCCGGGGTACGGGCCGGCTGC-3′, containing *Mun*I and *Eco*RI restriction enzyme recognition sequences, respectively (underlined). The amplified fragment was digested with *Mun*I and *Eco*RI, and inserted into the unique *Eco*RI site (second subcloning in Fig. 1) in the pB-spacer plasmid. Transformation of *Pichia pastoris* strain GS115, protein expression, and the affinity purification of CTB–COMP were as previously described [7]. CTB [7] and the COMP coiled-coil domain [25] were also expressed and purified as described previously.

2.2. Physicochemical stresses imposed on chimeric CTB–COMP fusion protein

To analyze the physicochemical stability of the CTB–COMP fusion protein, it was exposed to high temperature, a broad range of pHs, and sodium dodecyl sulfate (SDS) solutions. For the heat treatment, unfused CTB [7] or CTB–COMP in phosphate-buffered saline (PBS, pH 7) was incubated at 100 °C in a heating block for 0–30 min. For the acid and alkaline treatments, the proteins were incubated in solutions of pH 0.1–14 at 37 °C for 1 h. The solutions were neutralized before the products were analyzed. For treatment with a denaturant, the proteins were incubated in PBS containing 0–10% SDS at 37 °C for 1 h. The proteins were also analyzed with size-exclusion chromatography after the various treatments. Briefly, the proteins heat treated at 100 °C for 10 min, exposed to pH 0.1, or treated with 10% SDS were analyzed on a HiLoad 16/60 Superdex 200 pg column (0.8 ml/min flow rate; GE Healthcare, Little Chalfont, UK). Furthermore, the proteins were analyzed with GM1–enzyme-linked immunosorbent assay (ELISA) after the various treatments as described previously [5].

2.3. Intranasal immunization of mice

Seven-week-old female BALB/c mice (Japan SLC, Shizuoka, Japan) were administered 30 μg of CTB [7] or the CTB–COMP fusion protein that contained 30 μg of the CTB moiety *via* the intranasal (i.n.) route after the protein had been boiled for 0 or 10 min. Four mice per group were immunized three times, in weeks 0, 2, and 4. Immune sera were collected in week 6, and antigen-specific serum antibodies were analyzed as described previously [25]. The animal experiments were approved by the University of the Ryukyus Institutional Animal Care and Use Committee, and were conducted according to the Institutional Ethical Guidelines for Animal Experiments.

2.4. CT–GM1 binding inhibition assay by mouse antisera

To determine whether antisera induced by CTB or CTB–COMP fusion protein with or without heat treatment inhibit CT from binding its receptor GM1, 100 μl of the serially diluted antisera were incubated with 100 μl of CT (0.4 μg/ml in PBS, List Biological Laboratories, Inc.; Campbell, CA, USA) at 37 °C for 1 h. After the incubation, 50 μl of the CT/antisera mixtures were applied to microtiter plate wells (10 ng CT/well) (Sumilon; Sumitomo Bakelite Co., Ltd., Tokyo, Japan) precoated with GM1 (4 mg/ml). Then,

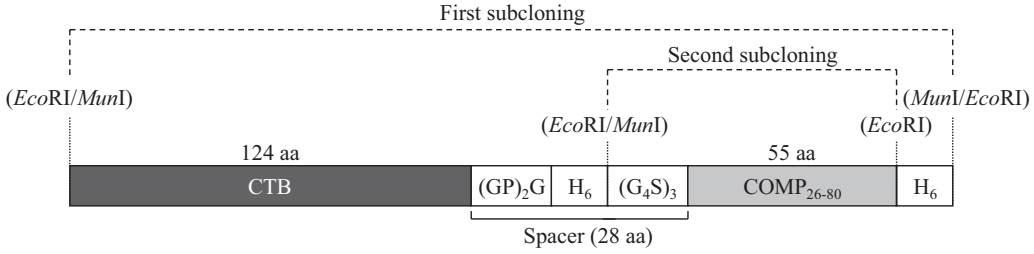

Fig. 1. Schematic representation of the chimeric CTB–COMP fusion protein engineered by genetically linking the cholera toxin B subunit (CTB) to the coiled-coil domain of cartilage oligomeric matrix protein (COMP) *via* a spacer. The fusion protein contains: (1) full-length CTB (124 amino acids; classical biotype 569B; NCBI GenBank: U25679); (2) a *de novo*-designed spacer containing an internal hexahistidine tag (28 amino acids; GPGPGHHHHHHELGGGSGGGGSGGGGS); (3) $COMP_{26–80}$ (55-amino acids coiled-coil domain of COMP: GGDLAPQMLRELQETNAALQDVRELLRQQVKEITFLKNTVMECDACGMQPARTPG [20]); and (4) a C-terminal hexahistidine tag. The entire fusion gene was inserted into the *Eco*RI site of pPIC3.5K (a *Pichia pastoris* expression vector).