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Different functional role of domain boundaries of Toll-like receptor 4

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ABSTRACT

Toll-like receptors (TLRs) recognize molecules representing danger signals via their ectodomain, while signal transduction is provided by the cytosolic TIR domain that recruits adapter proteins upon dimerization. Since in crystal structures both domains dimerize as rigid bodies, any structural adjustment must be provided by the intermediate segments between the domains. We investigated domain coupling by inserting flexible linkers between the structural domains of TLR4. Insertion of linkers between the transmembrane and cytosolic TIR domain did not affect activation, indicating that TIR domain dimerization is triggered by proximity. In contrast, insertion of a linker between the transmembrane and ectodomain or within the ectodomain decreased activation proportionally with the length of the linker. This suggests the requirement for tight coupling of the ectodomain to the membrane, which may facilitate its interaction with ligand, promote dimerization and prevent interaction with the cell–membrane surface. Native linker sizes of TLR4 orthologs support these conclusions.

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Introduction

Leucine-rich repeats (LRRs) represent a ubiquitous structural motif often used by receptors which activate protection from microbial infections [1,2]. Segments with LRRs provide the structural variability for recognition in the immune system of jawless vertebrates [3], they are also present in plant antiviral defense R-genes [4] and in cytosolic receptors of innate immunity, such as NLRs (nucleotide-binding domain and LRR containing family) [5]. Toll-like receptors (TLRs) represent a family of membrane anchored receptors containing LRRs, which are expressed on the cell surface of immune cells [6]. TLRs provide recognition of a wide variety of microbial and endogenous danger signals. The ectodomains of TLRs form a horseshoe-like shape [7] with cysteine-rich capping structures at the amino and carboxyl termini of each LRR block [8]. Conserved microbe-associated molecular patterns recognized by TLRs include bacterial lipopolysaccharide (LPS), peptidoglycan, lipopeptides, lipoteichoic acids, flagellin, bacterial and viral nucleic acids and others [9–12]. Binding of ligands to TLRs triggers complex downstream signaling, ultimately leading to the expression of proinflammatory genes [13]. In mammals, cell activation by bacterial LPS, a major component of the cell wall of Gram-negative bacteria, occurs via TLR4 [14]. TLR4 does not bind LPS directly but through an extracellular glycoprotein MD-2, which is bound to its ectodomain [15–18]. Activation of TLRs requires a process of ligand-induced dimerization. Recent crystal structures demonstrate that the extracellular domains remain rigid upon dimerization [19,20] and cytosolic TIR domains also associate without major structural rearrangement [21–23]. Therefore, the formation of a dimer allows structural adaptation only at the borders between the structured domains.

The crystal structure of TLR4 in complex with MD-2 and bound antagonist has been determined, and it showed that the TLR4 ectodomain consists of 22 LRRs with additional N- and C-terminal LRRs that are more tightly constrained through disulfides [24]. The TLR1/TLR2 ectodomains form a dimer crosslinked by triacylated lipopeptide and with additional interactions between ectodomains [25]. In the crystal structure of TLR3 in complex with the activating RNA duplex, additional interactions exist between the TLR3 ectodomains at the C-terminal segment, which is next to the membrane [26].

A transmembrane anchor separates the ectodomain LRR-containing segment from the intracellular signal transducing TIR domain. Our aim was to elucidate the role of the interface between protein domains of TLRs close to the cell membrane. We surmised that linkers between the structured domains of the hTLR4 molecule are important for its function and that insertions which increase the flexibility between domains would affect signaling of the receptor. We engineered flexible hydrophilic inserts at different juxtamembrane domain interfaces and examined their effects in a cell-based assay for TLR4 activation of downstream reporter expression. Results show that the domain interface on the extracellular side of the transmembrane segment has a significantly different role than on the cytosolic side, which allows us to propose a

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model to explain its significance for the mechanism of receptor activation.

Materials and methods

HEK293 cell activation assay. HEK293 cells were seeded in DMEM (Invitrogen, San Diego, USA) with 10% FBS (BioWhittaker, Walkersville, MD) in 96-well plates at 5×10^4 cells/well and incubated overnight in 5% CO_2 at 37 °C. Cells were then cotransfected for 4 h with pFLAG-CMV1-TLR4 and pEFBOS-huMD-2-FLAG-His together with NF-κB-dependant firefly luciferase and constitutive *Renilla* reporter plasmids using Lipofectamine 2000 (Invitrogen). The following day, cells were stimulated with S-LPS for 16 h. Cells were lysed in reporter assay lysis buffer (Promega) and analyzed for reporter gene activities using a dual-luciferase reporter assay system on a Mithras LB940 luminometer. Relative luciferase activity (RLA) was calculated by normalizing each sample's luciferase activity for constitutive *Renilla* activity measured within the same sample.

Site-directed mutagenesis. All mutations were introduced into a pFLAG-CMV1-TLR4 plasmid using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Several clones that resulted from the mutational procedure were sequenced to confirm the mutation. Primer sequences are available upon request.

Immunoblotting. HEK293T cells were transiently transfected with wt or mutant TLR4 using GeneJuice (Novagen) as a transfection reagent. Protein samples were resolved on 10% SDS–PAGE and transferred to nitrocellulose. The Flag epitope was detected with rabbit anti-Flag polyclonal antibodies (Sigma), followed by goat anti-rabbit IgG-HRP (Bio-Rad). Immunoblots were developed by chemiluminescence.

Molecular modeling for domain annotation. The ectodomain of TLR4 was modeled by splicing the segments of determined structures of the TLR4 ectodomain [24] using the SwissPDBView program. The model of TIR domains of TLR4 orthologs was selected from MODBASE [27] and used to determine the size of the cytosolic linker.

Results

Insertion of peptide linker between the transmembrane and cytosolic TIR domain of TLR4

We engineered flexible inserts between 2 and 10 residues long which increase flexibility between the studied protein domains on both sides of the membrane as shown schematically in Fig. 1. The TIR domain is linked to the cytosolic side of the transmembrane (TM) segment by the native linker that comprises, according to the domain annotation based on fold recognition, 11 residues in orthologs of TLR4. We expected that its extension might affect receptor activation by decreasing the amount of dimer formed by ectodomain crosslinking. The TIR domain dimer forms the scaffold for the assembly of adapters with TIR domains, such as Mal/TIRAP and MyD88 [28]. Our results showed that mutants with inserted linkers between the transmembrane and TIR domain (TM-TIR mu-

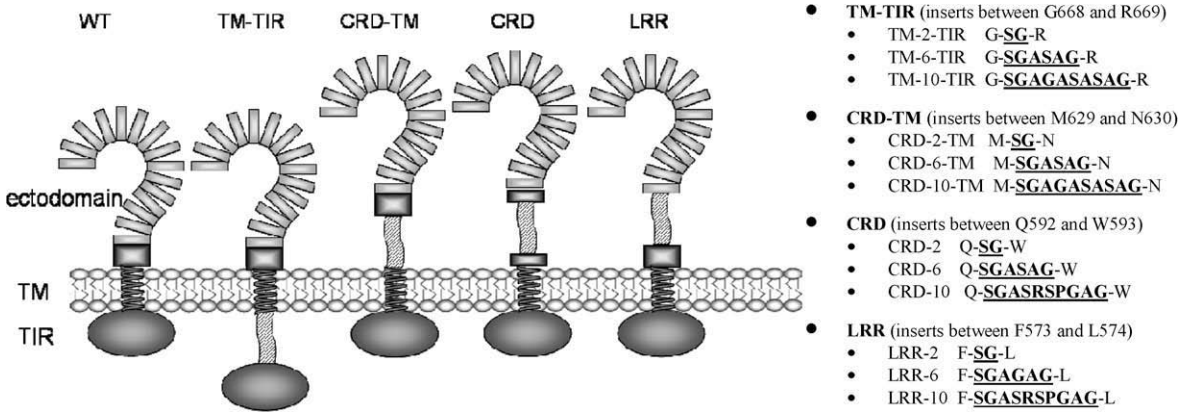

Fig. 1. Schematic representation of the hTLR4 insertion mutants. The blocks represent leucine-rich repeats (LRRs) of the ectodomain, with the larger block representing the "cysteine-rich domain" (CRD). The peptide linker insert is shown as a striped wavy form. TM, transmembrane domain; TIR, Toll/interleukin-1 receptor domain. Right, list of prepared mutants with inserts between domains of TLR4.

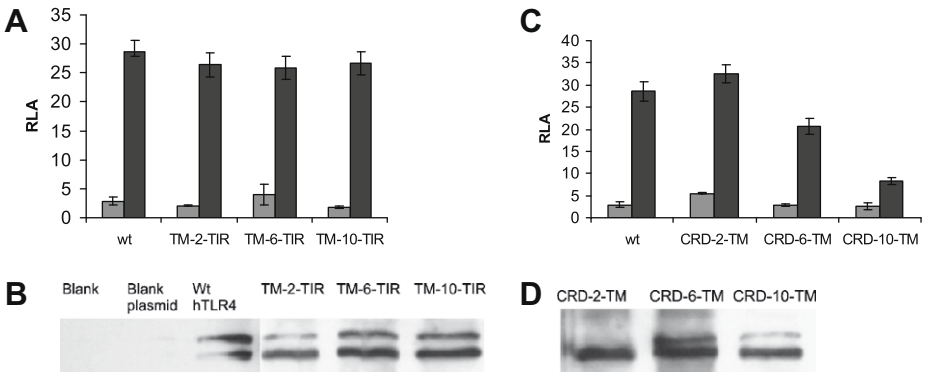

Fig. 2. Insertion of flexible linkers between the TM and TIR domain has no effect, while insertion between the TM and ectodomain impedes activation. (A,C) NF-κB activation of TM–TIR and CRD-TM TLR4 mutants in transfected HEK293 cells stimulated by 100 ng/ml of LPS (light bars – non stimulated samples). The results shown are representative of three experiments with similar results. (B,D) Western blot analysis of the expression of wtTLR4, TM–TIR and CRD-TM mutants.

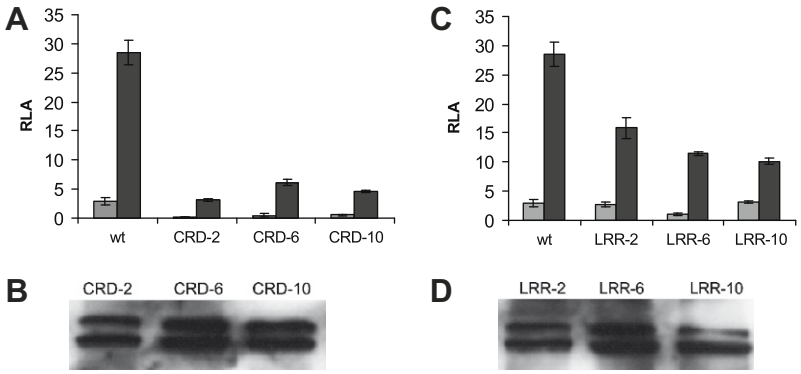

Fig. 3. Insertion of flexible linkers within the "cysteine-rich domain" (CRD) results in complete loss of activation and inserts upstream to the (CRD) inhibit activation proportionally to the insert length. (A,C) NF-κB activation of CRD and LRR mutants of TLR4 (light bars – non stimulated samples; dark bars – stimulated with LPS). The results shown are representative of three experiments with similar results. (B,D) Western blot analysis of the expression of CRD and LRR mutants.

tants) were expressed in HEK293 cells at comparable amounts as wtTLR4 (Fig. 2B). However, insertion of the linkers between 2 and 10 residues long had no effect on the ability of TLR4 to trigger LPS-induced cell activation (Fig. 2A), indicating that activation does not require tightly constrained orientation of TIR domains.

Linker insertion between the transmembrane segment and TLR4 ectodomain

The second group of insertion mutants was targeted into the border between the ectodomain and transmembrane segment (CRD-TM mutants). The amount of produced mutant protein was again comparable to the wild type (Fig. 2D). In this case we could, however, see a strong dependence of TLR4 activity on the length of the linker (Fig. 2C). The activity of the mutant decreased with increasing insert size and the mutant with an insertion of 10 residues retained less than 30% activity of wild type TLR4 responsiveness to LPS. This result indicates that, in contrast to the cytosolic side, flexibility is not tolerated at the border between the ectodomain and transmembrane segment.

Linker insertion into the C-terminal segment of the TLR4 ectodomain

Peptide linkers were also inserted within two positions of the C-terminal segment of the TLR4 ectodomain. One position was selected between Gln 592 and Trp 593, within the region which con-

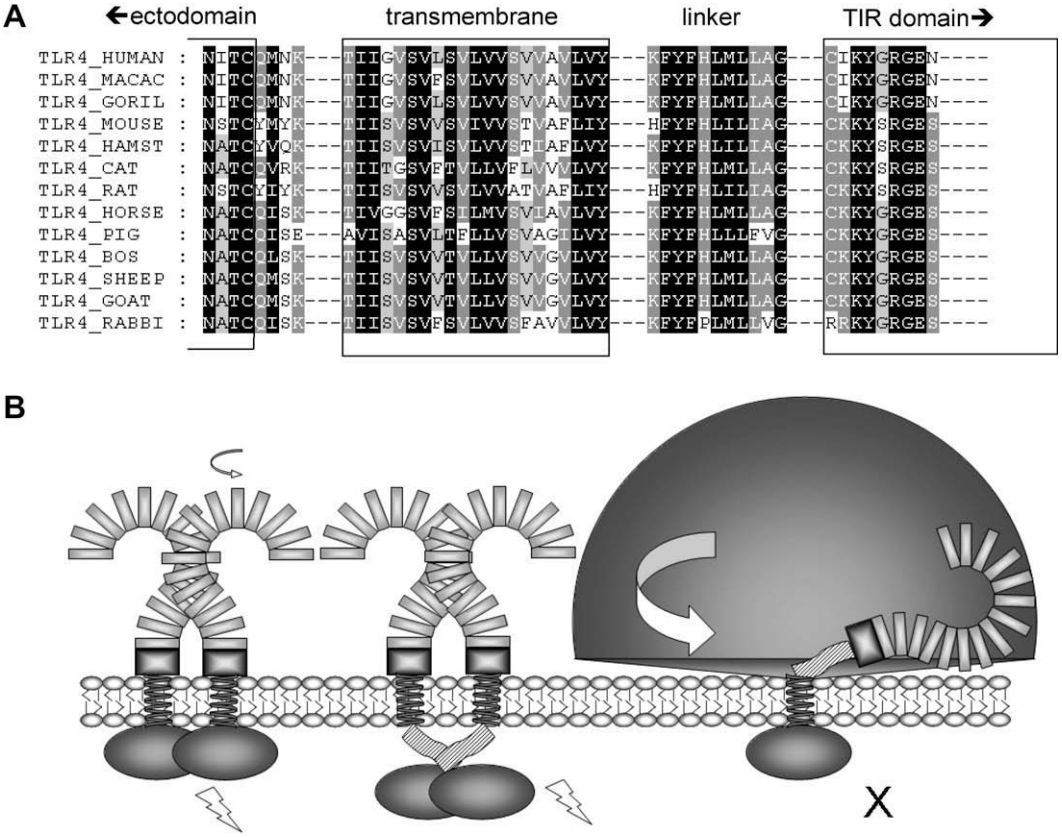

Fig. 4. (A) Alignment of TLR4 orthologs reveal a linker between the transmembrane and TIR domain, while the structured ectodomain extends to the transmembrane segment. The position of folded ectodomain and TIR domain was determined from the crystal structure and structural models [27]. (B) Model of the different effect of a flexible linker at different sides of the transmembrane segment: a flexible linker is tolerated on the cytosolic but not on the extracellular side of the transmembrane segment of TLR4. The linker on the cytosolic side allows TIR domain association. The linker in the ectodomain increases its flexibility, allows the ectodomain to interact with the membrane and hampers binding of ligand and dimerization.

tains four cysteine residues, the "cysteine-rich domain" (CRD mutants), while the second was inserted 20 residues further upstream of the "cysteine-rich domain" between Phe 573 and Leu 574 (LRR mutants). The CRD mutants resulted in almost complete loss of activation regardless of the insert size (Fig. 3A). The probable reason for this loss of activity is that the inserts might have interrupted the correct protein fold. This insert was positioned in the short helix, which is probably the reason why even a short insert has a strong effect. Nevertheless, the amount of produced protein was the same as for the wild type (Fig. 3B). LRR mutants (Fig. 3C and D) caused a reduction of activation proportional to the insert length, similar to the CRD-TM mutants, suggesting that the effect had the same cause.

Discussion

Insensitivity to the cytosolic insertions is in agreement with the suggestion of Núñez Miguel et al. [28], that the linkers between the membrane and the TIR domains have rotational flexibility. This seems plausible as the fairly long linkers (from 11 in TLR4 to more than 20 amino acid residues in other TLRs) contain several glycine residues. One possible explanation for the reduction of activation by extracellular insertions is that residues at the C-terminus of the ectodomain participate in dimer interaction. They have to be properly positioned at the membrane surface in order to form dimers. However, TLR4 ectodomain dimers are also formed in the presence of LPS and MD-2 in solution [24,29] and inserts within the ectodomain N-terminal to the "cysteine-rich domain", also decreased activation. A better explanation is that a rigid supposition of the ectodomain to the membrane results in positioning of the ectodomain in an erect arrangement relative to the membrane, "prearranged" for ligand binding and dimerization. Insertion of a flexible linker allows bending of the ectodomain, increases the sphere of accessible space and allows the ectodomain to interact with the membrane surface (Fig. 4B). Alignment of TLR4 orthologs around the transmembrane segment demonstrates that the structured ectodomain of TLR4 species variants extends all the way to the transmembrane segment (Fig. 4A). This is evident from the position of the cysteine residue, Cys627 in the case of human TLR4, which is disulfide bonded to Cys585 and participates in the well-defined fold of the ectodomain. In contrast, the polypeptide chain of TLR4 in the cytoplasm contains a linker between the transmembrane segment and TIR domain, whose position was predicted by structural modeling based on other TIR domains with determined crystal structure [27]. The same arrangement, i.e., tight supposition of ectodomain to the transmembrane segment and linker of more than 10 residues on the cytosolic side, is also present in other TLRs, where the linker is typically even longer than in TLR4.

We conclude that activation of TLR4, and likely also other TLRs, requires a tight coupling of the ectodomain to the membrane anchor. It has been previously shown that many different types of ectodomain crosslinking, regardless of the specific extracellular arrangement, can lead to activation of TLRs, either by replacement of the ectodomain with CD4 [30], replacement by pairs of integrin domains [31] or crosslinking with antibodies [32]. Therefore, the cytosolic domain requires only that two receptor molecules are brought in sufficient proximity to increase the local concentration of TIR domains and allow formation of a dimeric scaffold for recruitment of adaptors (Fig. 4B). The ectodomain, on the other hand, has to be positioned in the erect arrangement, which is achieved by rigid coupling to the membrane.

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