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) is a crucial adapter 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 phosphorylation 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-phil.ibcp.fr). Consensus secondary structure prediction was performed on the Predict-
Peptide-mediated Interference in MyD88 TIR Domain Dimerization

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

A structural alignment between the target sequence (GenBank™ accession number: NP_002459) and the sequence of MyD88 with known crystal structures was performed using FUGUE program and manually adjusted based on secondary structure prediction and conserved regions found (www.cyst.bioc.cam.ac.uk/~fuge/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.resb.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 the 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) (9-fluorenyl)methoxycarbonyl) solid phase method (16) on Rink-amide sites of either pCDNA3-N2-Myc or pGEX-3X expression vectors for Myc- or glutathione S-transferase (GST)-tagged TIR domain, respectively. Appropriate plasmids were diluted in SDS PAGE gel and transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore) using a semidyed blotting apparatus (Bio-Rad). Membranes were saturated with 5% dry milk in PBS containing 0.1% Tween 20 for 2 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 (Amer sham Biosciences) were incubated with the membranes for 1 h at room temperature. Membranes were washed with 3 times in PBS containing 0.1% Tween 20. Immunobosand bands were detected by the chemiluminescence method (Santa Cruz Biotechnology).

NF-κB Reporter Assay—HeLa cells or HEK293-TLR3 (2.5 × 10⁶) 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, AntennapediaΔ43-58-fused MyD88Δ161-295 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 (Promega) (dual-luciferase reporter assay system). Renilla luciferase activity, 100 μl of cell lysates were mixed with the response also abolishes the ability of TLR2 to interact 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 semidyed blotting apparatus (Bio-Rad). Membranes were saturated with 5% dry milk in PBS containing 0.1% Tween 20 for 2 h 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 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 contacting the second β-strand and second α-helix (BB in Fig. 1A), 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
Peptide-mediated Interference in MyD88 TIR Domain Dimerization

Table I. Peptide-mediated interference in MyD88 TIR domain dimerization.

| Protein  | Sequence | Accession N |
|----------|----------|-------------|
| MyD88   | 190 KLCVSL-RRVLPGCVCWISAIAS 209 | NP_002459 |
| IL18R   | 411 KLCIFERDVPVGGAVDZH2H 430 | NP_003846 |
| IL-1RaCP | 449 SLCLLEDRVAPGYYAEDIV 468 | NP_003844 |
| IL-1R1  | 422 KFFTGRDDVGEDIVKET 461 | NP_000969 |
| IL-1RaP | 436 KLCIFDREDLSGCVTDRTL 455 | NP_002173 |
| TLR1    | 665 QCLCHNRFVPGESIMII 694 | NP_003254 |
| TLR2    | 671 KLCLOHFIDPGBKIDNII 690 | NP_003255 |
| TLR4    | 704 QLCLOYRDFIPGVAIANII 723 | O00206 |

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. 3A). 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. 3A). In control experiments, a scrambled peptide (ST 2404) containing amino acids identical, though reshuffled, to those of MyD88196–202 did not significantly interfere with TIR domain dimerization (Fig. 3B).

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. Amino acid 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).
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 Antennapedia43–58-fused MyD88196–202 (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. 4C). Remarkably, the cell-permeable MyD88196–202 peptide (ST 2345) reduced NF-κB induction to 45-fold (35% reduction), whereas the scrambled peptide (ST 2403) had no significant effect (Fig. 4C). 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 MyD88196–202 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 MyD88196–202 peptide nor its scrambled control inhibited this activation, indicating that ST 2345 specifically interferes with MyD88-dependent signaling pathways (Fig. 4D).
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).

REFERENCES

1. Akira, S., and Takeda, K. (2004) Nat. Rev. Immunol. 4, 499–511
2. O’Neill, L. A. J. (2003) Biochem. Soc. Trans. 31, 643–647
3. Hardiman, G., Rock, F. L., Balasubramanian, S., Kastelein, R. A., and Bazan, J. F. (1996) Oncogene 13, 2467–2477
4. Akira, S. (2003) J. Biol. Chem. 278, 38105–38108
5. Burns, K., Martinon, F., Eslingler, C., Pahl, H., Schneider, P., Bodmer, J. L., Di Marco, F., French, L., and Tschopp, J. (1998) J. Biol. Chem. 273, 12203–12209
6. Janssens, S., and Beyaert, R. (2003) Mol. Cell. 11, 293–302
7. Janssens, S., and Beyaert, R. (2002) Trends Biochem. Sci. 27, 474–482
8. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Genes Dev. 13, 1297–1308
9. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakamichi, K., and Akira, S. (1998) Immunity 9, 143–150
10. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) Immunity 10, 293–302

Fig. 4. The cell-permeable Antennapedia43–58–MyD88196–202 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 Antennapedia43–58 sequence to favor delivery into cells. At the end of transfection, 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 interaction between TIR domains of peptides used in A. Results represent the average of three separate experiments. C and D, the cell-permeable Antennapedia43–58–MyD88196–202 peptide (ST 2345) inhibits NF-κB activation in vivo. HepG2 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 Antennapedia43–58–fused MyD88196–202 epta-peptide (ST 2345) or MyD88196–202 scrambled epta-peptide (ST 2403). After 2 h, cells were stimulated with or without 5 ng/ml IL-1 (C) or 5 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.
Peptide-mediated Interference in MyD88 TIR Domain Dimerization

11. Takeuchi, O., and Akira, S. (2002) Curr. Top. Microbiol. Immunol. 270, 155–167
12. O’Neill, L. A. J. (2004) Science 303, 1481–1482
13. Bjorkbacka, H., Kunjathoor, V. V., Moore, K. J., Koehn, S., Ordija, C. M., Lee, M. A., Means, T., Halmen, K., Luster, A. D., Golenbock, D. T., and Freeman, M. W. (2004) Nat. Med. 10, 416–421
14. Joosten, L. A., Koenders, M. I., Smeets, R. L., Heuvelmans-Jacobs, M., Helsen, M. M., Takeda, K., Akira, S., Lubberts, E., van de Loo, F. A., and van den Berg, W. B. (2003) J. Immunol. 171, 6145–6153
15. Choe, J. Y., Crain, B., Wu, S. R., and Corr, M. (2003) J. Exp. Med. 197, 537–542
16. Bodanszky, M. (1993) Principles of Peptide Synthesis, 2nd Ed., Springer-Verlag, Berlin
17. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
18. Schrodinger LLC (2003) MacroModel 8.5, Schrödinger LLC, Portland, OR
19. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
20. Katchalski-Katzir, E., Shariv, I., Eisenstein, M., Friesem, A. A., Aflalo, C., and Vakser, I. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2195–2199
21. Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., and Cao, Z. (1997) Immunity 7, 837–847
22. Dunne, A., Ejdeback, M., Ludidi, P. L., and O’Neill, L. A. (2003) J. Biol. Chem. 278, 41443–41451
23. Radons, J., Gabler, S., Wesche, H., Korherr, C., Hofmeister, R., and Falk, W. (2002) J. Biol. Chem. 277, 16456–16463
24. Poltorak, A., He, X. L., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) Science 282, 2085–2088
25. Xu, Y., Tao, X., Shen, B., Horng, T., Medzhitov, R., Manley, J. L., and Tong, L. (2000) Nature 408, 111–115
26. Kneelto, L. A., and Middaugh, C. R. (2003) J. Pharm. Sci. 92, 1754–1772
27. Jiang, Z., Zamanian-Daryoush, M., Nie, H., Silva, A. M., Williams, B. R., and Li, X. (2003) J. Biol. Chem. 278, 16713–16719
28. Bartfai, T., Behrens, M. M., Gaidarova, S., Pemberton, J., Shivanyuk, A., and Rebek, J. Jr. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7971–7976
29. Ulevitch, R. J. (2004) Nat. Rev. Immunol. 4, 512–520