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Determination of the physiological 2:2 TLR5:flagellin activation stoichiometry revealed by the activity of a fusion receptor

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ABSTRACT

Toll-like receptor 5 (TLR5) recognizes flagellin of most flagellated bacteria, enabling activation of the MyD88-dependent signaling pathway. The recently published crystal structure of a truncated zebrafish TLR5 ectodomain in complex with an inactive flagellin fragment indicated binding of two flagellin molecules to a TLR5 homodimer, however this complex did not dimerize in solution. In the present study, we aimed to determine the physiological stoichiometry of TLR5:flagellin activation by the use of a chimeric protein composed of an active flagellin fragment linked to the N-terminus of human TLR5 (SF-TLR5). This construct was constitutively active. Inactivation by the R90D mutation within flagellin demonstrated that autoactivation of the chimeric protein depended solely on the specific interaction between TLR5 and flagellin. Addition of wild-type hTLR5 substantially lowered autoactivation of SF-TLR5 in a concentration dependent manner, an effect which was reversible by the addition of exogenous *Salmonella typhimurium* flagellin, indicating the biological activity of a TLR5:flagellin complex with a 2:2 stoichiometry. These results, in addition to the combinations of inactive P736H mutation within the BB-loop of the TIR domain of TLR5 and SF-TLR5, further confirm the mechanism of TLR5 activation.

1. Introduction

Toll-like receptors (TLRs) are type I transmembrane glycoproteins structurally characterized by the presence of leucine-rich repeat (LRR) motifs in their horseshoe-shaped ectodomain (ECD), a single transmembrane helix and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain [1,2]. Binding of ligands to the ectodomain of TLR receptors induces formation of a receptor dimer [3–6] bringing the C-terminal regions of the ectodomains into close proximity, enabling intracellular TIR domains [7] to trigger downstream activation of signaling. Activation of TLRs proceeds through MyD88-dependent or TRIF-dependent signaling and synthesis of proinflammatory cytokines. Despite the conserved similar 'm' shaped TLR dimer formation, the active complexes of different TLRs differ in stoichiometry between the receptors and their ligands. TLR3 forms a symmetric 2:1 complex with dsRNA [3,8]. 2:1 stoichiometry has been determined for the asymmetric TLR1-TLR2-Pam_3CSK_4 and TLR2-TLR6-Pam_2CSK_4 complex [5,6]. TLR4/MD-2-LPS forms a symmetric complex with 2:2 stoichiometry [4,9].

TLR5 binds the monomeric form of flagellin which is the main structural component of bacterial flagella and functions as a virulence factor upon host infection. Flagellin consists of four linearly connected domains named D0, D1, D2 and D3, proceeding in this order from the core of the filament towards the exposed region [10,11]. The N- and C-terminal regions that form the D0–D1 domains are conserved among flagellins of different bacterial species, while amino acid sequences of D2–D3 domains are highly variable in sequence and length [12]. The region of flagellin recognized by TLR5 lies within the conserved D1 domain which is also necessary for filament assembly [13].

The determined crystal structure of the fragment zebrafish TLR5 with a D1–D2 domain of FliC from *Salmonella enetrica* serovar Dublin revealed that the TLR5-FliC heterodimer forms a 2:2 complex [14]. The buried interaction surface of this complex is quite low and the dimer has not been observed in solution. Moreover, since the flagellin fragment used in the crystal structure is biologically inactive there is a concern if the physiological ligand:receptor complex stoichiometry is the same as in the crystal structure.

The aim of the study was to test TLR5:flagellin activation stoichiometry on living cells. We aimed to solve this problem by the use of a flagellin-TLR5 chimeric protein (SF-TLR5) composed of

Abbreviations: SF-TLR5, short flagellin linked to TLR5; SFΔD0, short flagellin with a deletion of the D0 domain.

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active flagellin lacking the hypervariable region (termed SF) linked to the N-terminus of human TLR5. The selectivity of intramolecular flagellin:TLR5 interactions was demonstrated by the inactivity of a point mutant within flagellin and within the TIR domain of TLR5. Results of activation assays testing combinations of the chimeric protein and TLR5 demonstrate that the stoichiometry of the active TLR5:flagellin complex is 2:2.

2. Materials and methods

2.1. Cell cultures

The human embryonic kidney cell lines HEK293 and HEK293T were cultured in complete media (DMEM; 1 g/l glucose, 2 mM L-glutamin, 10% heat-inactivated FBS (Gibco)) in 5% CO_2 at 37 °C.

2.2. Plasmids and DNA construct preparation

Plasmid pUNO-hTLR5 coding for human TLR5 (InvivoGen) and control vector pcDNA3 (Invitrogen) were used. *Salmonella typhimurium* flagellin (bacteria provided by prof. Ignacio Moriyón, University of Navarra, Spain) was cloned into the *Xho*I and *Bam*HI sites of the pET-19b expression vector (Novagen) containing an N-terminal His-tag. SF-L27-TLR5 and SF-L57-TLR5 were prepared with PCR overlap extension technique from DNA encoding Short Flagellin (aa 2–176; 398-495), a 27 or 57 amino acid long linker and hTLR5 (aa 21-858) with an AU1-tag at the C-terminal end. Purified PCR fragments were cloned into the *Hin*dIII and *Bgl*II sites of the pFLAG-CMV-3 expression vector (Sigma) to obtain the N-terminal preprotrypsin leader sequence and N-terminal FLAG-tag. SFΔD0-TLR5 was prepared with PCR overlap extension technique from DNA encoding Short Flagellin lacking the D0 domain (SFΔD0) (aa 43-176; 398-456), a 27 amino acid long linker and hTLR5 (aa 21-858) and cloned into the *Hin*dIII and *Bgl*II sites of the pFLAG-CMV-3. R90D mutation was introduced into *S. typhimurium* flagellin/pET19b and SF-L27-TLR5/pFLAG-CMV-3 constructs using a QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. All plasmids were sequenced to confirm the mutation. Primers and sequences of chimeric proteins used in this study are listed in Supplementary Tables 1 and 2.

2.3. Production and isolation of S. typhimurium flagellin

Escherichia coli BL21 cells were transformed with the pET19b plasmid expressing His-tagged *S. typhimurium* flagellin and cultivated at 37 °C in LB (Luria-Bertani) medium supplemented with 50 μg/ml ampicillin. Overnight cultures were transferred to fresh media and induced with 1 mM Isopropyl β-D-thiogalactoside (IPTG) at an optical density of ~0.8 at 600 nm. Cells were grown at 25 °C for 8 h upon induction, harvested and lysed in buffer (10 mM TRIS pH 7.5, 1 mM EDTA, 0.1% DOC) containing a protease inhibitor cocktail (Sigma P8849). Supernatants were collected and His-tagged recombinant proteins were purified on Ni-NTA affinity agarose (Qiagen). Purity was confirmed with SDS–PAGE and immunoblotting.

2.4. Luciferase reporter assay

HEK293 cells were seeded in 96-well plates (Corning) and transiently transfected after 24 h using jetPEI transfection reagent (Polyplus Transfection) with indicated amounts of plasmids expressing chimeric or wild-type TLR5, 50 ng pELAM-1 (from C. Kirschning, Institute for Medical Microbiology, University of Duisburg-Essen, Essen, Germany) inducibly expressing NF-κB dependent firefly luciferase and 5 ng phRL-TK constitutively expressing *Renilla* luciferase (Promega). The total amount of DNA for each transfection was kept constant by adding appropriate amounts of the pcDNA3 plasmid. Cells were either lysed after 24 h or the media was changed and cells stimulated with purified flagellin were incubated for a following 18 h, lysed in passive lysis buffer (Promega) and analyzed for reporter gene activities using a dual-luciferase reporter assay. Relative light units (RLU) were calculated by normalizing firefly luciferase activity of each sample with constitutive *Renilla* luciferase activity measured within the same sample.

2.5. Immnuoblotting

HEK293T cells were seeded in 6-well plates (Techno Plastic Products) and transiently transfected after 24 h with 2 μg of plasmid DNA using jetPEI transfection reagent. Cells were lysed 48 h after transfection in lysis buffer (50 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 137 mM NaCl, 1% Triton X-100, 1% Na-DOC, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF) supplemented with protease inhibitors (Roche). Supernatants were collected and total protein concentration in supernatant was determined using the BCA assay (Pierce). Proteins from supernatant were separated by SDS–PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham Life Science). Primary antibodies used were rabbit anti-FLAG (F7425, Sigma) and rabbit anti-GFP (A11122, Invitrogen). Secondary antibodies used were horseradish peroxidase-conjugated goat anti-rabbit IgG (ab6721, Abcam). Blots were developed using ECL Western blotting detection reagent (Amersham Life Science) according to the manufacturer's protocol.

3. Results and discussion

3.1. Chimeric TLR5 with linked flagellin exhibits constitutive cell activation

The recently determined crystal structure TLR5-$N14_{VLR}$/FliC-ΔD0, in which the C-terminal region of the TLR5 ectodomain and the flagellin D0 domain are missing, implies that one flagellin molecule brings two TLR5 receptors together through binding to the primary binding interface of one TLR5 and through additional weaker interactions with the opposite TLR5 receptor. To define if only one flagellin molecule might be sufficient for TLR5 dimer activation (2:1 complex) or two are necessary as proposed in the crystal structure (2:2 complex) (Fig. 1A), we designed a cell-based assay that would discriminate between the two options. As previously reported by Hayashi et al. [15], TLR5 requires flagellin for its activation (Fig. 1C), so we assumed that short flagellin linked to TLR5 could activate TLR5 through binding and forming of active TLR5 dimer. Initially, we constructed a chimeric protein, linking flagellin from *S. typhimurium* lacking the hypervariable domain to the N-terminus of the full-length human TLR5 (Fig. 1B). The truncated form of *S. typhimurium* flagellin (named Short Flagellin (SF)) has the same structure as CBLB502 [16] which displays comparable stimulatory activity to the full-length recombinant flagellin. To exclude the possibility of impaired positioning of SF on the TLR5 receptor due to the short linker and to examine the importance of distance between the N-terminus of TLR5 and C-terminus of SF, we designed two chimeric receptors: SF-L27-TLR5 and SF-L57-TLR5, which differ in the length of the flexible linker between SF and hTLR5, being 27 or 57 amino acids long, allowing the separation of approx. 10 or 20 nm, respectively (Fig. 1B). We observed that both chimeric receptors were expressed similarly in HEK293 cells and were both constitutively active (Fig. 1D and E). Moreover, the length of the linker within this boundary has no effect on autoactivation, as both chimeric receptors exhibit constitutive activation detected even at very low

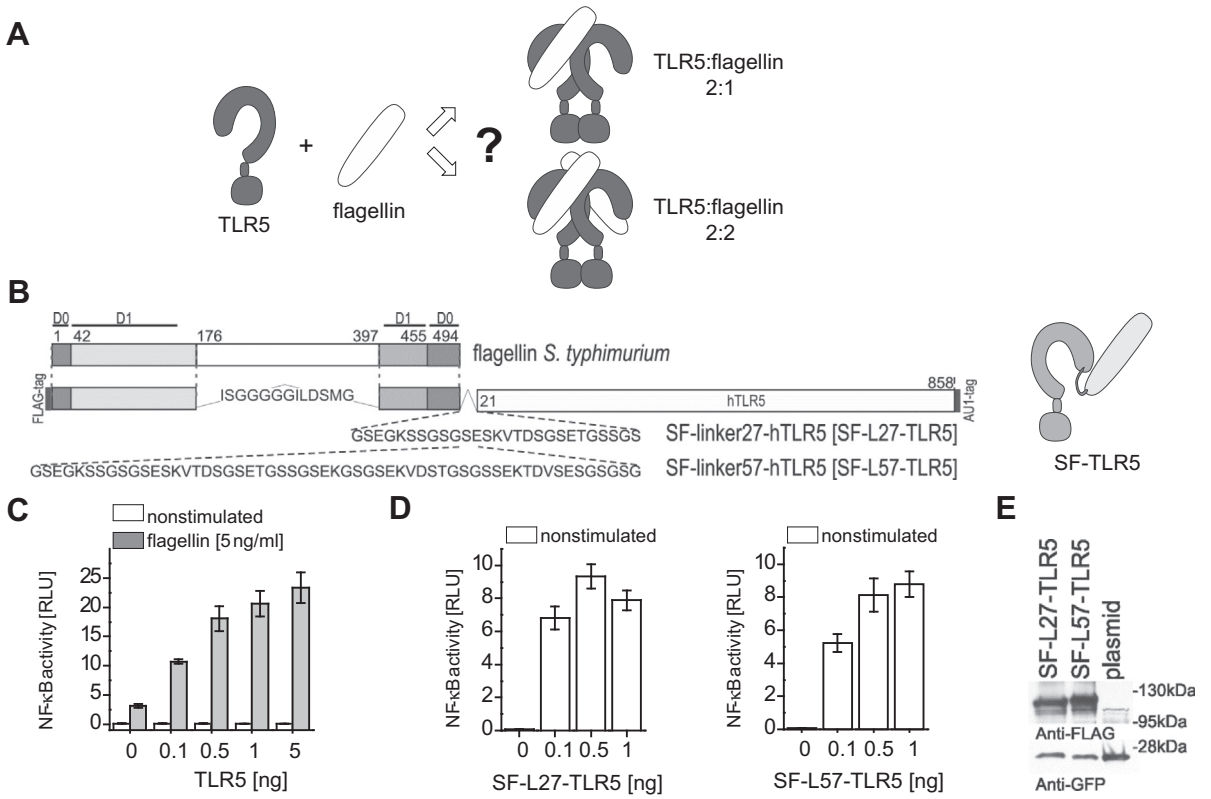

Fig. 1. TLR5 receptor with linked flagellin exhibits constitutive activation. (A) Upon stimulation with flagellin TLR5 could form 2:1 or 2:2 complexes in biological systems. (B) Scheme of SF-L27-TLR5 and SF-L57-TLR5 chimera in which flagellin lacking the hypervariable domain (Short Flagellin) is linked to hTLR5 via a 27 or 57 amino acid long linker. (C) NF-κB activation of hTLR5 in transfected HEK293 cells stimulated with *S. typhimurium* flagellin. (D) Both chimeric receptors are constitutively active even at low expression levels. HEK293 cells transfected with plasmids coding SF-L27-TLR5 and SF-L57-TLR5 express NF-κB activity without stimulation with flagellin. Luciferase activity data ($n = 4$, mean ± s.d.) are representative of at least three independent experiments. (E) Western blot analysis of the expression of SF-L27-TLR5 and SF-L57-TLR5 in HEK293T cells.

amounts of SF-L27-TLR5 or SF-L57-TLR5 (0.1 ng/well) transiently transfected into HEK293 cells.

3.2. Flagellin linked to the chimeric receptor governs TLR5 autoactivity

To verify that autoactivation of the chimeric receptor originates from the specific binding of flagellin to the receptor in the same manner as activation of wild-type TLR5 by free flagellin, we prepared a mutated chimeric protein SF^{R90D}-L27-TLR5 where substitution R90D was introduced into the SF of the chimeric protein. Smith et al. [13] showed previously that mutation of Arg90 to alanine significantly reduced the efficiency of flagellin to activate TLR5, thus we introduced the opposite charge on this position to intensify the effect of the mutation. This substitution rendered isolated recombinant *S. typhimurium* flagellin inactive (Fig. 2A). Moreover, the resulting SF^{R90D}-L27-TLR5 lost its constitutive activation that was not restored even after stimulation with flagellin (Fig. 2B).

Yoon et al. [14] showed that a deletion mutant CBLB502-ΔD0 where the D0 domain is deleted, binds to the TLR5 receptor but was unable to activate it. Therefore, we prepared a chimeric receptor with a ΔD0 deletion mutant linked to TLR5 termed SFΔD0-TLR5 in order to further confirm the role of SF in the chimeric TLR5 receptor (Fig. 2C). Although this truncated form of chimeric protein was expressed in HEK293 cells at comparable amounts as SF-L27-TLR5 Fig. 2E, the D0 deletion rendered the chimeric receptor SFΔD0-TLR5 inactive even when additionally stimulated with *S. typhimurium* flagellin (Fig. 2).

These results demonstrate that autoactivation of the chimeric TLR5 receptor is based on the specific binding of the linked flagellin to the TLR5 ectodomain in a similar way as binding of free flagellin to wild-type TLR5. In addition, the inability of the added free flagellin to trigger activation demonstrated that mutated R90D and truncated flagellin SFΔD0 bind to TLR5 in the chimeric receptor, yet cannot trigger receptor activation.

3.3. Two flagellin monomers are required for TLR5 activation

We examined whether one or two flagellin monomers are needed for formation of the physiologically active TLR5:flagellin complex (Fig. 1A). Theoretically if only one flagellin monomer is sufficient for TLR5 dimer activation (2:1 stoichiometry) (Fig. 3A), then the addition of an increasing amount of wild-type TLR5 should amplify the activity due to the formation of a SF-TLR5/TLR5 complex or, alternatively, the excessive SF domain from the chimeric receptor dimer (SF-TLR5)$_2$ should be able to activate the preformed TLR5 dimers formed between the wild-type TLR5 receptors, as described by Zhou et al [17]. Furthermore, addition of free flagellin should in this case only slightly enhance the activity of coexpressed SF-TLR5 and TLR5 by activating any inactive wild-type TLR5 dimers that did not recruit the SF domain from the fusion protein and thus remained unstimulated (Fig. 3A).

In the case of 2:2 stoichiometry, a decrease in SF-TLR5 activation might be expected by the addition of TLR5 (Fig. 3B) because the wild-type TLR5 could compete for the SF domain of the SF-TLR5 chimeras, hindering formation of the active SF-TLR5 dimers (Fig. 3B). The resulting TLR5/SF-TLR5 complex is inactive as it lacks an additional flagellin and this complex as well as the majority of free TLR5 could be activated by the additional stimulation with free flagellin. HEK293 cells were transiently transfected with the chimeric receptor or cotransfected with an increasing amount of hTLR5 (Fig. 3C and D). Decreased cell activation was recorded upon the addition of wild-type hTLR5 in comparison to the chimeric

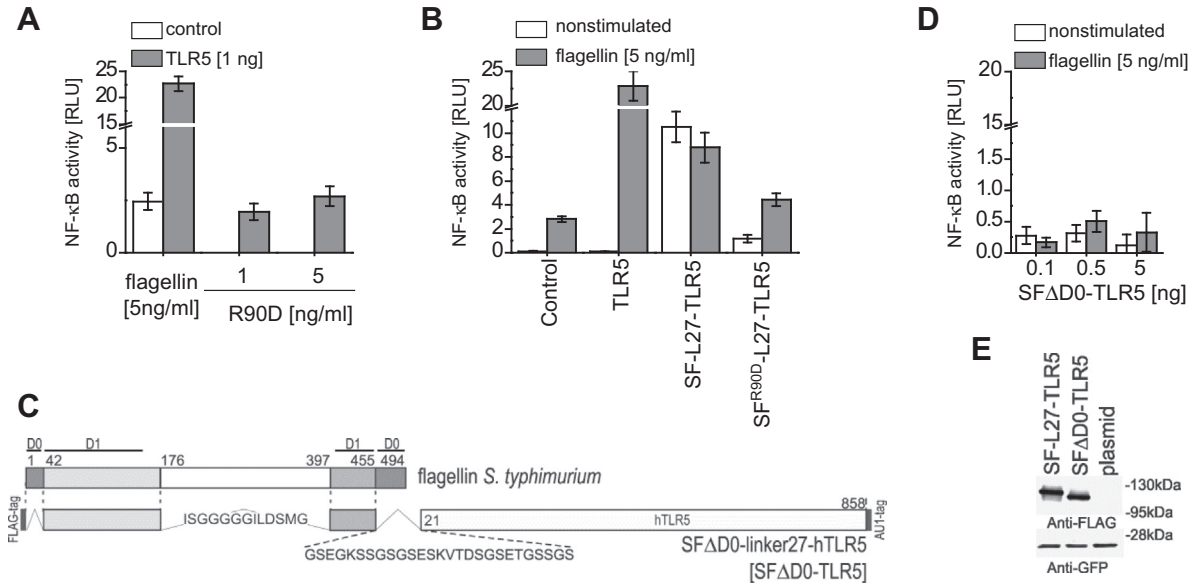

Fig. 2. The chimeric SF-TLR5 receptor activation is based on similar interactions as between soluble flagellin and wild-type TLR5. (A) Mutation R90D renders flagellin inactive for TLR5 activation. HEK293 cells transfected with hTLR5 were stimulated with *S. typhimurium* flagellin or the R90D mutant and luciferase assay was measured. (B) Mutation R90D in linked short flagellin results in complete loss of SF-TLR5 autoactivation. NF-κB activation of hTLR5, SF-L27-TLR5 and SF^{R90D}-L27-TLR5 (all 0.3 ng per well) in transfected HEK293 cells stimulated or nonstimulated with *S. typhimurium* flagellin was measured. (C) Scheme of the deletion mutant SFΔD0-TLR5. (E) Western blot analysis of the expression of SF-L27-TLR5 and SFΔD0-TLR5 in HEK293T cells. (D) NF-κB activation of SFΔD0-TLR5 in transfected HEK293 cells stimulated or nonstimulated with *S. typhimurium* flagellin. Luciferase activity data ($n = 4$, mean ± s.d.) are representative of at least three independent experiments.

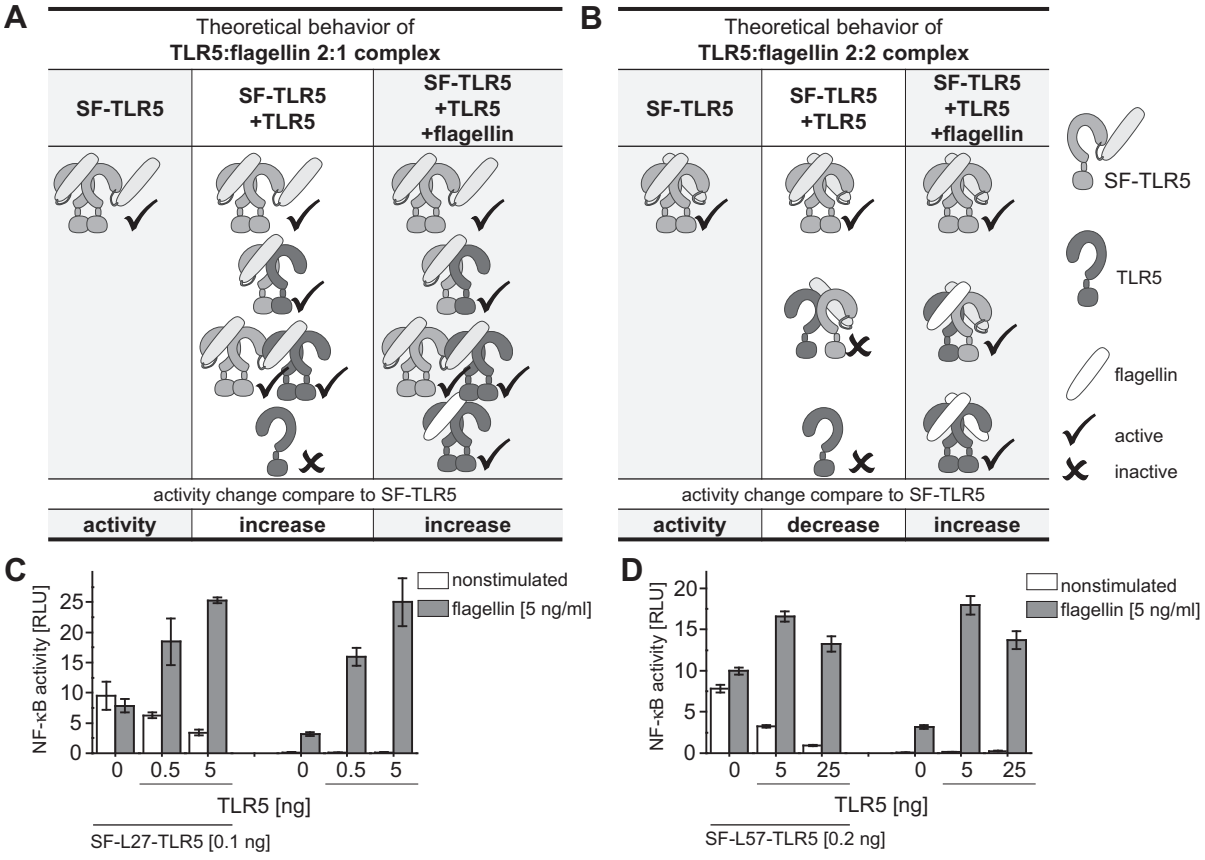

Fig. 3. Titration of chimeric SF-TLR5 receptor with wild-type TLR5 and soluble flagellin confirms the existence of 2:2 complexes. Scheme of the theoretical expected results of TLR5:flagellin 2:1 complex (A) and 2:2 complex (B). (A) In the case of the TLR5:flagellin 2:1 active complex, an addition of TLR5 should increase the TLR5 activity due to mixed active SF-TLR5/TLR5 dimers. Only wild-type TLR5 dimers are activated by the addition of soluble flagellin monomers. (B) Addition of TLR5 to the chimeric receptor titrates out the initial activity of SF-TLR5 if the TLR5:flagellin 2:2 complex is biologically relevant, because mixed inactive SF-TLR5/TLR5 heterodimers could be formed. The addition of soluble flagellin increases activity of inactive heterodimers and wild-type TLR5 homodimers. (C, D) hTLR5 inhibits autoactivation of SF-L27-TLR5 (C) and SF-L57-TLR5 (D) which is reversed with the addition of flagellin. HEK293 cells cotransfected with plasmid combination SF-L27-hTLR5 (C) or SF-L57-hTLR5 (D) and hTLR5 were stimulated with *S. typhimurium* flagellin, and NF-κB dependent firefly and *Renilla* luciferase activities were measured. Luciferase activity data ($n = 4$, mean ± s.d.) are representative of at least three independent experiments.

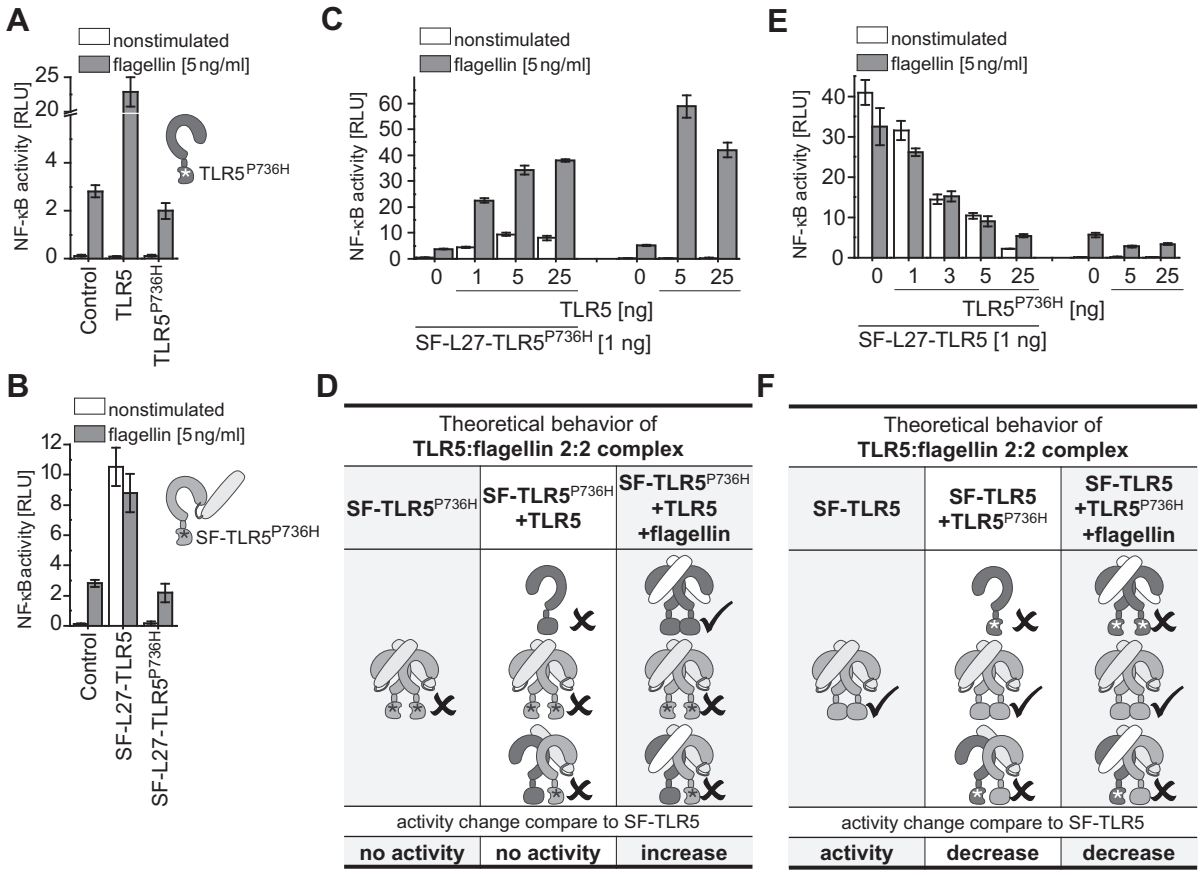

Fig. 4. A mutation in TIR BB-loop confirms the TLR5:flagellin 2:2 complex. Mutation P736H in the TIR domain of TLR5 renders hTLR5 (A) or the chimeric SF-L27-TLR5 (B) inactive even when stimulated with soluble *S. typhimurium* flagellin. NF-κB activation of hTLR5 and hTLR5^{P736H} (A) or SF-L27-TLR5 and SF-L27-TLR5^{P736H} (B) (all 0.3 ng per well) in transfected HEK293 cells was measured with or without *S. typhimurium* flagellin stimulation. (C) Activation of hTLR5 with flagellin is inhibited in the presence of SF-L27-TLR5^{P736H}. (E) hTLR5^{P736H} inhibits SF-L27-TLR5 autoactivation and is flagellin independent. HEK293 cells cotransfected with plasmid combinations SF-L27-TLR5^{P736H} and hTLR5 (C) or SF-L27-TLR5 and hTLR5^{P736H} (E), were stimulated with *S. typhimurium* flagellin, and NF-κB dependent firefly and *Renilla* luciferase activities were measured. Luciferase activity data (n = 4, mean ± s.d.) are representative of at least two independent experiments. The expected results of TLR5:flagellin 2:2 complex when SF-TLR5 or TLR5 is replaced with SF-TLR5^{P736H} (D) or TLR5^{P736H} (F).

receptor activity. The addition of soluble *S. typhimurium* flagellin however restored cell activation. These results match the theoretical prediction indicating 2:2 stoichiometry of the active complex (Fig. 3B).

3.4. Mutation in the TIR domain of TRL5 confirmed the existence of SF-TLR5/TLR5 dimers

To prove that SF-TLR5/TLR5 dimers are indeed formed, we introduced a P736H mutation into the TIR domain of SF-TLR5 and TLR5. This residue is positioned in the BB-loop and has been previously found to be essential for the activation of TLR2, TLR4 and chicken TLR5 based on affecting recruitment of the adapter protein MyD88 to the activated receptor TIR domain dimer [18–20]. The constructs SF-L27-TLR5^{P736H} and TLR5^{P736H} were both inactive and could not be activated by stimulation with *S. typhimurium* flagellin (Fig. 4A and B). SF-L27-hTLR5^{P736H} formed inactive dimers with wild-type TLR5 which was demonstrated through the reduced activity of TLR5 upon stimulation with flagellin (Fig. 4C and D). On the other hand, the constitutive activity of SF-L27-TLR5 was inhibited by the addition of an increasing amount of inactive TLR5^{P736H} and the addition of flagellin was not able to restore activity (Fig. 4E and F). This confirmed the formation of heterodimers between the chimeric receptor and TLR5 and that the same type of TIR domain interactions govern the activation as reported for other TLRs [18–20].

The importance of the binding stoichiometry lies in the sensitivity of the cell for detection of flagellin. We demonstrated in the biological assay that two flagellin molecules are required for the activation of a TLR5 dimer, while a single molecule is not sufficient despite binding to the two receptor molecules. The crystal structure lacks a substantial part of the receptor, which might therefore harbor additional interaction sites both for the interaction with flagellin as for the interaction between TLR5 ectodomains. The interaction between TLR5 and flagellin is strong, as the R90D mutant in the chimeric receptor could not be replaced with added soluble flagellin (Fig. 2B), however we cannot exclude the possibility that this is due to extremely high local concentration of the SF in the SF-TLR5. Our results cannot distinguish if the TLR5:flagellin 1:1 heterodimer complex first binds another heterodimer of TLR5:flagellin to form an active 2:2 complex or if the heterodimer TLR5:flagellin binds another TLR5, generating an inactive 2:1 complex which then binds an additional flagellin molecule forming an active 2:2 complex. Observations of inactive preformed TLR5 dimers on the other hand favors this second scenario [17]. Further research will be required to elucidate the sequence of the events governing TLR5 signaling in greater detail.

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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.2013.04.030.

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