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Peptide-mediated Interference of TIR Domain Dimerization in MyD88 Inhibits Interleukin-1-dependent Activation of NF-κB*

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Myeloid differentiation factor 88 (MyD88) plays a crucial role in the signaling pathways triggered by interleukin (IL)-1 and Toll-like receptors in several steps of innate host defense. A crucial event in this signaling pathway is represented by dimerization of MyD88, which allows the recruitment of downstream kinases like IRAK-1 and IRAK-4. Herein, we have investigated the function of the Toll/IL-1 receptor (TIR) domain in MyD88 homodimerization in cell-free and *in vitro* experimental settings by using epta-peptides that mimic the BB-loop region of the conserved TIR domain of different proteins. By using a pull-down assay with purified glutathione *S*-transferase-MyD88 TIR or co-immunoprecipitation experiments, we found that epta-peptides derived from the TIR domain of MyD88 and IL-18R are the most effective in inhibiting homodimerization with either the isolated TIR or full-length MyD88. Moreover, we demonstrated that a cell permeable analog of MyD88 epta-peptide inhibits homodimerization of MyD88 TIR domains in an *in vitro* cell system and significantly reduces IL-1 signaling, as assayed by activation of the downstream transcription factor NF-κB. Our results indicate that the BB-loop in TIR domain of MyD88 is a good target for specific inhibition of MyD88-mediated signaling *in vivo*.

Myeloid differentiation factor 88 (MyD88)[1] is a crucial adaptor protein that functions to recruit signaling proteins to receptors of the Toll-like or interleukin-1 receptor (TLR/IL-1R) superfamily (1, 2). Activation of signaling pathways downstream of this class of receptors is fundamental for several aspects of host defense.

The MyD88 protein has a modular structure composed of a death domain (DD) at the N terminus and a Toll/IL-1 receptor (TIR) domain at the C terminus separated by a short linker region, referred to as intermediary domain (3). Upon ligand stimulation, MyD88 is recruited to the membrane by interaction of its TIR domain with the analogous domain in the IL-1R or TLR receptors (4). It has been shown that MyD88 forms homodimers (5) and promotes the recruitment to the plasma membrane and the activation of two IL-1 receptor-associated kinases: IRAK-4 and IRAK-1. A homophilic interaction between MyD88 DD and the homologous DD found at the N terminus of the kinases is required for such event (6). A recent model proposes that MyD88 binds to IRAK-4 and promotes phoshorylation of critical IRAK-1 residues by IRAK-4 (7). These events stimulate IRAK-1 autophosphorylation and its interaction with TRAF6 (tumor necrosis factor (TNF) receptor-associated factor 6), leading to activation of both the inhibitory κB kinase (IKK) and the mitogen-activated protein kinases (MAPK) JNK and p38. These kinases are pivotal in the ultimate activation of several transcription factors, including NF-κB and activator protein 1 (AP-1), which elicit the production of essential effector molecules for immune and inflammatory responses (8). The generation of MyD88-deficient mice (9) has shown that this protein is required for the proliferative response of T-cells to IL-1, for the IL-18-mediated production of interferon-γ by Th1 cells, and for the activation of natural killer cells. Thus, MyD88 is an essential mediator for the response of several immune cells to cytokines. Moreover, MyD88-knock-out mice are insensitive to LPS-induced death and fail to secrete cytokines such as IL-6 and TNF-α *in vivo* (10). Most notably, this study also revealed that LPS-induced activation of both NF-κB and MAPK was delayed, rather than abolished, in these mice (11), highlighting the existence of a MyD88-independent pathway of TLR-4 signaling (12). On the other hand, loss of MyD88 expression has an anti-inflammatory effect in early atherosclerosis (13), whereas additional studies have further underlined the key inflammatory role of MyD88 in arthritis. Indeed, MyD88-deficient mice do not develop streptococcal cell wall-induced arthritis (14) nor a visually detectable synovitis after transfer of arthritogenic sera (15). Taken together, these results suggest that targeting the TLR/IL-1R pathway by interfering with the function of MyD88 may be a novel approach in the therapy of chronic inflammatory disorders. In the present study, we sought to investigate whether it was feasible to attenuate MyD88 signaling by means of peptide-mediated interference of MyD88 TIR domain homodimerization.

MATERIALS AND METHODS

Computational Methods—The MyD88 sequence was aligned by PSI-Blast to retrieve homologs and the selected sequences were aligned using ClustalW algorithm on the NPS server (npsa-pbil.ibcp.fr). Consensus secondary structure prediction was performed on the Predict-

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[1] The abbreviations used are: MyD88, myeloid differentiation factor 88; BSA, bovine serum albumin; DTT, dithio-DL-threitol; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; TLR, Toll-like receptor; IL, interleukin; DD, death domain; TIR, Toll/IL-1 receptor; TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; aa, amino acids; RP, reverse-phase; HPLC, high pressure liquid chromatography; HEK, human embryonic kidney; PLB, passive lysis buffer; RAcP, receptor accessory protein.

Protein server using different algorithms: Jpred, Psi-pred, PHD, Prof (cubic.bioc.columbia.edu/predictprotein).

A structural alignment between the target sequence (GenBank™ accession number: NP_002459) and the sequences with known crystal structures was performed using FUGUE program and manually adjusted based on secondary structure prediction and conserved regions found (www.cryst.bioc.cam.ac.uk/~fugue/prfsearch.html). The position of gaps and insertions was disfavored within conserved secondary structures.

By using a homology modeling technique and Swiss-PDBViewer software (17), the three-dimensional model of MyD88 TIR domain (aa 161–295) was built using TLR2 crystal structure (Protein Data Bank code 1fyx) as template, extracted from Protein Data Bank (www.rcsb.org). Molecular dynamics simulation, followed by simulated annealing technique was performed using MacroModel software (AMBER force field, GB/SA solvent model for water, 100 ps each step at different levels of constraint) (18). Quality evaluation of the model regarding variable regions and loops was done by the PROCHECK program (19). Models of MyD88 homodimers were generated using geometry and hydrophobicity complementary search algorithm GRAMM (20). The docking parameters were set to: step grid at 1.7 Å, repulsion potential at 30.0, and intervals of rotation at 10°. The 30 lower energy models were clustered to select the most representative ones and then they were minimized to obtain seven different homodimers.

Peptide Synthesis—The peptides were synthesized by the Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) solid phase method (16) on Rink-amide (amino methyl)-polystyrene resin using an ABi 431 A (Applied Biosystems) automatic peptide synthesizer and then cleaved from the resin using trifluoroacetic acid/H_2O/triisopropylsilane/1,2-ethanedithiol at 92.5:2.5:2.5:2.5 (v/v). The peptides were purified by reverse-phase high pressure liquid chromatography (HPLC-RP) on C12 reverse-phase Jupiter-Proteo (Phenomenex) semipreparative columns. The intermediates and product analysis were performed by liquid chromatography/mass spectroscopy by HPLC-RP on C18 reverse-phase Luna (Phenomenex) and mass spectroscopy with Thermofinnigan LCQ-Duo to confirm their molecular weight. The final peptide purity was 90–99%.

Plasmids—AU1-tagged MyD88 and AU1-tagged TIR domain of MyD88 expressing plasmids were a kind gift from Dr. Marta Muzio ("Mario Negri" Institute, Milan, Italy). cDNA encoding the TIR domain of MyD88 was amplified by PCR using pCDNA3-AU1-MyD88 as template and oligonucleotides 5′-AGGGATCCCCGACCCCCTGGGGCATATG-3′ (forward) and 5′-AGGAATTCTCAGGGCAGGGACAAGGC-3′ (reverse). The cDNA obtained was subcloned into the BamHI and EcoRI sites of either $pCDNA_3$-N_2-Myc or pGEX-3X expression vectors for Myc- or glutathione S-transferase (GST)-tagged TIR domain, respectively. For the NF-κB reporter assays, the NF-κB luciferase and *Renilla* luciferase constructs were used according to manufacturer's instructions (Promega).

Cell Culture and Transfections—The human embryonic kidney (HEK) 293 and HeLa cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), and grown in a 37 °C humidified atmosphere of 5% CO_2. HEK293-TLR3 cells (InvivoGen), stably transfected with human TLR3, were cultured in the same medium supplemented with 10 μg/ml blasticidin S (InvivoGen). The HEK293 cells were cultured in 10-cm-diameter dishes and transfected by the calcium phosphate method with 5–15 μg of the appropriate plasmids. The $Antennapedia_{43-58}$-fused $MyD88_{196-202}$ peptides were added to the medium 24 h after transfection.

GST-MyD88 TIR Domain Fusion Protein Synthesis and Purification—*Escherichia coli* cells (BL21) transformed with pGEX-3X-TIR construct were grown at 30 °C in LB medium to an optical density ($A_{600\ nm}$) of 0.6. GST-TIR fusion protein expression was induced by 3-h induction with 0.5 mM isopropyl β-thiogalactopyranoside (Sigma-Aldrich). Bacterial pellets were lysed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 1 mM DTT, protease inhibitors, by probe sonication. Bacterial lysates were centrifuged at 12,000 × *g*, and supernatant fractions were incubated with glutathione-Sepharose beads (Sigma-Aldrich) for 1 h at 4 °C under constant shaking. After several washes in PBS, GST-fusion proteins were eluted with 50 mM Tris-HCl, pH 8, 100 mM NaCl, containing 10 mM glutathione (Sigma-Aldrich). Purified proteins were stored at −80 °C in the same buffer containing 10% glycerol. Protein purity and integrity was analyzed by SDS-PAGE and Coomassie Blue staining.

Immunoprecipitation Assay—HEK293 cells were collected 24 h after transfection, washed in ice-cold PBS, and lysed in buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 15 mM $MgCl_2$, 15 mM EGTA, 1% Triton X-100, 10% glycerol, 20 mM β-glycerophosphate, 1 mM DTT, 0.5 μM $NaVO_4$, and protease inhibitors. After 10 min on ice, cell lysates were centrifuged at 10,000 × *g* for 10 min at 4 °C and cytosolic fractions collected for immunoprecipitation. Cell extracts (800 μg of total proteins) were incubated with 2 μg of anti-AU1 antibody (Babco) and protein A-Sepharose beads (Sigma-Aldrich) for 2 h at 4 °C under constant shaking. Following incubation, the beads were washed twice with lysis buffer, twice with PBS, and then incubated with 0.5 μg GST-TIR and 200 μM TIR peptides in PBS for 1 h under constant shaking at 4 °C. Sepharose bead-bound immunocomplexes were washed twice with PBS and eluted in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 0.7 M 2-mercaptoethanol, and 0.0025% (w/v) bromphenol blue) for Western blot analysis.

Co-immunoprecipitation Assay—Cell extracts (700 μg of total proteins) prepared as described above were precleared by incubation for 1 h with a mixture of protein A/G-Sepharose beads (Sigma-Aldrich) under constant shaking at 4 °C. For immunoprecipitation, 1 μg of mouse anti-Myc (E910, Santa Cruz Biotechnology) was preincubated for 1 h with a mixture of protein A/G-Sepharose beads (Sigma-Aldrich) in lysis buffer containing 0.05% bovine serum albumin (BSA, Sigma-Aldrich) under constant shaking at 4 °C. After incubation, the beads were washed twice with lysis buffer/0.05% BSA and then incubated with precleared cell extracts for 2 h at 4 °C under constant shaking. Sepharose bead-bound immunocomplexes were washed three times in lysis buffer and eluted in SDS-PAGE sample buffer for Western blot analysis.

Western Blot Analysis—Cell extracts or immunoprecipitated proteins were diluted in SDS sample buffer as described above and boiled for 5 min. Proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore) using a semidry blotting apparatus (Bio-Rad). Membranes were saturated with 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature and incubated with the following primary antibody (1:1000 dilution) overnight at 4 °C: mouse anti-AU1 (for AU1-TIR, from Babco), mouse anti-Myc (for Myc-TIR, from Santa Cruz Biotechnology), mouse anti-GST (for GST-TIR, from Santa Cruz Biotechnology). Secondary anti-mouse IgGs conjugated to horseradish peroxidase (Amersham Biosciences) were incubated with the membranes for 1 h at room temperature at a 1:10,000 dilution in PBS containing 0.1% Tween 20. Immunostained bands were detected by the chemiluminescence method (Santa Cruz Biotechnology).

NF-κB Reporter Assay—HeLa cells or HEK293-TLR3 (2.5×10^5) were cultured in 12-well plates and transfected with 0.5 μg of an NF-κB-dependent luciferase reporter gene and *Renilla* luciferase reporter gene (8 ng) as an internal control using the FuGENE 6 reagent (Roche Diagnostic) according to the manufacturer's instructions. Twenty-four hours after transfection, $Antennapedia_{43-58}$-fused $MyD88_{196-202}$ peptides were added to the medium. After 2 additional hours, either 5 ng/ml IL-1β (R&D Systems) (HeLa cells) or 25 μg/ml poly(I:C) (InvivoGen) (HEK293-TLR3) were added to the same medium. After additional 6 h, cells were harvested and lysed in 250 μl of passive lysis buffer (PLB) (dual-luciferase reporter assay system, Promega) for 15 min at room temperature. Cell lysates were cleared for 30 s by centrifugation at top speed in a refrigerated microcentrifuge and transferred to a fresh tube prior to reporter enzyme analysis. Ten μl of cell lysates were mixed with 100 μl of luciferase assay reagent II (Promega), and the NF-κB-firefly luciferase activity was determined using a biocounter luminometer. For the assessment of the *Renilla* luciferase activity, 100 μl of Stop & Glo® reagent were added to the same sample. Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of *Renilla* luciferase. Data are expressed as mean -fold induction ± S.D. from a minimum of three separate experiments.

RESULTS AND DISCUSSION

Recruitment of MyD88 to receptors belonging to the TLR/IL-1R superfamily requires a direct TIR-TIR domain interaction (21–23). Albeit most of the conserved residues in TIR domains lie within the core of the fold, some are solvent-exposed residues and may thus allow homotypic and/or heterotypic interactions to occur among different partners. Actually, a loop referred to as the BB-loop connecting the second β-strand and second α-helix (*BB* in Fig. 1*A*), and that includes a proline → histidine mutation, renders the mice harboring this mutation hyporesponsive to LPS (24). Moreover, the corresponding change also abolishes the ability of TLR2 to interact with MyD88 *in vitro* (25). Hence, we asked whether inhibition of MyD88 signaling might be achieved by interfering with its

A

Protein		Sequence		Accession N
		βB BB αB		
MyD88	190	KLCVSDRDVLPGTCVWSIAS	209	NP_002459
IL18R	411	KLCIFERDVVPGGAVVDEIH	430	NP_003846
IL-18RacP	449	SLCLLERDVAPGGVYAEDIV	468	NP_003844
IL-1RI	422	KLFIYGRDDYVGEDIVEVIN	441	NP_000868
IL-1RacP	436	KLCIFDRDSLPGGIVTDETL	455	NP_002173
TLR1	665	QICLHERNFVPGKSIVENII	684	NP_003254
TLR2	671	KLCLHKRDFIPGKWIIDNII	690	NP_003255
TLR4	704	QLCLHYRDFIPGVAIAANII	723	O00206

B

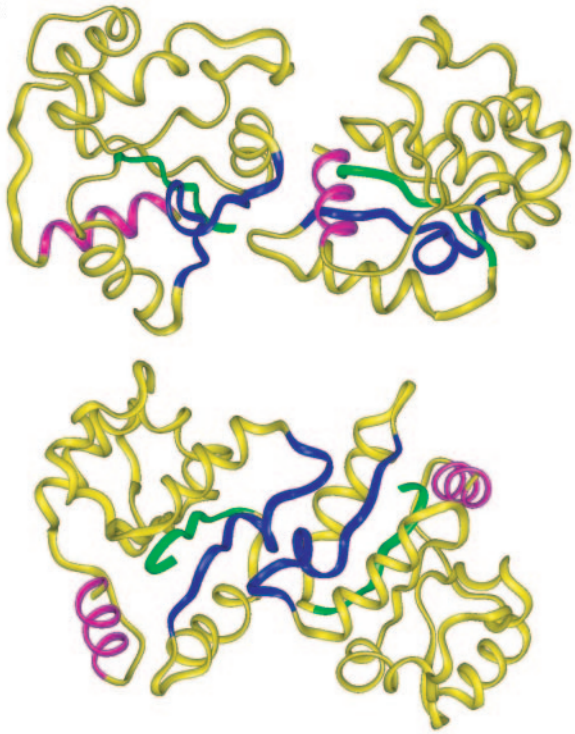

FIG. 1. **Amino acid sequence alignment of the conserved extended loop in the TIR domain of MyD88 and TLR/IL-1R receptors.** *A*, highlighted amino acids represent the BB-loop region in MyD88. Accession numbers of the corresponding sequences are listed on the *right*. Models of the MyD88 TIR domain homodimer are shown. *B*, best solution (*top panel*) and the eighth solution (*bottom panel*) of MyD88 TIR domain homodimer obtained using GRAMM software are represented in *yellow*. Conserved boxes in the TIR domain are shown in *green* (box 1), *blue* (box 2: BB-loop), and *purple* (box 3).

TIR homodimerization domain using specific peptides that mimic the BB-loop.

To better understand how the BB-loop could be located at the interface, we have modeled the TIR domain of MyD88 and generated homodimers using the GRAMM software. Shown in Fig. 1*B* is the best ranked and the eighth ranked solution, representing two different modes of interaction of the BB-loop, namely, docking either to Box 3 (aa 284–293, *top*) or to itself by an antiparallel packing (*bottom*). To experimentally assess the importance of this protein portion for homodimerization we produced a series of synthetic peptides (Table I).

As a first approach, we tested the ability of such peptides to interfere with the dimerization of MyD88 TIR domains *in vitro*. Recombinant AU1-tagged MyD88 was expressed in HEK293 cells and isolated by immunoprecipitation. Hence, a purified GST fusion protein containing the TIR domain of MyD88 (aa 152–296) was incubated with the beads preadsorbed to AU1-MyD88 in the absence or presence of the peptides described in Table I. As expected, GST-TIR was able to associate to full-length MyD88. Interestingly, we observed that an epta-peptide (ST 2348) with the MyD88 sequence comprising aa 196–202 strongly inhibited this interaction (71% inhibition, see Fig. 2, *A* and *B*), whereas a scrambled peptide (ST 2404) with the same aa composition was inactive (2% inhibition). In the same analysis, we found that an epta-peptide (ST 2350) based on the IL-18 receptor sequence (aa 417–423) was even more potent (86% inhibition), whereas similar epta-peptides based on the sequence of IL-1 receptor accessory protein (IL-1RAcP, aa 442–448) (ST 2349) or TLR1 (aa 670–676) (ST 2351) were either less effective (30% inhibition for IL-1RAcP) or completely inactive (0% inhibition for TLR1). The results of this analysis are listed in Fig. 2, *A* and *B*, and indicate an elevated sequence specificity in the inhibitory potential of TIR-domain based epta-peptides.

A comparison of peptides, referred to as ST 2348 (MyD88), ST 2349 (IL-1RacP), and ST 2350 (IL-18R), indicates that the 7 amino acids can be subdivided into three different portions: an N-terminal conserved charged portion (RD), a C-terminal conserved β-turn portion (PG), and a central linker in which the most relevant differences are found. In particular, a nonconservative substitution (V198S) distinguishes ST 2348 from ST 2349 whereas ST 2350 has a conservative substitution (L199V) in this position. According to our homodymer model (Fig. 1*B*, *bottom panel*), the first substitution might negatively affect the putative hydrophobic interaction between Val^{198} and Arg^{196}, while the substitution in ST 2350 would not affect the homodimerization. Thus, the experimental results shown in Fig. 2*B* lend great support to the model depicted in Fig. 1*B* (*bottom panel*).

To confirm the ability of the MyD88 and IL-18R epta-peptides to interfere with dimerization of the TIR domains, we sought an alternative method, namely, the co-immunoprecipitation procedure. The TIR domain of MyD88 (aa 152–296) was subcloned in expression vectors containing either a Myc or an AU1 epitope tag. When Myc-TIR and AU1-TIR were co-expressed in HEK293 cells they could specifically interact, as demonstrated by co-immunoprecipitation experiments (*lane 3* in Fig. 3*A*). Both the MyD88 (ST 2348) and the IL-18R (ST 2350) epta-peptides were capable of similarly inhibiting this interaction and only trace amounts of AU1-TIR were co-immunoprecipitated with Myc-TIR when these peptides were added to the cell extracts before immunoprecipitation (*lanes 4* and *5* in Fig. 3*A*). In control experiments, a scrambled peptide (ST 2404) containing amino acids identical, though reshuffled, to those of $MyD88_{196–202}$ did not significantly interfere with TIR domain dimerization (Fig. 3*B*).

Next, we tested the effect of the MyD88 epta-peptide (ST 2348) on live cells by fusing it to a short basic peptide sequence from the third helix of *Drosophila* Antennapedia homeodomain (aa 43–58, RQIKIWFQNRRMKWKK) to facilitate its delivery into cells (26). Peptide effectiveness was measured as its ability to interfere with dimerization of the MyD88-TIR domains expressed in HEK293 cells. Hence, Myc-TIR and AU1-TIR were co-expressed in the presence of either the cell permeable MyD88 epta-peptide (ST 2345) or its scrambled control (ST 2403). In agreement with the results from previous experiments, we found that the cell permeable MyD88 peptide (ST 2345) inhibited the dimerization of the TIR domains (61%), whereas the scrambled peptide (ST 2403) exerted only a minor effect (17% inhibition, Fig. 4, *A* and *B*). This experiment suggests that the epta-peptide $MyD88_{196–202}$ is capable of specifically interfering with dimerization of the TIR domains of MyD88 occurring in cells.

Since the MyD88 epta-peptide interferes with dimerization of the TIR domains in cells, we sought to determine whether it

TABLE I
Synthetic peptides mimicking the BB-loop

ID	Sequence	M_r	Notes
ST 2348	Ac-R-D-V-L-P-G-T-NH_2	797.9	Ac-$MyD88_{196–202}$-NH_2
ST 2404	Ac-P-T-D-L-V-R-G-NH_2	797.9	Ac-$MyD88_{196–202}$ scrambled-NH_2
ST 2350	Ac-R-D-V-V-P-G-G-NH_2	739.2	Ac-$IL\text{-}18R_{417–423}$-NH_2
ST 2349	Ac-R-D-S-L-P-G-G-NH_2	739.2	Ac-$IL\text{-}1RAcP_{442–448}$-$NH_2$
ST 2351	Ac-E-R-N-F-V-P-G-NH_2	859.0	Ac-$TLR1_{670–676}$-NH_2
ST 2345	Ac-R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K-R-D-V-L-P-G-T-NH_2	3026.7	Ac-$Antennapedia_{43–58}$-$MyD88_{196–202}$-NH_2
ST 2403	Ac-R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K-P-T-D-L-V-R-G-NH_2	3026.7	Ac-$Antennapedia_{43–58}$-$MyD88_{196–202}$ scrambled-NH_2

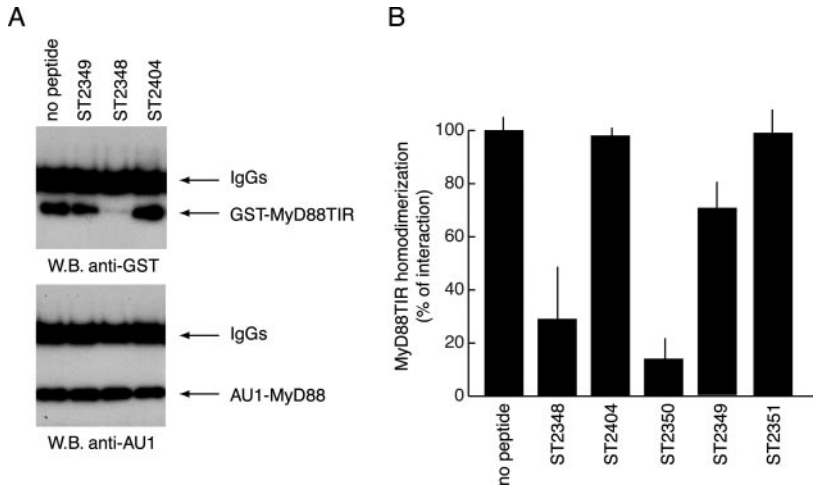

FIG. 2. **Interference of TIR domain dimerization by MyD88 selective epta-peptides.** *A*, representative experiment of the effect of peptides on the dimerization of the MyD88 TIR domains. Dimerization was tested in a pull-down assay using immunoprecipitated AU1-MyD88 as bait and GST-MyD88-TIR in the presence or absence of the indicated peptides by monitoring in Western blot (*W.B.*) the amount of GST-MyD88-TIR pulled down with AU1-MyD88. Data were quantified by densitometry, and a summary of the results obtained in four separate experiments is shown in *B*.

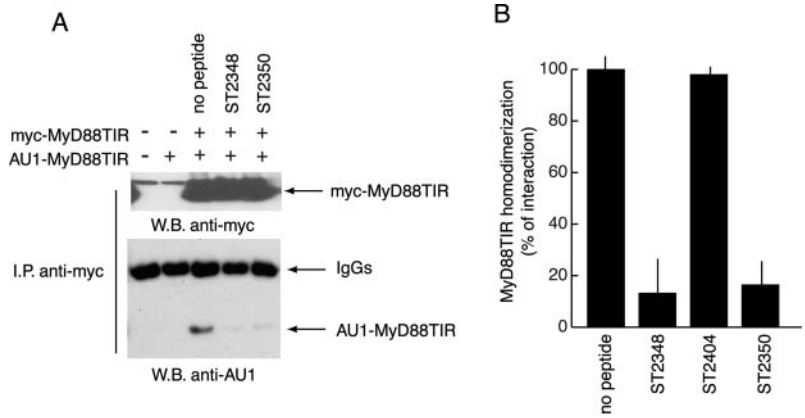

FIG. 3. **The MyD88 epta-peptide ST 2348 inhibits co-immunoprecipitation of the TIR domains.** *A*, HEK293 cells were transfected with either empty vectors (*lane 1*) or with AU1-MyD88-TIR alone (*lane 2*) or in combination with Myc-MyD88-TIR (*lanes 3–5*). Cell extracts were immunoprecipitated with anti-Myc antibody either without adding peptides (*lanes 1–3*) or adding 200 μM of either MyD88 epta-peptide (*lane 4*, ST 2348) or IL-18R epta-peptide (*lane 5*, ST 2350). *B*, densitometric analysis of the effect on the interaction between TIR domains of peptides used in *A*) and of a scrambled peptide (ST 2404) based on the MyD88 amino acid composition. Results represent the average of three separate experiments. IP, immunoprecipitation; *W.B.*, Western blot.

might interfere with the functional activation of the IL-1 signaling pathway. The downstream event of stimulation with IL-1 is transcriptional activation of NF-κB, and dimerization of MyD88 is crucial for efficient propagation of this signaling cascade. Hence, we transfected HeLa cells, which express IL-1R and MyD88 (data not shown), with a reporter NF-κB-luciferase construct and then treated cells with IL-1 (5 ng/ml) in the presence or absence of either $Antennapedia_{43–58}$-fused $MyD88_{196–202}$ (ST 2345) or its scrambled control (ST 2403). Activation of the pathway was determined by measuring luciferase activity in cell extracts 6 h after stimulation. We observed that IL-1 triggered a strong induction (70-fold) of luciferase activity in HeLa cells treated without peptide (Fig. 4*C*). Remarkably, the cell-permeable $MyD88_{196–202}$ peptide (ST 2345) reduced NF-κB induction to 45-fold (35% reduction), whereas the scrambled peptide (ST 2403) had no significant effect (Fig. 4*C*). This result indicates that the IL-1 biological response is selectively inhibited by interfering with dimerization of the TIR domain of MyD88. To determine whether the interfering action of the $MyD88_{196–202}$ peptide was specific, we checked its effect on the NF-κB activation exerted by the TLR-3 receptor, which signals through a MyD88-independent pathway (27). When HEK293 stably transfected with TLR-3 were stimulated with poly(I:C) (25 μg/ml), a 7-fold induction of NF-κB activity was observed. However, neither the $MyD88_{196–202}$ peptide nor its scrambled control inhibited this activation, indicating that ST 2345 specifically interferes with MyD88-dependent signaling pathways (Fig. 4*D*).

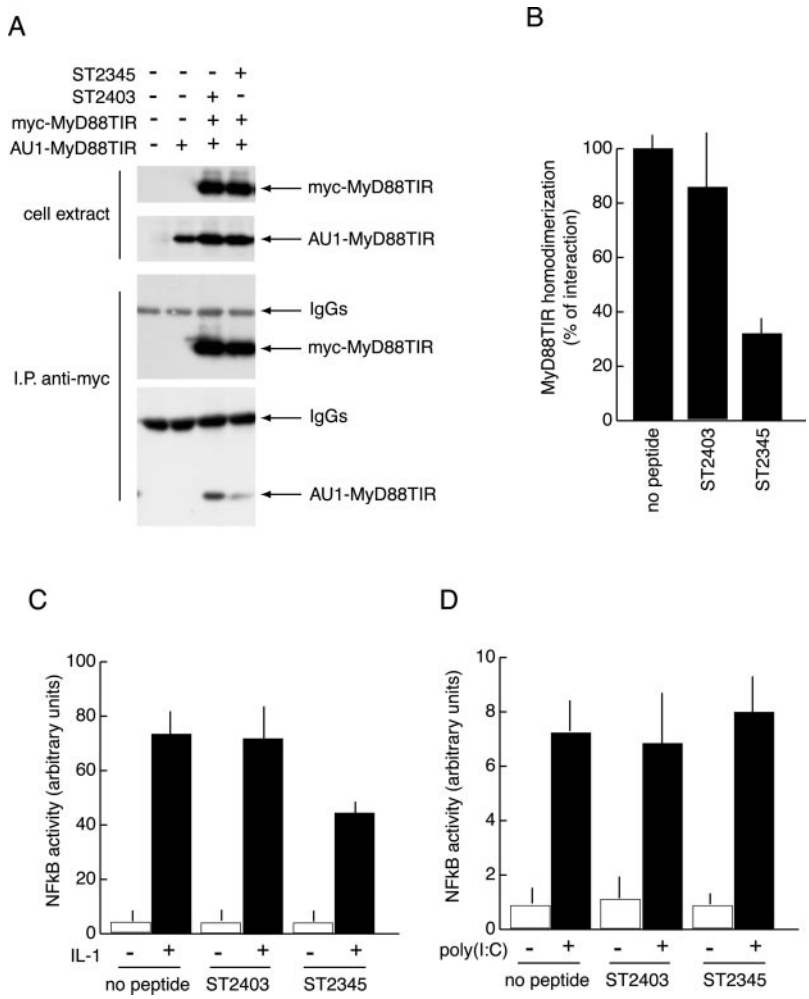

FIG. 4. **The cell-permeable Antennapedia$_{43-58}$-MyD88$_{196-202}$ peptide (ST 2345) inhibits dimerization of the TIR domains.** *A*, HEK293 cells were transfected with either empty vectors (*lane 1*) or with AU1-MyD88-TIR alone (*lane 2*) or in combination with Myc-MyD88-TIR (*lanes 3* and *4*). After transfection, cells were incubated for 24 h with epta-peptides (100 μM) fused to Antennapedia$_{43-58}$ sequence to favor delivery into cells. At the end of incubation, cells were collected, and dimerization of the TIR domains was assayed by co-immunoprecipitation. Briefly, cell extracts were immunoprecipitated (*IP*) with anti-Myc antibody and immunoprecipitated proteins were analyzed in Western blot with either the anti-Myc antibody or the anti-AU1 antibody to reveal the association. The cell-permeable peptide (ST 2345) strongly interfered with dimerization of TIR domains *in vivo*, whereas the cell-permeable scrambled peptide (ST 2403) exerted only a minor effect. *B*, densitometric analysis of the effect on the interaction between TIR domains of peptides used in *A*. Results represent the average of three separate experiments. *C* and *D*, the cell-permeable Antennapedia$_{43-58}$-MyD88$_{196-202}$ peptide (ST 2345) inhibits NF-κB activation *in vivo*. HeLa cells (*C*) or HEK293-TLR3 (*D*) were transfected with the NF-κB luciferase and *Renilla* luciferase constructs and 24 h after transfection were treated either without peptide or 200 μM Antennapedia$_{43-58}$-fused MyD88$_{196-202}$ epta-peptide (ST 2345) or MyD88$_{196-202}$ scrambled epta-peptide (ST 2403). After 2 h, cells were stimulated with or without 5 ng/ml IL-1 (*C*) or 25 μg/ml poly(I:C) (*D*) for 6 additional hours. At the end of incubation, cells were harvested and lysed in 250 μl of PLB lysis buffer, and luciferase activity was measured in soluble extracts using the luciferase assay reagent II and a biocounter luminometer. Data are normalized for transfection efficiency by dividing firefly luciferase activity with that of *Renilla* luciferase. Data are expressed as mean -fold induction ± S.D. from a minimum of three separate experiments.

Our results suggest that the region of MyD88 in the extended loop, which is highly conserved in the TIR domains of different proteins, is a good candidate for drug design aimed at interfering with MyD88 signaling. Interestingly, we observed by dimerization assays that the most effective epta-peptides for blocking MyD88 TIR/TIR homophilic association were those deduced from the sequence of the TIR domains of IL-18R and MyD88 itself.

A previous report (28) showed that a low molecular weight mimic of the three protruding amino acids in the BB-loop (consensus for several IL-1RI, MyD88, and Toll receptors) can successfully inhibit interactions between IL-1RI and MyD88. We have shown here that the extended loop, besides intervening in association with the receptor(s), is also important for dimerization of MyD88 as detected in both cell-free and *in vitro* cell systems. Moreover, since these active epta-peptides are capable of inhibiting the activation of NF-κB by IL-1, molecules that mimic the structure of MyD88 may become valuable tools for investigating the *in vivo* role of this protein in cytokine signaling possibly leading to the design of new therapeutics (29).

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Peptide-mediated Interference of TIR Domain Dimerization in MyD88 Inhibits Interleukin-1-dependent Activation of NF- κB

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