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Recombinant expression of TLR5 proteins by ligand supplementation and a leucine-rich repeat hybrid technique

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ABSTRACT

Vertebrate TLR5 directly binds bacterial flagellin proteins and activates innate immune responses against pathogenic flagellated bacteria. Structural and biochemical studies on the TLR5/flagellin interaction have been challenging due to the technical difficulty in obtaining active recombinant proteins of TLR5 ectodomain (TLR5-ECD). We recently succeeded in production of the N-terminal leucine rich repeats (LRRs) of *Danio rerio* (*dr*) TLR5-ECD in a hybrid with another LRR protein, hagfish variable lymphocyte receptor (VLR), and determined the crystal structure of its complex with flagellin D1–D2–D3 domains. Although the structure provides valuable information about the interaction, it remains to be revealed how the C-terminal region of TLR5-ECD contributes to the interaction. Here, we present two methods to obtain recombinant TLR5 proteins that contain the C-terminal region in a baculovirus expression system. First, production of biologically active full-length *dr*TLR5-ECD was substantially enhanced by supplementation of expression culture with purified flagellin proteins. Second, we designed TLR5-VLR hybrids using an LRR hybrid technology by single and double LRR fusions and were able to express diverse regions of *dr*TLR5-ECD, allowing us to detect a previously unidentified TLR5/flagellin interaction. The *dr*TLR5-VLR hybrid technique was also successfully applied to human TLR5-ECD whose expression has been highly problematic. These alternative TLR5 expression strategies provide an opportunity to obtain a complete view of the TLR5/flagellin interaction and can be applied to other LRR proteins.

1. Introduction

Toll-like receptors (TLRs) play a key role in the first line of defense against pathogens by recognizing common molecular structures of pathogenic microbes and subsequently activating innate immune responses [1]. Ten TLRs (TLR1–TLR10) have been identified in humans and are classified into 5 subgroups (TLR1/2/6/10, TLR3, TLR4, TLR5, and TLR7/8/9) depending on the nature of their ligands [2].

TLR5 is found in vertebrates from fish to mammals and directly binds a bacterial protein, flagellin, which polymerizes into a flagellar filament in a spiral manner [3,4]. Flagellin-induced TLR5 signaling activates NF-κB, a proinflammatory transcription factor, and promotes production of inflammatory cytokines for the activation of mucosal immunity against pathogenic flagellated bacteria in epithelial cells of the intestine and lung. TLR5 also exhibits anti-apoptotic radioprotective activity and protects the hematopoietic system and gastrointestinal tissues from radiation damages [5]. Thus, biochemical and structural studies on the TLR5/flagellin interaction is critical for the development and improvement of vaccines and vaccine adjuvants as well as therapeutics that would alleviate side effects of anticancer radiotherapy [5,6].

TLR5 is a type I receptor that consists of three domains including an N-terminal flagellin-binding ectodomain (ECD), a single-pass transmembrane domain, and an intracellular signaling domain. Biochemical and structural studies on the TLR5-ECD/flagellin interactions have been limited due to technical difficulty in obtaining TLR5-ECD proteins by conventional protein expression approaches. To overcome the expression challenges, we screened expression of diverse TLR5-ECD orthologs and found that only zebrafish (*Danio rerio*) TLR5-ECD (*dr*TLR5-ECD) proteins were produced in sufficient quantities in a baculovirus expression system [4]. However, the *dr*TLR5-ECD aggregated as a nonfunctional protein. To improve the TLR5-ECD protein behavior in solution, we utilized a leucine-rich repeat (LRR) hybrid technique that fuses two

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different LRR proteins, the TLR5 protein of interest with LRR repeats from a variable lymphocyte receptor (VLR), to produce TLR5-VLR hybrids [7–10]. As a result, several *dr*TLR5-VLR hybrid proteins were purified as functionally active monomers in substantially improved yields and used for structure determination of a complex between the N-terminal 14 LRR modules of *dr*TLR5 and the D1–D2–D3 domains of *Salmonella* flagellin, FliC [4]. The primary binding interaction is mainly mediated by a protruding loop of TLR5 LRR9 and the FliC D1 domain, contributing to formation of a high-affinity 1:1 TLR5/FliC heterodimer. Two copies of the heterodimers further assemble into a 2:2 complex using TLR5 LRR12–LRR13.

The structure provided important information about the TLR5/flagellin interaction at atomic resolution. Nevertheless, the complex lacks the C-terminal region of *dr*TLR5-ECD and the FliC D0 domain. Thus, it still remains to be revealed how the C-terminal region of TLR5-ECD contributes to TLR5/flagellin binding and TLR5 signaling. To provide a complete view on the interaction, we further screened and improved expression of *dr*TLR5-ECD and its fragments by ligand supplementation and an innovative protein engineering technique. First, we produced functional *dr*TLR5-ECD proteins in sufficient quantity for structural studies by adding exogenous purified FliC proteins into the *dr*TLR5-ECD expression culture. Second, we fused *dr*TLR5-ECD and hagfish VLR by single or double fusions and were able to express the C-terminal region of *dr*TLR5-ECD. Analogously, the *dr*TLR5-VLR hybridization strategy was successfully applied to expression and purification of human (*Homo sapiens*) TLR5-ECD (*hs*TLR5-ECD) fragments. These versatile approaches to recombinant TLR5 protein expression have already contributed to understanding of the structural and molecular mechanism for the TLR5/flagellin interaction and should enable advances in protein preparation of other challenging LRR receptors for biochemical or biophysical studies.

2. Materials and methods

2.1. Expression and purification of FliC and CBLB502

FliC (residues 1–504) of *Salmonella enterica* subspecies *enterica* serovar Dublin and its variant CBLB502 (FliC residues 1–175, a 16-residue linker, and FliC residues 401–504) were expressed and purified as previously described [4,5]. FliC and CBLB502 were produced in an *Escherichia coli* expression system using a modified expression vector, pET49b, which contains an N-terminal His_6 tag and an enterokinase or thrombin cleavage site. FliC proteins were obtained from soluble fractions of cell lysates, whereas CBLB502 was refolded from inclusion bodies with 2 M urea. FliC and CBLB502 proteins were purified by Ni-NTA and size-exclusion chromatography.

2.2. Construction of TLR5-ECD and TLR5-VLR hybrid expression vectors

TLR5-ECD and TLR5-VLR hybrids were expressed in a baculovirus expression system. A baculovirus transfer vector, pAcGP67A, that allows recombinant proteins to be extracellularly expressed under the control of the GP67 signal peptide, was modified to contain the thrombin cleavage site, Strep-Tactin II tag, and His_6 tag at the C-terminus and was used throughout [11]. To construct full-length TLR5-ECD expression vector, DNAs that encode *dr*TLR5b residues 22–652 [UniProt ID F8W3J5] or *hs*TLR5 residues 21–639 (UniProt ID Q32MI2) (Fig. S1) were amplified by PCR and ligated into the modified pAcGP67A vector between the N-terminal GP67 signal peptide and the C-terminal thrombin cleavage site.

To generate TLR5-VLR hybrid protein expression constructs, DNAs that encode the TLR5-VLR fusion proteins were inserted into the modified pAcGP67A vector. We prepared numerous inserts that can be grouped into three different types including an N-terminal single fusion, a C-terminal single fusion, and a double fusion at both N- and C-terminal regions (Fig. 1). The first type of insert is a single-fusion hybrid ^{T}N-x^{V} that contains the N-terminal fragment of TLR5-ECD (the N-terminal residue 22 to LRR module number x) and the C-terminal region of hagfish VLR B.61 (LRR4 to the residue 200). The C-terminal region of VLR was first amplified by PCR and the N-terminal part of TLR5 was then extended in the second PCR using the first PCR products as a reverse primer. The second type of insert is the C-terminal single fusion product ^{VT}x-C between the N-terminal region of VLR (the N-terminal residue 22 to LRR2) and the C-terminal fragment of TLR5-ECD (LRR module number x to the C-terminus). The inserts were created by two consecutive PCRs as described above for ^{T}N-x^{V}. The third type of TLR5-VLR hybrids is a double fusion product ^{VT}a-b^{V} where the central LRR fragments of TLR5-ECD from LRR 'a' to LRR 'b' is flanked by the N-terminal (the N-terminus to LRR2) and C-terminal (LRR4 to residue 200) regions of VLR. ^{VT}a-b^{V} inserts were also made by two consecutive PCRs but with DNA templates of ^{T}N-x^{V} or ^{VT}x-C for the second PCR.

2.3. Expression and purification of TLR5 proteins

TLR5-ECD and TLR5-VLR hybrids were expressed in a baculovirus expression system and purified by consecutive chromatography using Ni-NTA, Strep-Tactin II, and size-exclusion chromatography columns as previously described [4]. In some TLR5 constructs including TLR5-ECD and its VLR hybrid ^{VT}1-C, purified FliC proteins were added into expression culture at ~24 h postinfection at which proteins begin to be synthesized under a polyhedrin promoter of pAcGP67 expression vector and TLR5/FliC complexes were then purified in the same manner as TLR5-VLR hybrids.

2.4. Native PAGE

Binding of TLR5-VLR hybrids to FliC or CBLB502 was analyzed by native PAGE [11,12]. TLR-VLR hybrids, FliC (or CBLB502), and their complexes were loaded on 4–20% polyacrylamide gels and native PAGE was carried out without SDS in Tris–glycine buffer at pH 8.8 for 2–3 h at 100 V.

3. Results

3.1. Improvement in production of active drTLR5-ECD proteins by FliC supplementation

The major roadblock in *dr*TLR5-ECD expression is that a majority of the recombinant *dr*TLR5-ECD proteins are not properly folded, thereby forming aggregates by themselves or nonspecifically with cellular proteins resulting in non-functional proteins during expression even in a baculovirus expression system that uses eukaryotic insect cells. Only one third of the expressed proteins formed biologically active monomers by size-exclusion chromatography, even although the proteins had been previously purified by Ni-NTA and Strep-Tactin affinity chromatography (Fig. 2A). One litre expression culture provided a small quantity of purified *dr*TLR5-ECD proteins (<<50 μg from 1 L expression culture) that was not sufficient for structural studies in stark contrasts to a TLR5-VLR hybrid, ^{T}N-14^{V}, that was purified mainly as monomers (Fig. 2B) with a purification yield of ~1.4 mg per 1 L culture (Table S1).

Given that *dr*TLR5-ECD was expressible, we searched for a method that would improve TLR5-ECD protein stability to enhance the final yield of active *dr*TLR5-ECD proteins. For this purpose, we supplemented expression culture with purified FliC proteins at

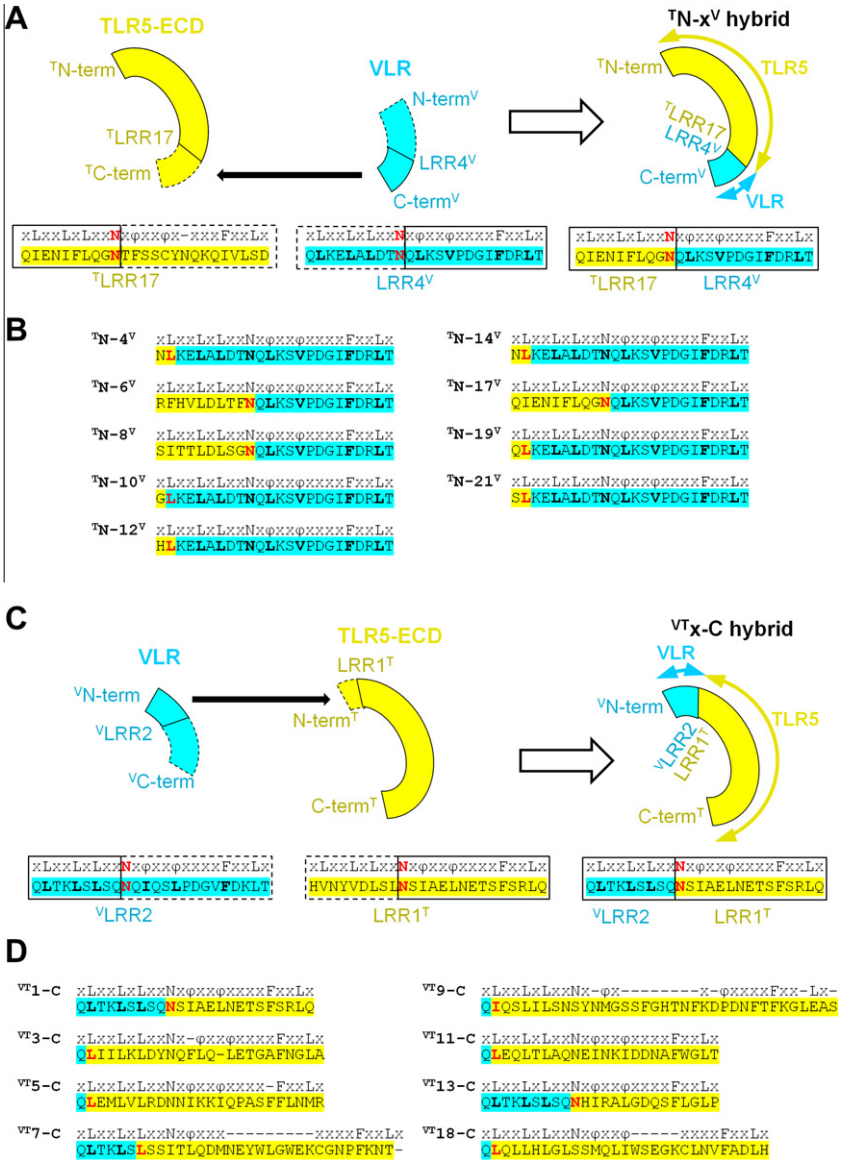

Fig. 1. ^{T}N-x^{V} (A, B) and ^{VT}x-C (C, D) hybrid designs that allow linkage of LRR fragments of TLR5 and VLR. (A) Schematic representation of ^{T}N-x^{V} hybrids. In ^{T}N-17^{V} as an example of ^{T}N-x^{V} hybrids, the TLR5 N-terminal region from the N-terminus to LRR17 (yellow in continuous lines) was fused with the VLR C-terminal region from LRR4 to the C-terminus (cyan in continuous lines) at the 10th consensus residue (asparagine in red). (B) Amino acid sequences of ^{T}N-x^{V} hybrids at a fusing LRR module. TLR5 and VLR sequences are highlighted in yellow and cyan, respectively. A consensus residue where fusion occurs is shown in red. (C) A schematic representation of ^{VT}x-C hybrids. In ^{VT}1-C as an example of ^{VT}x-C hybrids, the TLR5 C-terminal region from the LRR1 to the C-terminus (yellow in continuous lines) was fused with the VLR N-terminal region from the N-terminus to LRR2 (cyan in continuous lines) at the 10th consensus residue (asparagine in red). (D) Amino acid sequences of ^{VT}x-C hybrids in the fusing LRR module.

~24 h postinfection. As a result, a peak that corresponds to *dr*TLR5-ECD complexed with FliC became notably higher and larger in a FliC dose-dependent manner (Fig. 3). We obtained ~0.4 mg of *dr*TLR5-ECD/FliC complex after three consecutive purification steps (Ni-NTA, Strep-Tactin, and size-exclusion chromatography) from 1 L culture when 0.6 mg of FliC was added as a supplement. We conclude that the purification yield increased by at least four-fold by FliC supplementation. This substantial improvement in production of active *ds*TLR5-ECD proteins by FliC supplementation during culture enabled us to perform X-ray crystallographic studies on the TLR5/FliC complex, which required a large quantity of protein.

3.2. Expression of the drTLR5-ECD N-terminal fragments by a single LRR fusion

Both *dr*TLR5-ECD and VLR adopt a typical LRR domain structure consisting of 22 and 5 LRR modules, respectively, and are protected by N- and C-terminal capping motifs (LRRNT and LRRCT, respectively). The LRRNT and LRRCT motifs are indispensible for stability of the LRR domains and are one of determining factors for levels of protein expression. We hypothesized that the capping motifs of TLR5 and their connecting regions are responsible for aggregation and low protein yields. To improve solubility and yields of *dr*TLR5-ECD, we designed and screened *dr*TLR5-VLR hybrids, in which LRRNT or LRRCT of TLR5 was swapped with that of a high expresser LRR protein, VLR, using one of the 2nd (leucine), 7th (leucine), and 10th (asparagine) residues of the LRR consensus sequence ($xL_2xxL_5xL_7xxN_{10}$) as a fusion point (Fig. 1). Such technique has been already utilized in other TLRs, including TLR4 and TLR1/2/6 subfamilies, and enhanced protein expression levels and crystallizability for structural studies [7–9,13].

First, we designed nine ^{T}N-x^{V} hybrids (^{T}N-4^{V}, ^{T}N-6^{V}, ^{T}N-8^{V}, ^{T}N-10^{V}, ^{T}N-12^{V}, ^{T}N-14^{V}, ^{T}N-17^{V}, ^{T}N-19^{V}, and ^{T}N-21^{V}) that contain residues from the *dr*TLR5 N-terminal residue 22 through one of

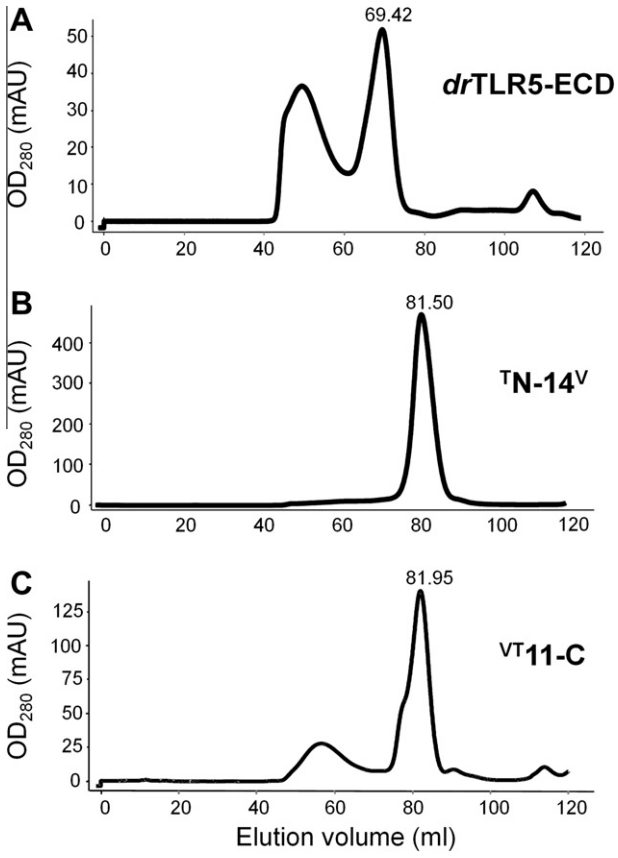

Fig. 2. Size-exclusion chromatography profiles of *dr*TLR5-ECD (A), ^{T}N-14^{V} (B), and ^{VT}11-C (C). Expressed proteins were first purified by Ni-NTA and Strep-Tactin affinity chromatography. The resulting proteins were digested by thrombin and injected into a Superdex 200 16/600 size-exclusion chromatography column.

the 2nd and 10th LRR consensus residues of LRR module 4–21 fused with VLR B.61 LRR4-LRRCT (Fig. 1A and B and Table S1) [14]. Six (^{T}N-4^{V}, ^{T}N-6^{V}, ^{T}N-10^{V}, ^{T}N-12^{V}, ^{T}N-14^{V}, and ^{T}N-17^{V}) of the nine tested ^{T}N-x^{V} LRR hybrids were successfully expressed in a baculovirus expression system. Except for ^{T}N-10^{V} that is very unstable with low purification yield (less than 0.1 mg from 1 L culture), ^{T}N-4^{V}, ^{T}N-6^{V}, ^{T}N-12^{V}, ^{T}N-14^{V}, and ^{T}N-17^{V} were stable in solution and were purified as monomers (Fig. 2B). ^{T}N-4^{V}, ^{T}N-6^{V}, and ^{T}N-14^{V} exhibited the highest expression levels with a purification yield of at least 1 mg from 1 L culture. ^{T}N-12^{V} and ^{T}N-16^{V} yielded 0.2–0.5 mg. The ^{T}N-x^{V} hybridization showed a high success rate of at least 60%.

We analyzed binding of purified ^{T}N-x^{V} hybrid proteins to FliC by native PAGE and size-exclusion chromatography (Table S1). Consistent with the critical role of a protruding loop of LRR9 in FliC binding by creating a binding groove [4], the ^{T}N-12^{V}, ^{T}N-14^{V}, and ^{T}N-17^{V} fusion proteins with the LRR9 loop formed a stable complex with CBLB502 that exhibited the same full TLR5-signaling activity as the full-length FliC (Fig. 4). In contrast, ^{T}N-4^{V} and ^{T}N-6^{V} that lack the LRR9 loop did not interact with CBLB502. ^{T}N-10^{V} did not bind FliC although ^{T}N-10^{V} contains the LRR9 loop, but this result is consistent with our prediction that the LRR9 loop in ^{T}N-10^{V} is not expected to adopt an optimal FliC-binding conformation for TLR5/FliC interaction due to significant steric clashes of LRR9 and its proximal region with VLR residues (Fig. S2).

3.3. Expression of the drTLR5-ECD C-terminal fragments by a single LRR fusion

To address the contribution of the TLR5 C-terminal region to flagellin binding, we designed LRR hybrid constructs that encode a

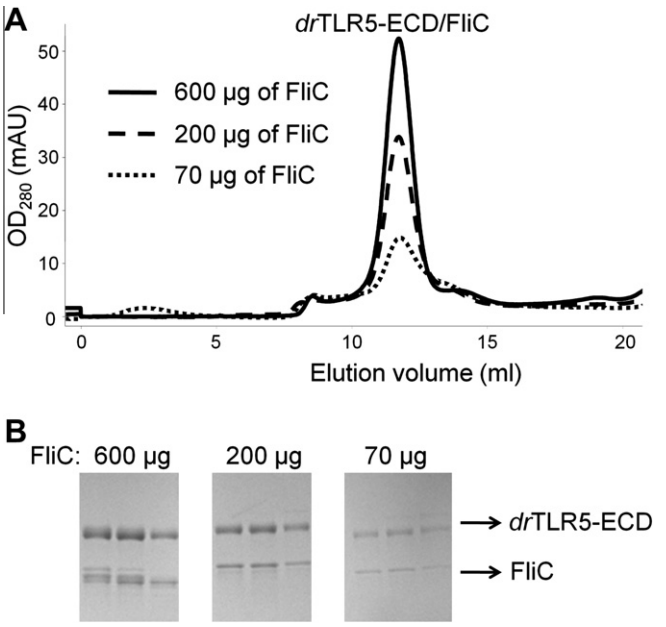

Fig. 3. Improved expression yield of functional *dr*TLR5-ECD proteins by FliC supplementation. Different amounts of purified FliC proteins (70 µg, 200 µg, or 600 µg) were added into 1 L of *dr*TLR5-ECD expression culture at ~24 h postinfection. At 48 h postinfection, culture medium that contains *dr*TLR5-ECD and FliC was harvested and subjected to purification by Ni-NTA affinity chromatography. The resulting material was analyzed using a Superdex 200 10/300 column to estimate the yield of *dr*TLR5-ECD/FliC complex (A). Peak fractions that correspond to the *dr*TLR5-ECD/FliC complex were analyzed by SDS–PAGE, indicating the presence of both *dr*TLR5-ECD and FliC in the peak (B). Peak height and width increased in a FliC dose-dependent manner, demonstrating that FliC supplementation is a crucial step to improve *dr*TLR5-ECD protein yield.

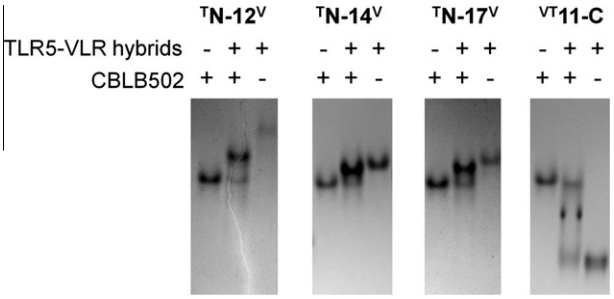

Fig. 4. Native PAGE analysis of interaction between *dr*TLR5-VLR hybrids and CBLB502. CBLB502 was added in molar excess compared to *dr*TLR5-VLR hybrids in each reaction. CBLB502 completely shifted ^{T}N-12^{V}, ^{T}N-14^{V}, and ^{T}N-17^{V} proteins by forming complexes. Noticeably, only a fraction of the ^{VT}11-C protein was complexed with CBLB502, suggesting a weak interaction.

fusion protein of the *dr*TLR5-ECD C-terminal region and the VLR N-terminal region, and tested their expression (Fig. 1C). We designed eight ^{VT}x-C hybrids (^{VT}1-C, ^{VT}3-C, ^{VT}5-C, ^{VT}7-C, ^{VT}9-C, ^{VT}11-C, ^{VT}13-C, and ^{VT}18-C) that extend either from the 2nd, 7th, or 10th LRR consensus residue in TLR5 LRR module number x through the C-terminal residue 652 of TLR5-ECD in fusion with VLR B.61 LRRNT-LRR2 (Fig. 1D and Table S1).

Only two ^{VT}x-C hybrids, ^{VT}1-C and ^{VT}11-C, were expressed, exhibiting a much lower success rate compared to ^{V}N-x^{T} hybridization. Although ^{VT}1-C was expressible, it formed aggregates as observed for *dr*TLR5-ECD. Similar to *dr*TLR5-ECD, aggregation formation was prevented by FliC supplementation during protein expression. The purification yield was enhanced to ~0.3 mg per 1 L culture in the presence of FliC. ^{VT}11-C was purified with a higher proportion of a monomeric form with much less aggregation

compared to *dr*TLR5-ECD (Fig. 2A and C). The final purification yield of ^{VT}11-C was ~0.6 mg from 1 L culture. Since ^{VT}11-C does not have the LRR9 loop, it did not form a tight complex with FliC as efficiently as other *dr*TLR5-VLR hybrids that contain the LRR9 loop, such as ^{T}N-12^{V}, ^{T}N-14^{V}, and ^{T}N-17^{V} (Fig. 4). Instead, only some of the ^{VT}11-C material formed complexes with CBLB502 even at molar excess of CBLB502, suggesting a weak interaction (Fig. 4). This previously unidentified TLR5/flagellin interaction is therefore localized on the C-terminal region of TLR5-ECD after LRR17, given that a TLR5-VLR hybrid, ^{VT}11-17^{V}, that contains TLR5 LRR11–LRR17 did not bind FliC (see below).

3.4. Expression of the central region of drTLR5-ECD by double hybridization with VLR

^{T}N-x^{V} and ^{VT}x-C hybridization screening provides valuable information about fusion points that are able to express stable monomeric TLR5 proteins. By combination of two sets of hybrids (the first set, ^{VT}1-C and ^{VT}11-C; the second set, N-12^{TV}, N-14^{TV}, and N-17^{TV}), we designed five double hybrids (^{VT}1-12^{V}, ^{VT}1-14^{V}, ^{VT}1-17^{V}, ^{VT}11-14^{V}, and ^{VT}11-17^{V}) that contain the central LRR region of TLR5-ECD flanked by N- and C-terminal regions of VLR (Table S1). For example, ^{VT}11-17^{V} contains TLR5 LRR11 through LRR17 for which the N- and C-terminal ends are fused with VLR LRRNT-LRR2 and LRR4-LRRCT, respectively. As expected, all the double hybrids were expressible with purification yields of 0.2–3.0 mg from 1 L culture. They exhibited expected FliC binding behavior depending on the presence of the LRR9 loop. ^{VT}1-12^{V}, ^{VT}1-14^{V}, and ^{VT}1-17^{V} formed complexes with FliC, whereas ^{VT}11-14^{V} and ^{VT}11-17^{V} did not.

3.5. Application of the drTLR5-VLR hybrid technique to hsTLR5

We also applied ligand supplementation and the LRR hybrid technique to *hs*TLR5-ECD to improve protein expression and purification. It is well known that *hs*TLR5-ECD is not expressible in a functional form even in a mammalian expression system. FliC supplementation did not significantly enhance *hs*TLR5-ECD expression, whereas VLR fusion allowed *hs*TLR5-ECD fragments to be expressed (Table S2). Nine *hs*TLR5-VLR hybrids (^{T}N-4^{V}, ^{T}N-6^{V}, ^{T}N-12^{V}, ^{T}N-14^{V}, ^{T}N-17^{V}, ^{VT}1-C, ^{VT}11-C, ^{VT}11-14^{V}, ^{VT}11-17^{V}) were generated using identical designs that provided high expression levels of *dr*TLR5, and their expression levels were analyzed (Table S2). ^{T}N-4^{V}, ^{T}N-6^{V}, and ^{VT}11-14^{V} hybrids that contain short fragments of human TLR5-ECD were expressed at high levels and purified as monomers with purification yields of ~0.3–0.8 mg from 1 L culture.

4. Discussion

Here, we present the use of alternative, powerful expression techniques to overcome the technical difficulty in production of functionally active recombinant proteins of an LRR innate immunity receptor. Since TLR5 was first identified as an innate immune receptor for bacterial flagellin in 2001 [3], biophysical characterizations, including structural studies, of TLR5 itself and its interaction with flagellin have been challenging due to the limited quantity of active TLR5-ECD proteins that could be obtained by conventional expression methods. Instead, mutational studies combined with cell-based singling assays were used to identify TLR5 residues that may be involved in flagellin interaction [15]. However, such results must be carefully interpreted since any decrease or a lack of cellular activity in mutants could not only be interpreted as disruption of TLR5/flagellin interaction but also as a result of mutation-induced protein misfolding [4]. Thus, it is essential that such mutation results should be confirmed by direct interaction studies that employ purified functional proteins, emphasizing the importance of studies to improve particularly challenging protein expression.

It has been reported that the yield of biologically relevant forms can be enhanced by external supplementation of culture medium with a small chemical ligand or by co-expression with protein ligands mainly in the *E. coli* expression system [16,17]. Binding of a ligand to the active site of an enzyme or a receptor results, in most cases, in stabilization of unstable region in a protein and improves the yield of active proteins. Consistent with that notion, we obtained active TLR5-ECD proteins in large quantity by supplementation of culture with a protein ligand, flagellin, in a baculovirus expression system. Given that flagellin buries extensive TLR5 surfaces (at least 1200 Å^2) upon complex formation, flagellin is likely to mask and stabilize some flexible regions, i.e. the protruding loops of LRR7 or LRR9, of *dr*TLR5 [4], providing a potential explanation for how flagellin supplementation improves the yield of biologically active *dr*TLR5-ECD proteins.

LRR domains are formed by successive assembly of multiple LRR modules and provide a scaffold that exerts versatile functions by modulating its surface residues. Each LRR is a structure motif that stacks consecutively with other LRRs into a solenoid-like shape with parallel β-strands on the concave side and an array of various secondary structures on the convex side. This LRR structure is stabilized at the N- and C-terminal ends by LRRNT and LRRCT capping motifs, respectively, that mask the hydrophobic ends of the solenoid. Protein engineering of LRR domains was first introduced in ribonuclease inhibitor [18]. The number of the central LRR modules was manipulated between the N-terminal and the C-terminal capping motifs. Later, more extensive LRR engineering was tested in TLR4 and TLR1/2/6 using LRR hybridization between two different LRR proteins to fuse an unstable LRR protein of interest and a more stable LRR protein, hoping to improve the stability and yield of the protein of interest [7–9]. By a single fusion that replaces either the N-terminal or C-terminal region of TLR-ECD with the corresponding region of a VLR, TLR-VLR hybrids exhibited substantial improvement in protein expression and crystallizability without adversely affecting the intrinsic biological activity of the TLR. In our study, we extended this methodology to a double fusion by inserting TLR5 LRR modules between the N-terminal and the C-terminal regions of a VLR. The double fusion hybrids were expressible if each of their two fusions yielded high expression levels. Using this double fusion, we were able to expand the crystallization search space and crystallize ^{VT}11-17^{V}.

We further extended the LRR hybrid technique to *hs*TLR5-ECD. *hs*TLR5-ECD is extremely difficult to express in an active form even in a mammalian expression system. Because *dr*TLR5 and *hs*TLR5 contain homologous sequences (but with relatively low sequence identity, 37%), we tested expression of *hs*TLR5-VLR hybrids using an identical fusion strategy as with *dr*TLR5-ECD. We were able to express and purify fusion proteins in around a third of designed *hs*TLR5-VLR hybrid constructs. *hs*TLR5 LRRNT-LRR6 or LRR11–LRR14 regions were expressible using ^{T}N-4^{V}, ^{T}N-6^{V}, and ^{VT}11-14^{V} *hs*TLR5-VLR hybrids, suggesting that other regions, such as LRR6–LRR11 and LRR14-LRRCT, are the main cause of poor expression of *hs*TLR5-ECD.

The improvement of TLR5 expression levels and purification yields by LRR hybridization with a VLR has been essential for structural and binding studies. TLR5-VLR hybrids provide an important tool to biochemically and biophysically study the TLR5/flagellin interaction. In particular, the LRR hybrid technique was critical for determination of a crystal structure of the ^{T}N-14^{V}/flagellin complex [4]. Furthermore, this LRR hybrid technique provides highly versatile applications for expression of LRR-containing immune receptors whose heterologous expression has been elusive. For

examples, TLR7/8/9-ECD can be fused with relatively stable VLR or TLR3 proteins. NOD-like receptor (NLR) hybrids can be designed in fusion with its structurally homologous LRR protein, ribonuclease inhibitor (RI), for expression screening. Along with the LRR hybrid technique, the increase in production of active TLR5-ECD proteins by flagellin supplementation would benefit ongoing structural studies that require a large amount of protein sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.021.

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