An Alanine-to-Proline Mutation in the BB-Loop of TLR3 Toll/IL-1R Domain Switches Signalling Adaptor Specificity from TRIF to MyD88

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An Alanine-to-Proline Mutation in the BB-Loop of TLR3 Toll/IL-1R Domain Switches Signalling Adaptor Specificity from TRIF to MyD88

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A functionally important proline residue is highly conserved in the cytosolic Toll/IL-1R signaling domains of human TLRs. The antiviral Toll, TLR3, is unusual because it has alanine instead of proline at this position and is the only human TLR that associates directly with the adaptor molecule TIR domain–containing adaptor inducing IFN-β (TRIF) rather than MyD88. In this article, we report that a mutant TLR3 that substitutes the BB-loop alanine for proline (A795P) enhances NF-kB activation but is incapable of mediating TRIF-dependent IFN response factor 3 responses. Wild-type and A795P TLR3 associate constitutively with both TRIF and MyD88, and activation induces additional binding of TRIF to the wild-type and of MyD88 to the A795P mutant receptors, respectively. In addition, activation of A795P, but not wild-type TLR3, leads to the recruitment of TRAF6, a downstream signal transducer of the MyD88-dependent pathway. These results show that adaptor specificity can be conferred by minimal determinants of the Toll/IL-1R domain.


Toll-like receptors (TLRs) play an important role in innate immunity by recognizing specific molecular patterns expressed by microbes. TLR signaling requires dimerization of the receptor extracellular domains, mediated by microbial products, leading to the dimerization of the receptor cytoplasmic Toll/IL-1R (TIR) domains (1, 2). The activated conformation of the receptor provides a scaffold for the recruitment of the downstream signaling molecules that activate transcription factors including NF-κB and IFN response factors (IRFs), leading to the production of proinflammatory cytokines and type 1 IFNs.

TLRs use four signaling adaptor proteins (3). Of these, TIR domain–containing adaptor inducing IFN-β (TRIF) and MyD88 adaptor molecules are required for the activation of IFR3/7 and NF-κB, respectively. TRIF is the largest member of the adaptor family and is required for TLR3-induced signaling and, together with TRIF-related adaptor molecule (TRAM), the MyD88-independent signaling pathway of TLR4, whereas MyD88 couples to all the human TLRs except TLR3. Acting as a platform, TRIF accumulates other signaling molecules to the “TRIF signalingosome” and activates a range of cellular responses mediated by other modules in TRIF, as well as the TIR (4). TRAF3 binds to the N terminus of TRIF leading to phosphorylation of IRF3 and IRF7 by the TANK-binding kinase-1 (TBK-1). In contrast, receptor-interacting protein-1 (RIP-1) can bind to the C terminus of TRIF, causing both Fas-associated death domain–dependent apoptosis and activation of NF-κB by the IkB kinase complex. The role of TRAF6 in TRIF signaling is controversial. Although some studies found that TRAF6 functions exclusively in MyD88-directed signaling, others suggest a secondary role in TRIF-mediated activation of NF-κB (5–7).

The TIR domains of the TLRs and their cognate adaptors are ∼200 aa and fold into a characteristic α-β structure (8). The loops connecting these secondary structure elements mediate receptor–adaptor interactions. Of particular interest is the BB-loop that links the second β-sheet to the second α-helix of the TIR. A naturally occurring mutation of the conserved proline residue in the BB loop of TLR4 was identified as the cause of LPS hyporesponsiveness in C3H/HeJ mice. This missense mutation substituted proline with a histidine residue (P712H) and displayed dominant-negative properties (9, 10). The importance of the BB-loop in TLR and adaptor function is further emphasized by the inhibitory properties of cell-permeable peptide mimetics (11, 12).

The BB-loop proline is conserved in all human TLRs except for TLR3, which has alanine at this position, and previous studies suggest that this residue plays an important role in recruiting TRIF into the signaling complex (13). To test this idea further, we have studied the signaling characteristics of a mutant TLR3 that replaces the BB-loop alanine for proline. We show that this single amino acid change switches TLR3 from a TRIF to a MyD88-directed signaling pathway.

Materials and Methods

Plasmids and reagents

Full-length pCMV-Myc-Mal, pCMV-MyD88-Myc, and pcDNA3.1-TLR7 were a kind gift from Dr. Alexander Weber (University of Tübingen, Tübingen, Germany); pcDNA3.1-Myc-TRIF was a kind gift from Dr. Rongtuan Lin (McGill University, Montreal, QC, Canada); pcDNA3.1-Flag-TLR2,-Flag-TLR3,-Flag-Mal, and -HA-Mal were a kind gift from Dr. Ashley Mansell (Monash Institute of Medical Research, Clayton, VIC, Australia); pcDNA3.1-TBK1-Myc was a kind gift from Dr. Ivan Dikic (Institute of Biochemistry, Frankfurt Medical School, Frankfurt, Germany); pcDNA3-Myc-TRAF6 was a kind gift from Dr. William Dalton ( Moffitt Cancer Centre, Tampa, FL); pcDNA1–RIP–1–Myc was a kind gift from guest on July 15, 2017 http://www.jimmunol.org/ Downloaded from
from Dr. Michelle Kelliher (University of Massachusetts Medical School, Worcester, MA), and Gal4-IRF3-IRF5, -IRF7, and Gal4-luciferase constructs were a kind gift from Dr. Kate Fitzgerald (University of Massachusetts Medical School). Vector pEYFPN1-TLR5-YFP was obtained from Clontech, and reporter gene constructs NF-κB-, IFN-β-luciferase, and Renilla luciferase-thymidine kinase encoding plasmid were a kind gift from Dr. Clare Bryant (University of Cambridge, Cambridge, U.K.). Myc-TRAM was cloned into the pCMV-Myc vector at sites HindIII and KpmI, and Flag-TRAF6 into the pEFBOS vector at sites Xhol and HindIII. All BB-loop mutants were generated using the QuickChange II site-directed mutagenesis kit with PfuTurbo (Stratagene) using the above mentioned plasmids.

Cell culture and reagents

Human embryonic kidney (HEK) 293, HEK293T, and HEK293-derived MyD88-deficient (I3A) (14) cells were cultured in DMEM medium supplemented with 10% FCS (Invitrogen) and 100 U/ml penicillin/streptomycin, and maintained in a 37 °C humidified atmosphere. Pam3Cys4K was obtained from EMD microcollections and CL097 imidazoline compound, FLA-STA Ultrapure Flagellin from Salmonella typhimurium and polyinosinic-polycytidylic acid [poly(I:C)] HMW synthetic analog of dsRNA; all were sourced from Invivogen.

Luciferase reporter assay

HEK293 or HEK293 MyD88-deficient (I3A) cells were seeded at 1 × 10^5 cells/well in a 96-well plate 36 h before transfection with jetPEI (Polyplus). NF-κB-, IFN-β-, or upstream activation sequence (UAS)-Gal4-dependent gene expression was determined using luciferase reporter constructs concomitantly with indicated vectors. Gal4-fusions IRF3, IRF5, and IRF7 were used to analyze activation of IRF activity. The Renilla luciferase-thymidine kinase encoding plasmid was used to normalize for transfection efficiency, and pG-DNA 3.1 empty vector was used to maintain constant DNA. Transfected cells were lysed using Passive lysis buffer (Promega), and assayed for luciferase and Renilla activity using luciferase assay reagent (Promega). Luminescence readings were corrected for Renilla activity and expressed as fold increase over nonsaturation and subtracting background. Values were expressed as a fold increase over nonstimulated control values. Data are presented as mean ± SE of one of three independent experiments. Statistical analysis was performed using two-way ANOVA where significance is relative to wild-type TLR3 plasmid transfection: *p < 0.05, **p < 0.01, ***p < 0.001.

Immunoprecipitation and immunoblot analysis

HEK293T cells were seeded at 1.5 × 10^5 cells/well in a 6-well plate and were transfected using JetPEI with the indicated plasmids where the total amount of DNA (3 μg/well) was kept constant. Twenty-four hours after transfection, cells were lysed in KabiB buffer as described (15). EZview Red Anti-Flag M2 affinity beads (25 μl, 50% slurry; Sigma) were incubated with the cell lysates for 2 h at 4°C. The immune complexes were precipitated, washed, and eluted by addition of sample buffer for resolution by SDS-PAGE and immunoblotting with either anti-Myc (Abcam), anti-HA (Abcam), or anti-Flag Ab (Sigma). Immunocomplexes were visualized by using SuperSignal West Pico chemiluminescent substrate solution (Pierce) followed by exposure to X-ray film (Hyperfilm ECL, Amersham Pharmacia) to detect chemiluminescence.

Densitometry analysis for immunoprecipitated Western band intensities was calculated using the Total Lab software (Nonlinear Dynamics), verifying for nonsaturation and subtracting background. Values were expressed as a percentage of the total area of each band normalized to the strongest band.

Small interfering RNA knockdown of TRIF, RNA extraction, and quantitative PCR

The 25-bp duplex of targeting stealth small interfering RNA (siRNA) or nontargeting (NT) siRNA (Invitrogen) was transfected into subconfluent HEK293/293T cells, using siRNA jetPRIME (Polyplus). Double-stranded siRNAs containing equal parts of the following antisequences was used to knock down TRIF: siRNA2, 5′-CCCAUUGACGCCGCUUUCGGA-CUGGA-3′; siRNA3, 5′-C CCAUCUCAUGCCGCUUUCGGA-CUGGA-3′; siRNA4, 5′- CC CAUCUCAUGCCGCUUUCGGA-CUGGA-3′; and GAPDH: 5′-GAAGGTGAAAGGTGAGATC-3′. NT siRNAs were low GC and medium GC stealth RNAi negative control duplexes (Invitrogen). Forty-eight hours after transfection, the cells were either transfected with the relevant constructs or analyzed by real-time quantitative PCR (qPCR) or Western blot using anti-TRIF (Cell Signaling) and β-actin (Ambion). Total RNA from cells were purified using RNeasy Plus kit and Qiagen RNeasy (Qiagen). First-strand cDNA was synthesized from the total RNA using SuperScript II reverse transcriptase (Invitrogen) with random hexamer (Promega) as described in the manufacturer’s instructions.

The cDNA served as template for real-time qPCR analysis to determine the level of gene knockdown using Maxima SYBR Green/ROX qPCR SuperMix (Thermo Scientific). Relative gene levels of TRIF were determined and normalized against internal control of GAPDH to determine the relative gene knockdown using Pfaffl method. The gene-specific primers for TRIF: 5′-TCTTCCTCCTGTSCCTCC-3′, 5′-CTGGAATTCCTCGGCA-3′; and GAPDH: 5′-GAAGGTGAAAGGTGAGATC-3′. 5′-GAAGATGGTG-ATGCGATTCC-3′ were designed to span the exon–exon junction to eliminate amplification of genomic DNA.

Results

The BB-loop alanine residue of TLR3 confers specificity for TRIF-directed activation of IRF-3 and IFN-β

The 10 human TLRs all have a proline-glycine dipeptide in the BB-loop except for TLR3, which has alanine-glycine (Supplemental Fig. 1). In view of this, we asked whether the BB-loop alanine and proline residues played a role in receptor selectivity for TRIF and MyD88, respectively. To this end, we mutated A959 of TLR3 to a proline and also to histidine, a mutation previously shown to disrupt TRIF recruitment and IRF3-directed signaling (13). HEK293 cells were transfected with either wild-type TLR3, TLR3 A795P, or TLR3 A795H, and downstream activation of the IFN-β and NF-κB reporter genes was assayed. After poly(IC) stimulation, wild-type TLR3 strongly activated the IFN-β promoter, whereas mutants A795P and A795H were not responsive (Fig. 1A). As expected, wild-type TLR3 supports a small but sustained induction of NF-κB, but surprisingly the A795P potentiated this response by almost 20-fold (Fig. 1B). A795H was unable to activate the NF-κB-driven reporter and exerted a dominant negative effect on the endogenous response to poly(I:C).

We next sought to investigate the effect of the TLR3 A795P mutant on downstream IRF activity. Because TLR3 signaling activates IRF3 and IRF7, but not IRF5 (15), we measured activation of these transcription factors by wild-type and A795P mutant TRIF. MyD88, respectively. To this end, we mutated A959 of TLR3 to a proline and also to histidine, a mutation previously shown to disrupt TRIF recruitment and IRF3-directed signaling (13). HEK293 cells were transfected with either wild-type TLR3, TLR3 A795P, or TLR3 A795H, and downstream activation of the IFN-β and NF-κB reporter genes was assayed. After poly(IC) stimulation, wild-type TLR3 strongly activated the IRF-3 and IFN-β promoters, whereas mutants A795P and A795H were not responsive (Fig. 1A). As expected, wild-type TLR3 supports a small but sustained induction of NF-κB, but surprisingly the A795P potentiated this response by almost 20-fold (Fig. 1B). A795H was unable to activate the NF-κB-driven reporter and exerted a dominant negative effect on the endogenous response to poly(I:C).

Constitutive and inducible binding of MyD88 and TRIF to wild-type and TLR3 A795P mutant

We next asked whether a switch in adaptor specificity from TRIF to MyD88 accounts for the observed enhancement of NF-κB activation by TLR3 A795P. Both wild-type and A795P, but not A795H TLR3, associate with MyD88 in the absence of stimulation. However, treatment with poly(IC) induces a 2.5-fold increase in MyD88 binding to A795P, but not to wild-type TLR3 (Fig. 2A, 2B). To investigate the adaptor specificity of the A795P receptor further, we used a derivative of the HEK293 cell line (I3A) that does not express MyD88. As shown in Fig. 2C, A795P mutant induces a 2.5-fold increase in NF-κB activity, but the 20- to 30-fold enhancement seen in Fig. 1B is not observed. Interestingly, reconstitution of MyD88 into the HEK293 I3A cells caused a suppression of this low-level response by both the wild-type and A795P TLR3. This suggests that overexpression of MyD88 exerts a dominant negative effect on TRIF-mediated activation of NF-κB, an effect that was also demonstrated in another study in which MyD88 negatively regulated TLR3/TRIF-induced corneal inflammation (17).

To further explore the mechanism by which the A795P TLR3 mutant upregulates NF-κB activation, we asked whether the adaptor molecule TRIF can still associate with this mutant receptor. These
experiments show that TRIF can bind constitutively to both wild-type and A795P TLR3. However, after stimulation, TRIF binding to the wild-type TLR3 is enhanced 2.5-fold compared with the mutant A795P, with only a 1.5-fold increase. This is the reciprocal pattern to that observed with MyD88 (Fig. 3A, 3B, compared with Fig. 2A, 2B). To determine whether the enhanced signaling to NF-κB by A795P TLR3 is TRIF dependent, activation of NF-κB was examined in the presence and absence of targeted functional gene silencing of TRIF. In HEK293 cells, endogenous TRIF protein was constitutively expressed in the presence of an NT siRNA control, whereas TRIF protein levels were markedly reduced after treatment with two TRIF-targeted siRNAs (Fig. 3C). Expression of TRIF mRNA as measured by qPCR also demonstrated a significant reduction when compared with NT siRNA control levels (Fig. 3D). As shown in Fig. 3E and 3F, silencing of TRIF abolished signaling to both IFN-β and NF-κB by wild-type TLR3 in response to treatment with ligand. By contrast, A795P TLR3 retained the ability to activate NF-κB in the absence of TRIF protein, a finding that supports the conclusion that the mutant receptor signals to NF-κB by a MyD88-dependent pathway.

We also examined the association of other signal transducers that are involved in the TRIF- and MyD88-directed pathways. We found that the death-domain kinase RIP-1 that is required for TRIF-dependent activation of NF-κB binds constitutively to wild-type
and A795P TLR3 (Fig. 4A, 4B). In contrast, TBK-1, required for TRIF-dependent IRF3 activation, is present at much lower levels in complexes with the A795P mutant (Fig. 4C, 4D). Interestingly, TRAF6, an E3-ubiquitin ligase recruited to the myddosome complex in MyD88-directed signaling (5), formed a complex with the A795P receptor upon poly(I:C) stimulation, whereas wild-type TLR3 did not (Fig. 4E, 4F). The bridging adaptors MyD88 adaptor-like (Mal) and TRAM were unable to bind either wild-type TLR3 or the A795P/H mutants (Fig. 4G, 4H). Taken together, these results indicate that the mutant A795P shifts the adaptor specificity of TLR3 toward MyD88.

BB-loop mutations within the TIR domain of other TLRs alter signaling

In view of the striking properties of the TLR3 A795P mutant and a recent finding that TRIF can mediate activation of NF-κB and MAPKs by TLR5 (18), we investigated the effect of mutating the BB-loop proline residue in otherTLRs. As expected, upon treatment of HEK293 cells with the TLR2 agonist Pam3CSK4, transfected wild-type TLR2 induced strong NF-κB reporter gene activity over time, whereas the BB-loop TLR2 P681H mutant inhibited this response (Fig. 5A). Interestingly, the TLR2 P681A mutant significantly enhanced NF-κB activity by 3-fold at early time points when compared with wild-type TLR2. By contrast, IFN-β-driven reporter gene activity for both mutant TLR2 constructs showed no response after Pam3CSK4 stimulation, whereas a minor increase over time was observed for wild-type TLR2 (Fig. 5B). For TLR5, an expected NF-κB response was induced by flagellin, and like TLR2 P681A mutant, TLR5 P736A mutant also significantly enhanced NF-κB reporter gene activity, whereas mutant TLR5 P736H was at levels similar to that of wild-type TLR5 response (Fig. 5C). Both TLR5 mutants induced a small IFN-β signal, but wild-type TLR5 remained unresponsive (Fig. 5D). In the case of TLR7, neither BB-loop mutant drove NF-κB or IFN-β activity in response to imidazoquinolines, whereas wild-type receptor signaled normally (Fig. 5E, 5F). These results demonstrate that a BB-loop alanine is not sufficient to confer TRIF-directed signaling to MyD88-dependent TLRs.

Discussion

The activity of human TLR3 is required for protection against encephalitis caused by HSV-1, and although not essential, probably participates in host defense against viral infection in other tissues (19, 20). Activation of TLR3 by viral nucleic acid leads to the production of IFN-β and -λ, which inhibit viral replication. The antiviral functions of TLR3 are mediated by IRFs that require
TRIF for activation. In this article, we report the remarkable finding that the specificity of TLR3 for TRIF depends critically on a single residue A795 in the TIR domain “BB-loop.” Interestingly, IRF-5, which is not a target of the TLR3 signaling pathway (15), was activated by the A795P mutant, a response that is also observed in MyD88-dependent signaling by TLR7 and TLR8. We also show that the mutant receptor associates with TRIF and MyD88 constitutively, either directly or perhaps as part of detergent-resistant membrane microdomains (21). Treatment with the ligand poly(I:C) enhances the binding of TRIF to wild-type TLR3, and MyD88 to the A795P mutant receptor, providing further evidence that this mutation alters adaptor specificity. Furthermore, the activation of NF-κB by the A795P receptor was found to be dependent on MyD88 and largely independent of TRIF. The partial inhibition observed in NF-κB activity induced by the A795P mutant in cells targeted with TRIF siRNAs (Fig. 3E) suggests that this receptor may retain the ability to

**FIGURE 3.** TLR3 A795P signaling response is not TRIF dependent. (A) HEK293T cells were transfected with 3 μg total DNA containing 1 μg full-length Myc-tagged TRIF with either 2.0 μg Flag-TLR3 (lanes 1, 2), Flag-TLR3 A795P (lanes 3, 4), or Flag-TLR3 A795H (lanes 5, 6) in the presence or absence of poly(I:C) (100 μg/ml) for 30 min. Results are representative of three independent experiments. (B) Densitometry analysis for immunoprecipitated Western band intensities. (C-F) Two different TRIF-targeted siRNAs or NT siRNA at 10 nM were transfected into HEK293 cells and left for 48 h. (C) After siRNA transfection, cell lysates were probed with anti-TRIF Ab for endogenous TRIF detection. (D) Total RNA was extracted, and relative expression levels of TRIF were determined by qPCR. (E and F) HEK293 cells were transfected with empty vector or expression plasmid for TLR3, TLR3 A795P, or TLR3 A795H mutants (10 ng) with the NF-κB (10 ng) or IFN-β (25 ng) promoter reporter. After 24 h, cells were stimulated with 20 (E) or 200 μg/ml (F) poly(I:C) for 8 h followed by harvesting of the cell lysates and assessment of luciferase reporter gene activity. **p < 0.001.
activate NF-κB by the TRIF pathway, or that the presence of TRIF enhances the MyD88-directed response. The inability of TLR3 A795H to complex with TRIF confirms another study in which this mutant was unable to recruit TRIF, highlighting the BB-loop of TLR3 as a critical determinant for TRIF–TIR interaction (13). In addition to alterations in TIR adaptor specificity, the A795P receptor, but not the wild-type, recruits the downstream signal transducer TRAF6, further evidence of a switch to MyD88-directed signaling. Previous studies showed that TRAF6 is essential for MyD88 signaling in murine macrophages, but not for TLR3 signaling. In contrast, the related signal transducer TRAF3, but not TRAF6, is essential for the

**FIGURE 4.** TLR3 A795P suppresses TBK-1 but enhances TRAF6 recruitment. HEK293T were transfected with 3 μg total DNA containing either (A) 0.5 μg full-length Myc-tagged RIP-1 and Myc-TRIF, (C) 0.5 μg Myc-tagged TBK-1 and Myc-TRIF, (E) 0.5 μg Myc-tagged TRAF6 and Myc-TRIF, (G) 0.2 μg Myc-tagged Mal, or (H) 1 μg Myc-tagged TRAM, with either 2.0 μg Flag-TLR3 (lanes 1, 2), Flag-TLR3 A795P (lanes 3, 4), or Flag-TLR3 A795H [lanes 5, 6 for Mal and TRAM (G, H) only] in the presence or absence of poly(I:C) (100 μg/ml) for 30 min. Results are representative of three independent experiments. (B, D, and F) Quantitation by densitometry.
induction of type 1 IFNs and the anti-inflammatory cytokine IL-10 (5, 7). Our finding that MyD88 binds constitutively to TLR3 indicates that adaptor usage by the TLRs is more promiscuous than previously thought. In this regard, recent reports have provided evidence that Mal and MyD88 have regulatory roles in normal TLR3 signaling (17, 22). In cells lacking MyD88, TRIF–TLR3 activation of IFN-β is significantly enhanced, although TRAM-TRIF signaling by TLR4 is suppressed (22). Thus, the repressive function of MyD88 is specific for TLR3, and our finding that MyD88 is constitutively associated with TLR3 suggests that this is dominant negative effect acts at the level of adaptor recruitment. Another study revealed that the absence of Mal also enhances signaling by TLR3, consistent with Mal serving as a bridging function to bring MyD88 to the membrane (23), although in this study we could not detect a direct interaction of Mal with TLR3. A possible mediator of TLR3 regulation by Mal/MyD88 is IRAK-2. IRAK-2 can associate with MyD88 and TRAF6 to activate the transcription factor NF-κB (24, 25) and also indirectly associates with TLR3 (26). Thus, formation of TLR3–TRIF complexes may allow for recruitment of TRAF6, which then forms a complex with IRAK-2 and MyD88, and signals to NF-κB. In the case of the A795P mutant, a conformational change in TRIF might inhibit TBK-1 binding and allow for enhanced TRAF6 recruitment, which, in turn, recruits more IRAK-2 and MyD88 resulting in a strong upregulation of NF-κB activity. However, our finding that TRIF is not required for NF-κB activation by the A795P receptor argues against such an indirect model.

Different stoichiometric combinations of receptors and adaptors cluster into membrane microdomains and regulate a specific signaling response against a particular pathogen (21). Therefore, our results may suggest that in the absence of stimulus, TLR3 is primed in microdomains with MyD88, TRIF, or both adaptors, and that binding of ligand leads to the formation of an active signalosome with TRIF and wild-type TLR3, or with MyD88 in the case of the A795P mutant receptor.

Currently, the molecular mechanisms by which postreceptor complexes form during TLR signaling are unclear. The variability of different receptor and adaptor TIR surfaces and potential electrostatic complementarity may underlie the functional specificity of different modules in the TLR signaling response (27). In this regard, we describe BB-loop mutants of TLR2 and TLR5 that exhibit similar upregulation of NF-κB to that seen with the TLR3 A795P mutant. It may be that the adaptor molecules bound to these mutants undergo a conformational change that favors enhanced recruitment of downstream molecules. Conversely, the mutation may also inhibit or restrict further recruitment of other adaptor molecules that negatively regulate NF-κB. Because both TLR2 and TLR5 do not signal from endosomes, it may be that altering their BB-loop to an alanine is not sufficient for it to switch adaptor specificity with TRIF. By contrast, in the case of endosomal TLR7, both mutations within the BB-loop disrupted NF-κB signaling. However, the P935A mutant also inhibited IFN-β activity, suggesting that switching to TRIF may require more than just a single amino acid substitution. Crystal structures of several isolated TIR domains are known, but there are no structures of receptor–adaptor TIR complexes (28). However, modeling studies suggest that ligand binding to the receptor ectodomains causes dimerization of the TIR domains in a symmetrical arrangement (29–31). The receptor BB-loops are predicted to form...
a critical part of this homodimerization interface, a conclusion that is supported by site-directed mutagenesis and studies with cell-permeable peptide inhibitors. Comparison of the crystal structures of TLR2 TIR and the inactive mutant P681H (32) shows that although the BB-loops adopt a similar overall conformation (Supplemental Fig. 2), the histidine residue protrudes from the loop in a way that the proline cannot because of geometrical constraints. It seems likely that this either prevents dimerization of the mutant or forms a scaffold that is unable to support the binding of adaptors. By contrast, it is likely that substitution of proline for alanine would not cause such a steric hindrance of dimerization or binding interfaces but could confer a different adaptor specificity or preference. It is interesting to note that the Drosophila Toll receptors also have a hydrophobic residue at this position, usually valine.

In view of the functional consequences of the proline substitution in the human TLR3, we carried out a phylogenetic analysis to discover whether Ala295 is conserved across species. As shown in Supplemental Fig. 3, alanine is found at this position in TLR3 of all vertebrates with the exception of fish, which have proline. Fish TLR3 is also activated by dsRNA, and this leads to the production of IFNs and the activation of NF-κB (33). In contrast with mammals, the BB-loop of the fish TRIF adapter has alanine, and mutation of this residue to proline inactivates signaling to both IFNβ and NF-κB, suggesting that reciprocal mutations in the receptor and adaptor TIRs can complement each other (34). Fishes have a paralog of TLR3, TLR22, which is also involved in antiviral responses mediated through TRIF (35). TLR22 has proline in the BB-loop and like TLR3 detects dsRNA (36). It may be that TLR22 is specialized to recognize dsRNA at the cell surface. Mammals may have lost this TLR when they began to live on land and it became dispensable because of redundancy (37).

In conclusion, this study shows that a single residue in the BB-loops of TIR domains plays a critical role in adaptor selectivity. Structural studies of complex formation by TLR3 with TRIF and other TLR–adapter interactions will provide a detailed understanding of the molecular topology that governs downstream signaling responses. The study emphasizes the importance of TIR–TIR interactions for inflammatory signaling processes and that BB-loop-mediated protein–protein interactions are important targets for the development of new anti-inflammatory drugs.

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Disclosures

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