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Targeting TLR4 Signaling by TLR4 Toll/IL-1 Receptor Domain-Derived Decoy Peptides: Identification of the TLR4 Toll/IL-1 Receptor Domain Dimerization Interface

Vladimir Y. Toshchakov,* Henryk Szmacinski,† Leah A. Couture,* Joseph R. Lakowicz,† and Stefanie N. Vogel*

Agonist-induced dimerization of TLR4 Toll/IL-1R (TIR) domains initiates intracellular signaling. Therefore, identification of the TLR4–TIR dimerization interface is one key to the rational design of therapeutics that block TLR4 signaling. A library of cell-permeating decoy peptides, each of which represents a nonfragmented patch of the TLR4 TIR surface, was designed such that the peptides entirely encompass the TLR4 TIR surface. Each peptide was synthesized in tandem with a cell-permeating Antennapedia homeodomain sequence and tested for the ability to inhibit early cytokine mRNA expression and MAPK activation in LPS-stimulated primary murine macrophages. Five peptides—4R1, 4R3, 4BB, 4R9, and 4cE—potently inhibited all manifestations of TLR4, but not TLR2 signaling. When tested for their ability to bind directly to TLR4 TIR by Förster resonance energy transfer using time-resolved fluorescence spectroscopy, Bodipy-TMR-X–labeled 4R1, 4BB, and 4cE quenched fluorescence of TLR4-Cerulean expressed in HeLa or HEK293T cells, whereas 4R3 was partially active, and 4R9 was least active. These findings suggest that the area between the BB loop of TLR4 and its fifth helical region mediates TLR4 TIR dimerization. Moreover, our data provide direct evidence for the utility of the decoy peptide approach, in which peptides representing various surface-exposed segments of a protein are initially probed for the ability to inhibit protein function, and then their specific targets are identified by Förster resonance energy transfer to define recognition sites in signaling proteins that may be targeted therapeutically to disrupt functional transient protein interactions. The Journal of Immunology, 2011, 186: 4819–4827.

Toll-like receptors sense conserved microbial and endogenous danger molecules to elicit a primary innate immune response (1). Each TLR is comprised of an extracellular domain with multiple leucine-rich repeats, a single transmembrane helix, and an intracellular region (2, 3). The intracellular region of a TLR is ~150 aa long and is comprised largely of a Toll/IL-1R (TIR) resistance domain. TIR domains, also present in TLR adapters, are typical of interaction domains that function to mediate transient interactions of signaling proteins to enable signal transduction (4, 5). Multiple interactions of TIR domains of TLRs and their adapters are pivotal in the early stages of TLR signaling complex formation (reviewed in Refs. 5, 6). Interaction of a TLR agonist with cognate TLR ectodomains induces TIR domains of two TLRs to come into direct physical contact to dimerize. The TLR TIR dimer serves as a nucleating center for the recruitment of TIR domain-containing TLR adapters. Posttranslational modifications of interacting proteins apparently do not play a role in the earliest stages of TLR complex assembly (i.e., receptor TIR dimerization and initial stabilization of the dimer by adapter recruitment), as neither TLRs nor TIR-containing TLR adapters possess intrinsic enzymatic activity. Adapter recruitment is achieved through a cooperative interaction in which the TIR of the recruited adapter binds two (or more) TIRs simultaneously within the initial complex (5, 7), thus enlarging and stabilizing it. Although TIR domains demonstrate a strong preference for establishing interactions within the structural family, functional TIR–TIR interactions are specific, as indicated by the observations that: 1) specific pairs of TLR TIR domains either homodimerize or heterodimerize; and 2) specific combinations of TIR-containing adapters are recruited to these dimers and mediate signaling in response to TLR activation (1). Growing evidence indicates that TIR domains interact through topologically diverse surface areas (7–13). Nonetheless, no specific TIR-binding motif has been identified within TIR domains.

TLR4 has as its prototype agonist LPS, a main component of the outer membrane of Gram-negative bacteria. TLR4 is the most complex of TLRs in that four TIR-containing adapter proteins (i.e., MyD88, TIR domain-containing adapter protein [TIRAP], TIR domain-containing adaptor inducing IFN-β [TRIF]–related adaptor molecule [TRAM], and TRIF) participate in signal transduction (reviewed in Ref. 1). TIRAP and TRAM have a simpler, single-domain architecture, whereas MyD88 and TRIF have additional, non-TIR domains that are responsible for recruitment of additional signaling proteins that possess enzymatic activity required for propagation of signal to downstream targets (14).
Despite the fact that the constituents of the TLR4 signaling complex have long been identified, the architecture of the complex, as well as the stoichiometry of components in the complex, continue to remain a matter of debate.

One approach to the identification of protein–protein interfaces in transient signaling complexes is the decoy peptide approach, in which cell-permeable decoy peptides that represent various surface-exposed segments of a signaling protein are examined for the ability to inhibit cellular signaling (6). Inhibition of signaling by a decoy peptide is thought to indicate that the specific region of protein’s surface represented by the decoy is a functionally important protein–protein interface because its ability to signal is presumably competitively inhibited. Previously, we compared several cell-permeable decoy peptides derived from a conserved structural feature, the BB loop, of several TLRs (10) and TLR adapters (9) and found that these BB-loop peptides differ markedly in the ability to inhibit TLR4 and TLR2 signaling. In this study, we sought to use this same approach to map TLR4 TIR regions that serve as protein interfaces by designing a library of decoy peptides that collectively encompass the entire surface of the TLR4 TIR. Each peptide in the library was synthesized in tandem with a cell-permeating peptide sequence and then tested first for its ability to inhibit LPS signaling in primary murine macrophages. Five of 12 peptides strongly inhibited all manifestations of LPS signaling examined, and these were further tested for the ability to bind directly to the TLR4 TIR domain using the Förster resonance energy transfer (FRET) approach. TLR4, labeled with Cerulean fluorescent protein (Cer) at the C terminus (15), was used as an energy donor, whereas peptides labeled with Bodipy-TMR-X (BTX) served as spectrally matching energy acceptors. Direct binding to the TLR4 TIR was observed for several inhibitory peptides tested, as evidenced by the ability of their BTX-labeled analogs to quench the fluorescence of cells that express the TLR4-Cer fusion protein (TLR4-Cer). One of the TLR4-binding peptides, 4R1, also bound to the TLR2-Cer fusion protein (TLR2-Cer); however, 4R1 failed to inhibit TLR2-mediated signaling. Collectively, the TLR4-binding, inhibitory decoy peptides represent a large, contiguous area on the TLR4 TIR surface and thus are likely to comprise the functional dimerization interface of TLR4 TIR. The data presented in this study identify decoy peptides that target TLR4 TIR, thereby providing direct evidence for the mechanism of decoy peptide action, and suggest the position of the functional dimerization interface on the TLR4 TIR.

Materials and Methods

Animals and cell culture and treatment

C57BL/6j mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Harvesting, culturing, and stimulation of peritoneal macrophages were as described in our previous publications (9, 10, 16). The inhibitory activity of various decoy peptides was measured in primary thiglycollate-elicited mouse peritoneal macrophages cultured overnight in RPMI 1640 medium supplemented with 2% FCS. Peptides were added to cells 30 min before LPS stimulation. Protein-free Escherichia coli K235 LPS was used at a final concentration of 10 ng/ml to stimulate peritoneal macrophages. The lipopeptide TLR2 agonists S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-Cys-Ser-Lys4-OH (P3C) and S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys4-OH (P2C) and S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys3-OH (P1C) (EMC Microcollections, Tubingen, Germany) were used at 50 and 500 ng/ml, respectively.

Preparation of slides for fluorescence spectroscopy

HeLa and HEK293T cell lines were used as transfection hosts to measure the binding of peptides to TLR4 TIR domain by FRET. Both cell lines were cultured in 10% FCS DMEM. Slides for fluorescence spectroscopy were prepared somewhat differently for HeLa and HEK293T cells. HeLa cells (3.5–4 × 10⁵/well) were plated in a six-well plate on day 1. HEK293T cells were plated into eight-well Lab-Tek II chamber glass slides (Nunc International, Naperville, IL) (5 × 10⁴ cells/well). The next day, cells were transfected with TLR4-Cer–encoding pcDNA 3.1 Zeo (−) vector using SuperFect transfection reagent per the manufacturer’s recommended protocol (Qiagen). On day 3, the transfected HeLa cells were trypsinized, resuspended, and counted. Seven thousand HeLa cells per well were plated into 3-mm silicon gasket wells mounted on a coverglass (CultureWell; Grace Bio-Labs). Following transfection with TLR4-Cer, HEK293T cells were allowed to rest for 24 h. On day 4, the cells were mock-treated or treated with BTX-labeled decoy peptides (40 μM) or LPS (500 μg/ml) for 1 h. After this incubation period, the gasket was removed and the cells washed with PBS. Cells were fixed with fresh 4% paraformaldehyde in 0.1 M PBS for 5 min, washed with PBS again, and the slides mounted using the DABCO-based antifading fluorescent mounting medium.

TLR4-Cer and TLR2-Cer pcDNA3.1 plasmid description

The pcDNA3.1 vector that encodes hemagglutinin (HA)-tagged murine TLR4 was obtained from DM Assabbi, University of Maryland Baltimore (UMB). The HA tag was removed and replaced by wild-type sequence of murine TLR4 using NheI and NotI sites. The wild-type sequence encoding the N terminus of TLR4 was amplified from murine macrophage cDNA library using primers 5′-tatGCTGACccagatgtcgcctggc-3′ and 5′-ATCCGCAAGCGAATGGAACCT-3′. The vector encoding Cer was a kind gift of Dr. Mark Rizzo, UMB. Cer coding sequence was cloned from the phage cDNA library using modifying primers 5′-tatGCTGACccagatgtcgcctggc-3′ and 5′-tatCCTGAGGagctgagcaaggg-3′ (restriction sites are capitalized) and the right primer 5′-tagatgtaaaCGCCGCTattcgtgatt-3′ designed to conserve a Ncol site already present in the original construct shortly after the stop codon. Cer coding sequence was inserted in the obtained untagged murine TLR4 using Bsa36I and NotI sites. Next, the stop codon of TLR4 (-TGA-) was replaced by the serine triplet (TCA) by introducing a single point mutation using the QuikChange Lightning site-directed mutagenesis kit from Stratagene and primers 5′-cgccacagtgaaggcgcttggaggtg-3′ and 5′-GCTCCACATGCTTGGAGCtTTGGCGG-3′. This mutation created a TLR4-Cer fusion protein in which Cer is attached to the C terminus of TLR4 TIR domain through a short Ser-Gly linker (underlined in the forward mutagenesis primer). Finally, the sequence of TLR4-Cer fusion was recloned into pcDNA3.1 Zeo (−) vector (Invitrogen) using Nhel and NotI sites.

The TLR2 coding sequence was PCR amplified from a mouse macrophage cDNA library using modifying primers 5′-ATATGCTGAGCCACATGCTACGAGCTTTCTT-3′ and 5′-TTACCTGAGGATTTGATCTAGTCTCA-3′. The PCR product obtained was recloned into TLR4-Cer pcDNA3.1 Zeo (−) vector to replace the TLR4 coding sequence using Nhel and Bsa36I sites. All expression vectors were verified by sequencing.

Peptide design and synthesis

Decoy peptides representing different surface-exposed structural elements of murine TLR4 TIR and a previously described control peptide (CP) (9) (Table I) were synthesized in tandem with the cell-permeating sequence derived from the Drosophila Antennapedia homeodomain (RQIKIWIQRMKWK). Peptide synthesis, purification, and verification were performed at the Biopolymer and Genomics Core Facility (UMB). The desired peptides were synthesized on a Prelute peptide synthesizer (PFI Instruments, Boston, MA) using Fmoc coupling strategy.

To synthesize fluorescently labeled peptides, each peptide was derivatized on resin with BTX. 6-[4-(4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propionyl)amino] hexanoic acid, succinimidyl ester [Invitrogen, Carlsbad, CA]. To prepare an N-terminal derivatizing the dye was added to the completed peptide resin (1.6:1 dye/resin) in 100 μM N-Methylmorpholine in N,N-dimethylformamide.

Peptide purification was performed on a Beckman Gold system consisting of two 110B pumps and a 166 detector (215 nm) using a Dynamax reverse-phase C18 column (8 × 25.6 mm × 250 mm) (Varian, Walnut Creek, CA). Peptide verification was performed using a Beckman System 32 Karat Gold containing a model 126 pump with a Beckman 168 diode array detector (215 and 540 nm) on a Beckman reverse-phase C18 column (ODS, 5 μ, 4.6 × 250 mm). Peptide mass was verified by MALDI-TOF measurements carried out on a Waters Analytical AxyPrep Gold C18 (7 μm 90A) using a saturated solution of α-cyano-4-hydroxycinnamic acid in water/aceto-nitrile (1:1) containing 0.5% trifluoroacetic acid.

Evaluation of TLR4 and TLR2 signaling in primary macrophages

Methods used for isolation and quantification of cytokine mRNA and Western analysis procedures are provided in our prior publications (9, 10). Phosphorylation of ERK and JNK MAPKs was detected using the
Fluorescence spectroscopy

To detect energy transfer, we measured the effects of BTX-labeled decoy peptides on TLR4-Cer and TLR2-Cer fluorescence lifetime. Time-resolved measurements were carried out using frequency domain fluorometry using the K2 fluorometer (ISS, Champaign, IL) attached to the fluorescence microscope (Axiovert TV130, Zeiss); intensity decays were measured by collecting phase-modulation data over the modulation frequency range 5–300 MHz. Cer fluorescence was excited using the blue light-emitting diode combined with the excitation band pass filter 426–446 nm and detected through the 480/40 emission band pass filter. Frequency-domain fluorometry and intensity decay analysis was performed as described in detail elsewhere (17). Briefly, intensity decays of Cer fluorescence were preliminary analyzed using the multiexponential intensity decay model:

\[ I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i), \]

where \( \tau_i \) is the decay time and \( \alpha_i \) is the amplitude of a component. The FRET efficiency (\( E \)) was calculated from the average lifetimes of the donor as:

\[ E = 1 - \frac{\langle t_{DA} \rangle}{\langle t_D \rangle} \]

where the average lifetimes are amplitude weighted lifetimes given by the sum of the \( \alpha_i \tau_i \) products. The lifetimes were calculated for each treatment in the absence of acceptor (\( \langle t_D \rangle \)), and in the presence of acceptor (\( \langle t_{DA} \rangle \)). The three-exponential model was used to calculate the average lifetimes. These average lifetimes are proportional to the steady-state intensities.

Prior to detection of FRET, both expression of Cer-labeled TLRs as well as the efficient permeation of each labeled decoy peptide into cells was verified using the inverted Nikon Eclipse TE2000-S microscope (Nikon) equipped with the epi-fluorescence attachment and a mercury lamp illuminator.

Modeling studies

Structural models of mouse TLR4 TIR domain were built, compared, and verified using the Swiss-Model Workspace homology modeling server and the DeepView program (Swiss-ProtViewer) (18, 19). We also used a structural model of murine TLR4 deposited in the ModBase repository (20) to evaluate the obtained structures. Modeling of TLR4 TIR dimers was performed using the GRAMM-X server (21).

Results

Design of TLR4 TIR-specific library of decoy peptides: identification of inhibitory peptides

A receptor TIR domain is predicted to have more than one functional interface (e.g., a receptor dimerization interface and at least one adapter-interacting surface). Our prior work demonstrated that the decoy peptide derived from the BB loop of TLR4 TIR, 4BB, potently inhibited LPS signaling (10). Based on the rationale for the existence of multiple interfaces in a single TIR domain, we hypothesized that additional TLR4 TIR-derived peptides would inhibit LPS signaling. To test this hypothesis, we designed a library of decoy peptides, with each peptide in the library being a surface-exposed segment of the TLR4 TIR primary sequence. Analysis of TIR domain structures available in the Protein Data Bank (http://www.rcsb.org/pdb) suggested that a set of 11 decoy peptides of approximately equal length is sufficient to represent the entire TIR surface. Table I provides the sequences of TLR4 TIR-derived decoy peptides and indicates structural regions they represent. The cell-permeating sequence of Antennapedia homeodomain was placed at the peptide’s N terminus, and each cell-permeating peptide was examined for the ability to inhibit LPS signaling in primary murine macrophages. The data in Fig. 1 confirmed our initial hypothesis that multiple peptides would inhibit TLR4 signaling. Five of the 11 peptides strongly inhibited all manifestations of TLR signaling examined. Peptide 4R1 (Region 1

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
<th>Structural Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>4R1</td>
<td>AGCKYSRGESIYD</td>
<td>Linker to the transmembrane region</td>
</tr>
<tr>
<td>4R2</td>
<td>SGNEDMVRHELKVN</td>
<td>AA* and aA</td>
</tr>
<tr>
<td>4R3</td>
<td>EBGVPPFHLCAA</td>
<td>AB and BB</td>
</tr>
<tr>
<td>4R4</td>
<td>LHYRDF1PGAV1AA</td>
<td>BB, BB, and oB</td>
</tr>
<tr>
<td>4R5</td>
<td>NIQEGFHSRKSV</td>
<td>oB and BC</td>
</tr>
<tr>
<td>4R6</td>
<td>RHFIQSNCIFPEYE</td>
<td>CC and oC</td>
</tr>
<tr>
<td>4R7</td>
<td>IAQTWQFLSRGSI</td>
<td>CD</td>
</tr>
<tr>
<td>4R8</td>
<td>VLVERKSL</td>
<td>DD</td>
</tr>
<tr>
<td>4R9</td>
<td>LRQVHEILRLSR</td>
<td>oD</td>
</tr>
<tr>
<td>4R10</td>
<td>NTYLEWDENPGLGR</td>
<td>DE, BB, and EE</td>
</tr>
<tr>
<td>4E</td>
<td>HIFVRLEGNALDD</td>
<td>aE</td>
</tr>
<tr>
<td>4R12</td>
<td>ASNPQETAEEQET</td>
<td>C-terminal tail</td>
</tr>
<tr>
<td>CP</td>
<td>SLHGRGDPEAFII</td>
<td>Randomized sequence</td>
</tr>
</tbody>
</table>

*Structural regions of TIR domain are designated as follows: helices are designated by Greek \( \alpha \) (e.g., \( \alpha A \) is helix A); strands are indicated by Greek \( \beta \) and loops are indicated by two capital letters (e.g., AB is the loop that connects helix A and strand B).

The Cer-BTX FRET system for detection of direct TIR–peptide binding in cells

A typical FRET system for studying the interaction of two proteins in a cellular system is comprised of these two proteins genetically phosphorylated Abs from Cell Signaling Technology. Anti-actin Ab was from Santa Cruz Biotechnology.
labeled with an enhanced variant of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) that serve as energy donor and acceptor, respectively. We engineered murine TLR4 C-terminally fused with Cerulean (Cer) fluorescent protein (15) (TLR4-Cer) to use as the energy donor. Labeling a peptide with a fluorescent protein, however, would be impractical, as such a large label is likely to affect peptide permeability and/or binding. Therefore, we used a fluorescent dye, BTX, as a peptide-associated energy acceptor. The Förster distance, \( R_0 \), is the donor-acceptor distance at which the energy transfer is 50% efficient. This parameter is often used to predict the FRET performance of a particular pair of fluorophores. \( R_0 \) calculated for the Cer/BTX pair based on donor-acceptor spectral overlap, donor quantum yield, and acceptor extinction, and assuming the random donor-acceptor orientation is 5.89 nm, whereas \( R_0 \) similarly estimated for the CFP/YFP pair is 5.21 nm. Therefore, the Cer/BTX FRET system comprised of a Cer-tagged protein and a BTX-labeled decoy peptide is predicted to yield better FRET performance than in the case of the classical CFP/YFP pair. In addition to these advantageous spectral properties, the choice of BTX as the energy acceptor for this FRET system is based on electroneutrality of this fluorophore, as an uncharged dye is less likely to affect peptide cell permeability.

We first confirmed that transfection of cells with TLR4-Cer or incubation of cells in the presence of BTX-labeled peptide for 30 min prior to stimulation with LPS (10 ng/ml) (A, B). TLR2 agonists P2C and P3C were used at 50 and 500 ng/ml, respectively. Cytokine mRNA expression was measured 1 h after stimulation with a TLR agonist and is normalized to the expression of Hprt gene. Protein extracts for Western analysis were obtained 30 min after LPS or P3C stimulation. After stimulation with P2C, ERK activation was detected 15 min after addition of the agonist. Data in A show means and SEM of six independent experiments; the Western blot shown in B is representative of four separate experiments. Data presented in C and D were obtained from four independent experiments.

Occurrence of FRET decreases donor fluorescence lifetime (17). Therefore, measurement of TLR4-Cer fluorescence lifetime can be used to detect donor/acceptor binding. TLR4-Cer was expressed in cells, and the time-resolved frequency-domain measurement of TLR4-Cer fluorescence lifetime was carried out. Similar average lifetimes were observed whether the protein was expressed in HeLa or HEK293T cells (i.e., 1.77 ± 0.12 ns and 1.67 ± 0.15 ns, respectively).

To validate this FRET measurement system, we investigated the effects of TLR4 agonist LPS on the TLR4-Cer fluorescence lifetime. This experiment is based on the rationale that fluorophores with significantly overlapping absorption and emission spectra can exchange the excited-state energy with an identical molecule, provided that two molecules of the fluorophore, in this case, Cer, are brought into sufficiently close proximity. Such a phenomenon is a particular type of FRET called fluorescence resonance energy mi-
Next, using BTX-4BB and BTX-CP as a donor-binding and nonbinding energy acceptor, respectively, we sought to confirm that the binding acceptor quenches donor fluorescence. TLR4-Cer was expressed in HEK293T cells. Cells were preincubated without a labeled peptide or in the presence of 40 μM BTX-CP or BTX-4BB and frequency-domain TLR4-Cer fluorescence lifetime measurements were carried out by collecting the phase-modulation data in the 5–300 MHz range of excitation modulation frequencies. Fig. 2F shows the effect of BTX-4BB on the TLR4-Cer fluorescence intensity decays. The shorter lifetime of TLR4-Cer fluorescence in the presence of the acceptor is demonstrated by the shift of phase-modulation data toward higher excitation modulation frequencies (Fig. 2F). This shift demonstrates that BTX-4BB decreases the TLR4-Cer fluorescence lifetime and provides confirmatory evidence that the donor and acceptor molecules are in close proximity, and FRET occurs between these molecules. Increasing the BTX-4BB concentration to 100 μM did not induce stronger quenching of TLR4-Cer signal compared with 40 μM (data not shown), thus confirming our functional inhibition data that 40 μM is a saturating concentration of the inhibitor. BTX-CP did not change TLR4-Cer fluorescence lifetime significantly (not shown), thereby indicating that no binding occurs between CP and TLR4-Cer (9, 10 and data not shown).

Effects of TLR4 TIR-derived inhibitory decoy peptides on TLR4-Cer fluorescence lifetime

Having established a FRET technique suitable for detection of peptide–TIR binding in a cellular system, we next sought to identify additional inhibitory peptides that target TLR4. The rationale for this was that, even if 4BB was confirmed to bind TLR4 TIR directly, additional peptides might also target the TLR4 TIR, based on the published modeling of TIR–TIR complexes that suggests that a TIR–TIR interface may be formed by several nonconsecutive segments of TIR primary sequence (7, 8, 12, 24). An additional possibility is that a TIR–TIR interaction may not be isologous [i.e., topologically distinct surfaces may mediate an interaction of two TIRs; for example, the back-to-face model presented previously by Jiang et al. (11) for interactions of TIR domains of TLR2 and MyD88].

Dimerization of receptor TIR domains initiates intracellular TLR signaling. Therefore, peptides that target the receptor dimerization surface would be expected to inhibit all manifestations of TLR signaling. In addition to peptide 4BB, peptides 4R1, 4R3, 4R9, and 4αE similarly inhibited both cytokine induction and MAPK activation (Fig. 1), and these peptides were BTX-labeled at the N terminus and examined for the ability to quench TLR4-Cer fluorescence by time-resolved fluorescence spectroscopy. Fig. 3 shows the average lifetimes of TLR4-Cer expressed in HeLa cells that were incubated in the presence of BTX-labeled peptides and the FRET efficiencies calculated for each donor/acceptor pair. Efficient binding was observed for 4R1 and 4αE; in addition to 4BB, these peptides induced significant quenching of TLR4-Cer fluorescence, with FRET efficiencies being ~40%. Peptide 4R3 and 4R9 did not quench TLR4-Cer fluorescence as potently as 4R1, 4BB, and 4αE; although we observed slightly decreased values of fluorescence lifetime when TLR4-Cer–expressing cells were incubated in the presence of these peptides. The CP did not bind the TLR4 TIR, as evidenced by its failure to quench TLR4-Cer fluorescence (Fig. 3).

To understand more fully the specificity of decoy peptide binding, we also examined if TLR4 TIR-derived peptides bind to the TLR2-Cer fusion protein. We generated the TLR2-Cer fusion protein expression vector, transfected it to HeLa cells, and treated the transfected cells with BTX-labeled TLR4 TIR-derived decoy

FIGURE 2. Cer-BTX FRET system for detection of intracellular TIR–peptide binding. A. A fluorescent image of HeLa cells expressing TLR4-Cer. B. HeLa cells incubated in the presence of BTX-4BB for 1 h. These photomicrographs were taken with an inverted fluorescent microscope using CFP (A) or G-2E/C (B) filter block (Nikon). Cells were fixed with 4% paraformaldehyde 2 d after transfection with TLR4-Cer (A) or after 1 h treatment with BTX-4BB peptide (40 μM) (B). BTX-labeled 4BB inhibits LPS-induced ERK activation (C) and IL-1β mRNA (D). See Fig. 1 for experimental details. E. LPS decreases TLR4-Cer fluorescence lifetime. HeLa cells expressing TLR4-Cer were stimulated with 500 ng/ml LPS for 1 h prior to fixation. Frequency-domain fluorometry was carried out using a K2 fluorometer attached to the fluorescence microscope. In photomicrographs were taken with an inverted fluorescent microscope using CFP (A) or G-2E/C (B) filter block (Nikon). Cells were fixed with 4% paraformaldehyde 2 d after transfection with TLR4-Cer (A) or after 1 h treatment with BTX-4BB peptide (40 μM) (B). BTX-labeled 4BB inhibits LPS-induced ERK activation (C) and IL-1β mRNA (D). See Fig. 1 for experimental details. E. LPS decreases TLR4-Cer fluorescence lifetime. HeLa cells expressing TLR4-Cer were stimulated with 500 ng/ml LPS for 1 h prior to fixation. Frequency-domain fluorometry was carried out using a K2 fluorometer attached to the fluorescence microscope. In

flourescence lifetime. Stimulation of TLR4 with an agonist induces (23) demonstrated EM for Cer and observed that EM decreases Cer migration (EM). Using DNA vectors that encode two Cer molecules tethered by a polypeptide chain of varied length, Koushek and Vogel distance between two Cer moieties of the TLR4 TIR dimer. Our data confirmed this expectation (Fig. 2E). This LPS-induced decrease of TLR4-Cer fluorescence lifetime is evidenced by the fact that the increase in phase shift, and the decrease of fluorescence modulation occurs at higher excitation modulation frequencies. TLR4-Cer was expressed in HEK293T cells preincubated in the absence of the acceptor (black squares) or in the presence of BTX-4BB (40 μM) for 1 h (blue squares). Lines on the graph illustrate the results of fitting of experimental data using the three-exponential model. The FRET efficiency, 0.43 in this experiment, was calculated using Eq. 2 as described in the data using the three-exponential model. The FRET efficiency, 0.43 in this experiment, was calculated using Eq. 2 as described in the

Materials and Methods
peptides. Peptides 4R3, 4BB, 4R9, and 4aE do not bind the TLR2-Cer fusion protein, as evidenced by the fact that they did not affect TLR2-Cer fluorescence significantly (Fig. 3C). Surprisingly, peptide 4R1 quenched TLR2-Cer almost as effectively as it did with respect to TLR4-Cer fluorescence. Although the data obtained suggest that peptides 4BB and 4aE target the TLR4 TIR with specificity, 4R1 appears to bind both TLR4 and TLR2, despite the fact that it failed to inhibit TLR2-mediated signaling (Fig. 1C, 1D). The interaction of 4R1 with TLR2 is unlikely to signify a functionally meaningful TIR–TIR interaction because TLR4 and TLR2 TIRs do not form a functional heterodimer. In general, the high binding selectivity would not be expected for the inhibitory peptides, as these large molecules are low-affinity binders that act effectively in the micromolar concentration range (i.e., to inhibit signaling they need to be present in significant molar access over the amount of their target). Yet, the high relative binding affinity of 4R1 with respect to TLR4, together with the fact that this peptide inhibits TLR4 signaling, suggests that this region, in addition to 4BB and 4aE regions, is important for TLR4 TIR dimerization.

The relative positions of TLR4 TIR-derived, inhibitory peptides are shown in Fig. 4 and illustrate that 4BB, 4R1, and 4aE comprise the large contiguous region of TLR4 TIR surface. The 4R3 region lies adjacent to 4R1 and the other two TLR4-binding peptides, 4BB and 4aE (Fig. 4). Thus, the data presented in this study suggest that the TLR4 TIR surface region formed by 4R1, 4BB, and 4aE peptides, with the additional possible smaller contribution of 4R3, mediates the signaling-initiating TLR4 TIR dimerization. The data also provide the first demonstration, to our knowledge, that TIR-derived decoy peptides do indeed target the TIR domain predicted to establish a functional interaction with a decoy’s prototype protein.

Discussion

Agonist-induced dimerization of TLR TIR domains is the first intracellular event that initiates a complex chain of protein interactions and modifications that ultimately results in background-specific cellular activation (reviewed in Refs. 1, 5, 25). The mechanism by which dimerization is translated into signaling is that the TLR TIR dimer acts as the nucleating center for recruitment of TLR adapter proteins through multiple interactions of TIR domains present in TLRs and TIR adapters. A functionally competent receptor TIR dimer exposes a surface to which an adapter TIR docks so that the recruited TIR interacts with both TIRs of the dimer simultaneously, thereby stabilizing the initial dimer (5–7) (Fig. 5A, 5B). The signal dependency of the TLR signaling complex assembly is achieved through the cooperativity of initial TIR domain interactions that mediate adapter recruitment to the receptor TIR dimer. The cooperative mode of TIR domain interactions is marked by the substantial enlargement of the cumulative contact area between the complex’s subunits due to an additional interface(s) that is (are) established in this cooperative mode. The simplest model of a cooperatively formed trimeric TIR complex, as well as a noncooperative, bridging mode of interaction, is shown in Fig. 5B and 5C. Once assembled, the initial complex composed of TLR TIRs and TIR domain-containing adapters initiates assembly of the Mydosome, a signaling complex composed of MyD88, IL-1R–associated kinase ( IRAK) 2, and IRAK4 that assembles through interactions of death domains present in MyD88 and IRAKs (26, 27).

The structures of several TIR domains have been resolved based on x-ray crystallography or NMR spectroscopy (28); the structure of other TIR domains can be reasonably reliably predicted by current homology modeling tools. Nevertheless, the available knowledge of individual TIR structures has not yet resulted in a commonly accepted model of an oligomeric TIR complex that assembles as a consequence of agonist-induced signaling. Several intrinsic properties of TLR signaling complexes complicate experimental analysis of their structural details. First, these signaling complexes are transient, intrinsically unstable heterocomplexes that are comprised from both the membrane-spanning proteins (i.e., TLRs, and cytosolic proteins, e.g., MyD88). Such complexes are especially difficult to isolate from a cell in an intact state for subsequent investigation. Second, these complexes are also difficult to recreate in an in vitro system using recombinant proteins because these heterocomplexes are composed of more than two different proteins, the stoichiometry of which in the complex is uncertain. Third, methods that permit indirect analysis of protein interfaces in cells, such as mutagenesis at the putative interface site, complemented with analysis of the mutant’s functionality,
The interface area is 2
complexes. The total interface area equals ∼3xs. The bridging mode of interaction in a TIR trimer. Total interface area is 2s. The complex is as stable as the comprising binary complexes. S, average size of TIR–TIR interface area.

Often rely on overexpression of one or two individual proteins, whereas the temporary protein complexes formed in response to TLR stimulation in cells are likely to be a result of cooperative interaction of more than two proteins, where additional proteins play an essential role of stabilizing the initial, relatively weak interaction between the first two (Fig. 5A versus 5B). In addition, ectopic expression of proteins often leads to false-positive results caused by abnormal expression levels. Several examples of controversial findings obtained through overexpression of TIR-containing proteins have been reviewed (6).

Numerous attempts to predict the positions of TIR–TIR interfaces computationally have not revealed a unified topology for a TIR–TIR dimer either (8, 11–13). Moreover, a typical result of an in silico docking experiment is a number of closely scoring TIR–TIR dimer models that differ considerably in interface positions and rotationally, thus leaving a choice of the physiologically relevant TIR dimer conformation to the investigator’s subjective decision (8, 11, 29) (V. Toshchakov, unpublished observations). The failure of computational methods to distinguish a single functional TIR dimer conformation may be attributable to the relatively small size of any binary TIR–TIR interface. A survey of available models of TIR–TIR complexes built based on experimental data or in silico suggests that the size of an interface in a binary TIR–TIR complex typically ranges between 550–700 Å² (5, 7, 11, 30). An atypically large TIR–TIR interface was found upon crystallographic resolution of the TLR10 TIR dimer with a buried area of 974 Å² (24). This is probably as large as a TIR–TIR interface can be, given the geometry of a TIR domain, its small size (3–3.5 nm in diameter), and globular and generally convex shape of a TIR domain. The typical interface size in a binary TIR–TIR complex is less than the average size of interfaces found in transient, nonobligate protein complexes (31, 32).

Pepitides 4R1, 4BB, and 4eE represent adjacent areas of the TLR4 TIR surface (Fig. 4A, 4B). 4R1 is positioned in the center of the interface; 4BB and 4eE lie on the opposite sides of 4R1. It is noteworthy that 4R1 represents a partially structured region of TLR4. Only the C-terminal portion of the homologous region was found structured in TLR2 and TLR1 TIR crystals (35). The N-terminal part of the 4R1 region is the distal portion of the presumably unstructured region that connects the transmembrane helix to the TIR domain. Strong TLR4 inhibitory activity of the 4R1 decoy peptide, together with the fact that it binds to the TLR4 TIR directly, suggests that this region plays an essential role in experimentally or predict computationally; 4) several similarly stable dimer conformations may exist and be functional for each particular binary TIR–TIR interaction; 5) pre-existing spatial and orientational constraints, as well as the presignaling binding state of the proteins, may influence significantly the signaling outcomes; and finally, 6) the complex is assembled through the cooperative mechanism of TIR domain interactions, in which only those binary TIR–TIR interactions are stabilized and in which relative interface positions are geometrically amenable to the cooperative mode of the complex assembly (Fig. 5B versus 5C). Currently available computational algorithms have been developed to predict a binary interaction of two proteins, not a cooperative protein interaction in which more than two proteins are required to form a stable complex.

This report represents a novel, two-tiered approach to identification of areas that mediate protein–protein interactions in signaling complexes. We tested this approach with the goal of identifying the TLR4 TIR dimerization interface. A library of cell-permeable, TLR4 TIR-derived decoy peptides, each of which represents a nonfragmented patch of the TLR4 TIR surface, was generated and probed for the ability of individual peptides to inhibit TLR4 signaling in primary macrophages. Only 5 of 12 peptides potently inhibited all manifestations of LPS-signaling examined. Based on the rationale that the blockage of TLR4 TIR dimerization surface should inhibit all signaling pathways activated by the TLRs, these five peptides were further tested for their ability to bind directly the TLR4 TIR in a cell culture assay. Three inhibitory peptides—4R1, 4BB, and 4eE—targeted the TLR4 TIR directly. Although 4R1 also binds TLR2, it did not inhibit TLR2-mediated signaling, thereby suggesting that 4R1 binds TLR2 outside of a functionally important site.

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![FIGURE 5. Schematic representation of different modes of TIR domain interactions. A, A binary TIR–TIR interaction. B, A cooperative interaction of three TIR domains leads to the formation of a stable complex. Each TIR domain interacts with two other TIR domains of the trimer. Total interface area equals ∼3xs. C, The bridging mode of interaction in a TIR trimer. Total interface area is 2s. The complex is as stable as the comprising binary complexes. S, average size of TIR–TIR interface area.](http://www.jimmunol.org/)

![FIGURE 6. A, Superimposition of 25 best scoring models for TLR4 TIR dimer in which the BB loop of one TIR directly contacts the E helix of the other. In A and B, TIR domains that interact via the BB loop are superimposed and shown space filled. Only backbones are shown for TIR domains that interact via E helix. Color coding of the space-filled TIR model is as in Fig. 4. B, The first (blue) and 24th (green) scoring models of TLR4 TIR dimers demonstrated in A are shown in B. E helices involved in dimerization are shown in ribbon style. The selected models differ significantly in the position of intermolecular contacts within the specified areas as well as in the mutual orientation of comprising TIR domains.](http://www.jimmunol.org/)
formation of a functional TLR4 TIR dimer. An example of such a case in which an unstructured region adjacent to an interaction domain plays a role in complex formation could be found in the complex of death domains (DD) of Drosophila proteins Pelle and Tube. In this complex, the C-terminal region of Tube DD abuts Pelle DD and thus contributes substantially to the intermolecular Pelle–Tube contacts (27, 36). Peptide 4R3 demonstrated weak quenching of TLR4-Cer compared with 4R1, 4BB, and 4xaE; however, 4R3 is intimately juxtaposed on the surface of TLR4 TIR to the peptides that bind TLR4 with higher affinity (Fig. 4B). For this reason, the area represented by 4R3 is also likely to belong to the TLR4 dimerization interface; however, the actual intermolecular contact area within the region represented by this peptide is predicted to be less. Thus, four peptides that are strong inhibitors of TLR4 signaling are clustered on the surface of TLR4 TIR. The total area represented by these peptides is large and convex (Fig. 4A, 4B). The convex shape of the interface suggests that it represents a superimposition of two interfaces with actual intermolecular contact areas being different in the two TIRs that form the functional dimer. Our current hypothesis is that an area within the BB loop of one TLR4 TIR establishes the intermolecular contacts with the E helix of another TLR4 TIR. 4R1 region apparently plays a role stabilizing the interaction between 4BB and 4xaE regions. Although 4R9 inhibit TLR4 signaling; it does not bind TLR4 TIR. The actual target of this peptide is yet to be determined. Because 4R9 is located on the opposite side of the TIR domain relative to the putative TLR4 dimerization interface (Fig. 4C), it is tempting to speculate that this region is a docking site for one of the adapter proteins. Future FRET analyses with TIR-containing adapter proteins will be required to elucidate its function and binding partner.

Available site-directed mutagenesis data support functional importance of regions represented by 4R1, 4BB, and 4xaE peptides. The following mutations within these regions have been reported to affect severely the signaling ability of human TLR4: YD674–675AA (4R1 region), HYR708–710AAA (4BB), DFI711–713AAA (4BB), PGV714–716AAA (4BB), I718A (4BB), FWR807–809AAA (4xeE), R809STOP (4xeE), RLR810–812AAA (4xeE), LR811–812AA (4xeE), and L815A (4xeE) (37). It is noteworthy that all of these functionally important residues are conserved in mice and humans, except for the synonymous replacement of R812 in the human gene for lysine in mice. Additional data supporting functional importance of residues within the BB loop region have been reviewed (6).

Nunez Miguel et al. (7) were the first to develop a computational model of a higher-order TIR complex comprised of four TIR domains. Using the structure of TLR10 dimer observed in the asymmetric unit of TLR10 TIR crystal as a template, the authors first built the TLR4 TIR dimer model and then docked the TIR domains of TRAM and TIRAP onto the TLR4 dimer. This excellent theoretical work provided the first structural model that explained how adapter recruitment stabilizes a TLR dimer (i.e., through the cooperative interaction of an adapter with two receptor TIRs simultaneously). However, the starting point for building this model was the assumption that structurally homologous regions mediate the contact in a functional TLR4 dimer and in the TLR10 TIR asymmetric unit in crystal. Our data do not support this assumption. Although we confirm the involvement of the TLR4 TIR BB loop in TLR4 TIR dimerization, our data suggest that this region establishes contact with the E helix of TLR4, rather than the C helix as found in the structure of TLR10 TIR crystals. In our opinion, this discrepancy highlights the fact that the functional dimer does not necessarily represent the most stable among possible dimer conformations. The critical feature of a functional dimer is that it exposes a composite binding site so that the adapter interacts with both TIRs simultaneously, thereby stabilizing the dimer. Indeed, if modeling of a binary TIR–TIR complex typically gives an estimate for the TIR–TIR interface area of 550–750 Å² (5, 7, 11), the estimate of the cumulative buried area in the trimeric TIR complex composed of two receptor TIRs and an adapter TIR is ~3 times larger and varies in the range of 1750–1850 Å² (7) (Fig. 5, as an illustration).

We built a structural model of TLR4 TIR dimer constraining the interface positions within the BB loop area on one TIR domain and the E helix on the other as suggested by our experimental data. Fig. 6A depicts the 25 best scoring models for the TLR4 TIR dimer. Not surprisingly, the obtained dimer conformations are quite diverse both rotationally and with respect to the positions of actual intermolecular contacts within the specified areas. A survey of individual structures suggests that the E helix may contact both lateral sides of protruding BB loop as well as different subregions along the BB loop (Fig. 6B). Similarly, the BB loop may contact different sections or sides of the E helix. Such a result agrees with the notion of weakness (or plasticity) of individual TIR–TIR interactions of which only those conformations can be stabilized that expose the composite adapter-docking site so that the adapter can bind both TIRs simultaneously, thereby cementing the initial interaction. Although our data identify the specific regions that mediate TLR4 TIR dimerization, they are not sufficient to distinguish the functional dimer conformation. Yet, we propose that this goal can be achieved using this decoy peptide approach through the identification of the adapter-interacting areas in the TLR4 TIR complemented by reciprocal identification of receptor-interacting areas within the TLR adapters.

In summary, our findings clearly demonstrate usefulness of the decoy peptide approach to identification of interfaces that mediate transient interactions of signaling proteins. We also identified several inhibitory decoy peptides and, for some of them, identified the specific target within TIR domains involved in signaling. Each of these peptides can serve as a template for the development of lead therapeutics designed to treat diseases in which excessive TLR4 signaling has been implicated. This gains importance in light of the emerging field of peptidomimetics in which drug candidates are created to mimic a functional protein epitope rather than the more conventional approach of targeting a small molecule into a functional pocket on a protein surface (38–40).

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