MyD88 has a modular organization, an N-terminal death domain (DD) related to the cytoplasmic signaling domains found in many members of the tumor necrosis factor receptor (TNF-R) superfamily, and a C-terminal Toll domain similar to that found in the expanding family of Toll/interleukin-1-like receptors (IL-1R). This dual domain structure, together with the following observations, supports a role for MyD88 as an adapter in IL-1 signal transduction; MyD88 forms homodimers in vivo through DD-DD and Toll-Toll interactions. Overexpression of MyD88 induces activation of the c-Jun N-terminal kinase (JNK) and the transcription factor NF-κB through its DD. A point mutation in MyD88, MyD88-lpr (F56N), which prevents dimerization of the DD, also blocks induction of these activities. MyD88-induced NF-κB activation is inhibited by the dominant negative versions of TRAF6 and IRAK, which also inhibit IL-1-induced NF-κB activation. Overexpression of MyD88-lpr or MyD88-Toll (expressing only the Toll domain) acted to inhibit IL-1-induced NF-κB and JNK activation in a 293 cell line overexpressing the IL-1RI. MyD88 coimmunoprecipitates with the IL-1R signaling complex in an IL-1-dependent manner.

The myeloid differentiation protein (MyD88) has no known biological function (1). Sequence analysis, however, suggests that it may have signaling capabilities; MyD88 is predicted to have a modular organization consisting of an N-terminal death domain (DD) separated by a short linker from a C-terminal Toll domain (2–7).

The N-terminal DD is related to a motif of approximately 90 amino acids that was initially defined as the region of similarity between the cytoplasmic tails of the FAS/Apo1/CD95 and TNF receptors required for their induction of cytotoxic signaling (8, 9). The DD, which has in recent years been found in many additional proteins, is now known to mediate protein–protein interactions with other DD sequences forming either homo- or heterodimers (10). This property is utilized by many members of the TNF superfamily (i.e. FAS, TNF-R1, DR3/Apo3/WSL-1/TRAMP, and TRAIL-Rs 1 and 2), in response to ligand activation, to establish interactions that form the foundation for building signaling complexes that can induce responses such as cytotoxicity, activation of the c-Jun N-terminal kinase (JNK)/stress-activated protein kinases, and/or activation of the transcription factor nuclear factor κB (NF-κB) (11).

MyD88’s C-terminal Toll domain is comprised of approximately 130 amino acids (5). This domain was originally described based on the homology between the cytoplasmic signaling regions of the Drosophila melanogaster transmembrane protein Toll and the IL-1RI, but is now found in an expanding family of proteins, most of which are cell surface receptors (5, 12–14). MyD88 is the only reported mammalian protein with a Toll domain that is not predicted to be a transmembrane. The Toll domain lacks an intrinsic signaling capacity and thereby transduces signals by recruiting associated proteins. It is not known whether Toll domains function in an analogous manner to DDs by mediating Toll-Toll interactions. However, the discovery that the Toll-containing IL-1 receptor accessory protein (IL-1RacP) acts as a co-receptor for IL-1RI and is an indispensable molecule in the IL-1RI signal transduction complex suggests that interactions between like domains may have a role in the formation of signaling complexes (15–17).

In recent years some of the proteins involved in the proximal signaling events associated with IL-1RI-induced activation of NF-κB have been identified. This has revealed the striking similarity between the IL-1RI and Drosophila Toll signaling pathways. Toll induces Dorsal activation (a homolog of NF-κB) which like NF-κB is normally held in an inactive state in the cytoplasm by the 1b-like inhibitory protein, Cactus. Following stimulation, these inhibitory proteins become phosphorylated, ubiquinated, and degraded via proteasome-mediated pathways, which frees NF-κB/Dorsal to translocate into the nucleus and begin transcription. The Drosophila Ser/Thr kinase Pelle is believed to be involved in the phosphorylation of Cactus (18). In the IL-1 pathway, a Ser/Thr kinase that is rapidly recruited to the IL-1RI complex, within seconds of IL-1 binding, has recently been identified (19). This kinase, the IL-1 receptor associated kinase (IRAK), is highly homologous to Pelle but not to other mammalian Ser/Thr kinases. Interestingly, both Pelle and IRAK have N-terminal DDs (19). In the Toll pathway a second protein exists with a DD, Tube, which regulates the activity of Pelle through DD-DD interactions (20–22). Additional DD-containing adapter molecules may, therefore, also exist in the IL-1 pathway. With its dual domain organization MyD88 has ideal properties to function as an adapter linking Toll and death modules. Here, we examine this intriguing possibility by analyzing the functional role(s) of the MyD88 domains.
EXPERIMENTAL PROCEDURES

Cell Culture—The 293T human embryonic kidney cell line or 293 cells (ATCC CRL 1572) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin (100 μg/ml of each) and grown in 5% CO2 at 37 °C. Northern Blot Analysis—Isolation of total RNA from various mouse tissues was carried out as described elsewhere (23, 24). A MyD88 antisense probe was synthesized by in vitro transcription in the presence of 12.5 μCi [32P]UTP (400 Ci/mmol; Amersham International, Amersham, UK). Following extensive washing of the Northern blot, the membrane was exposed to X-ray films (XAR; Eastman Kodak Co.) for 4 days.

Expression Vectors—Mouse MyD88 was first identified as a transcript encoding a protein of 243 residues (1). However, since then several lines of evidence suggest that the transcript for MyD88 is 54 amino acids longer (predicted molecular mass of ~33 kDa) and starts at Met minus 53 (4, 25, 26). To obtain full-length mMyD88 cDNA, total RNA isolated from murine bone marrow was reverse transcribed using a cDNA cycle kit from Promega, amplified by PCR (JT478 5′-ggc ggg ggg cgc-3′ and JT479 5′-ctc atc agt ctc atc ttc-3′) and subcloned into PCR-TA-cloning vector (Invitrogen). A modified pCRII (Invitrogen) mammalian expression vector was constructed by cloning a Kozak consensus sequence (GCCACC) and the Flag epitope (MDYKDDDDK) between the BamH I and EcoRI sites of PCR-III to yield pMet-Flag-V63. MyD88 was subcloned as an EcoRI fragment into pMet-Flag-V63 to give pFlag-MyD88 or into pMet-Myc (27) to give pMyc-MyD88. MyD88-F56N (referred to as MyD88-Ipr) and MyD88 lacking the DD (MyD88-ΔDD) were generated by PCR using the following primers: MyD88-lpr, JT317 5′-gaa gtt ctc cat-3′; Toll-C, JT478 5′-gaa ttc atg gcc ggg ggg ccg-3′; Toll-N, JT318 5′-gaa ttc atg gcc ggg ggg ctc gga gta ctc gag-3′; Toll-C, JT318 and JT319 were then cloned as EcoRI fragments into pMet-Flag-V63. MyD88 encoding the Toll domain (amino acids 161–296) and deletions within this region, Toll-N (amino acids 161–296), Toll-C (amino acids 230–296), and Toll-2292–296 (amino acids 161–281) were amplified by PCR with the following primers: Toll, JT749 5′-tct ggc ttc att ttc-3′ and JT318; Toll-N, JT749 and JT752 5′-ctc cag gag gaa ctc gag-3′; Toll-C, JT749 and JT752 5′-ctc cag gag gaa ctc gag-3′ and JT318 and Toll-2292–296, with primers JT749 and JT750 5′-ctc cag gag gaa ctc gag-3′ and JT318 and Toll-2292–296. MyD88-N was made by removing the EcoRV fragment from pFlag-MyD88. All constructs were confirmed by sequencing. Yeast expression vectors for MyD88 were prepared by cloning DNA for MyD88 and the deletion mutants (indicated above) as EcoRI fragment into the LexA DNA-binding domain vector, pBTM116 (28), GALA DNA-binding domain vector, pGBT9 (CLONTECH), and into the GALA activation domain vector, pGAD10 (CLONTECH).

The following plasmids were obtained from the indicated sources: NF-κB luc (Vitor Jungenele, Lausanne, CH); Myc-IL-1RI, IRAK and IL-1RαP (Keith Ray and Filippo Volpe, Glaxo Welcome, UK); Flag-JNK (Christian Widman, Denver, CO); TRAF6 (28–522) and TRAF2 (67–501) (Hanfeld Wajant, Stuttgart, Germany). IRAK encoding the DD (amino acids 161–167) was amplified using the following primers: PC1-1 5′-atc cca atc acc cca caa ggg gtt-3′ and JT651 5′-gaa ttc atg ggc ggg cag cag-3′ and JT902 5′-cag ttt ctg ggg cgg-3′. Yeast-Two-Hybrid Interaction Analysis—Protein/protein interactions were analyzed by cotransforming plasmids encoding the LexA-DNA binding (LexA-db) fusion proteins or GALA-DNA binding (GALA-db) fusion proteins with plasmids encoding the various GALA-activation domain (GALA-db) fusion proteins (2.5 μg of each subclone) into Saccharomyces cerevisiae strain CTY10–5d (used with LexA-db constructs) or Y190 (used with GALA-db constructs) following the Two-Hybrid System protocol (CLONTECH). Filter lift assays for colony color development were done as described previously (29).

Generation of Glutathione S-transferase (GST)-MyD88 and in Vitro Binding Assays—MyD88 was cloned as an EcoRI fragment into pGEX-4T-1 (Pharmacia Biotech Inc.). GST fusion proteins were induced with isopropyl-thiogalactoside and purified on glutathione-agarose beads as described elsewhere (30). 35S-Labeled MyD88 and mutant versions were generated with the TNT coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions. Following translation 35S-labeled reticulocyte lysates (2 μl) were incubated with 290 μl of GST beads (2.5 μg/ml of each) and subcloned into pGEX-4T-1. After incubation the GST beads were washed for 2 min at low speed and then washed six times with binding buffer. The washed beads were boiled in SDS sample buffer and loaded onto 12% SDS-polyacrylamide gels which were enhanced by incubation in En'HanceTM (Du Pont) prior to fluorography at ~80 °C.

Antibodies/Western Blot Analysis—Monoclonal antibodies used for immunoprecipitations and Western blotting include anti-Flag M2 anti-body (Kodak Biosciences) used at a concentration of 5 μg/ml antibody against the Myc epitope (9E10, Sigma) used at a concentration of 1 μg/ml, anti-UVS antibody (Sigma) used at a dilution of 1:20,000, anti-JNK2 antibody (Santa Cruz Biotechnology) used at a dilution of 1:1,000, and antibodies against the active phosphorylated form of JNK, Anti-ACTIVETM (Promega) used at a dilution of 1:5,000. Antiserum against MyD88 (AL126) was generated using a peptide spanning amino acids 54–77 (MGFEYLEIRELETRPDPTRSLLDA), which was synthesized using the multiple antigen technology (31). The antiserum was affinity-purified on the MyD88 peptide coupled to CNBr-Sepharose 4B (Pharmacia) and used at a dilution of 1:500. For Western analysis protein extracts were separated by SDS-PAGE and transferred to Hybond nitrocellulose membrane (Amersham Life Science). Blots were incubated with the antibodies in blocking buffer (PBS, 0.5% Tween 20, 5% skim milk) followed by horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoResearch Labs Inc.) diluted 1:2000 in blocking buffer. Bound antibody was detected using the enhanced chemiluminescence kit (Amersham International) according to the protocol of the manufacturer.

Gel Permeation Chromatography—293T cells (1 × 107) were transi- tionally transfected with Flag-MyD88 (12 μg) 24 h after transfection the cells were harvested and lysed in PBS (300 μl) containing Complete™ protease inhibitor mixture by mild sonication (four times for 5 s each). The soluble cellular extract (150 μl) was mixed with the internal standards catalase and ovalbumin, then loaded onto a Superdex-200 HR10/30 column, and the proteins were eluted in PBS at 0.5 ml/min. Every second fraction (250 μl) was precipitated with trichloroacetic acid and then analyzed by Western blotting with anti-Flag antibody. The column was calibrated with the following standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

Tissue Extracts, and Coimmunoprecipitation—Tissue extracts were prepared from BalB/c mice by homogenization in PBS containing 1% Nonidet P-40 and Complete™ protease inhibitor mixture (Boehringer Mannheim). The extracts were spun at 13,000 rpm for 20 min at 4 °C, and the supernatants were collected for Western blotting analysis. Cellular extracts were obtained from 293T cells harvested 26 h after transfection and lysed as described previously (37). For coimmunoprecipitation of MyD88 and its deletion mutants, transfected 293T cells (1 × 107), were lysed in 200 μl of lysis buffer (50 mM Tris, pH 7.8, 150
MyD88 Mediates JNK and NF-κB Activation

Expression of MyD88—The murine MyD88 transcript was originally reported to be expressed in myeloid precursor enriched murine bone marrow cells and not in nonmyeloid tissues (1). Since then, MyD88 mRNA has been detected in a number of nonmyeloid cell lines (4, 26). Here we extend this analysis to show that the murine MyD88 transcript (approximately 2.2 kilobase pairs) is present in many tissues, in fact in all tissues tested except for the brain (Fig. 1A). In order to examine the expression pattern of the MyD88 protein, we generated antibodies against a synthetic peptide (corresponding to residues 54–77) of murine MyD88, which specifically recognized the 30-kDa Flag-MyD88 protein expressed in 293T cells (Fig. 1B). MyD88 was detected in many tissues with highest levels found in the brain and with the empty vectors (pETM116 and pGAD10) served as negative controls. Interaction of the empty vector with Trp1 and Fas-ID with Fas-ID served as positive controls (data not shown).

NF-κB Activation Assays—Electrophoretic mobility shift assays were carried out as described previously (32). In brief, total cellular extracts prepared from transfected 293 cells (2 × 10⁶) were first treated with IL-1β (200 ng/ml) for 3 min. The complex was then precipitated by the addition of 3 μl of antibody containing 20 μg of bovine serum albumin (Sigma), 2 μl of poly(dI-dC) (Boehringer Mannheim), 0.5 μl of a buffer containing 0.1% Nonidet P-40, 0.1% phenylmethanesulfonyl fluoride, 1% aprotinin. The cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma), and resuspended in four cell volumes of drawing buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 14 mM MgSO₄, 4 mM EGTA, 1 mM DTT). Where indicated IL-1 (50 μl of anti-Flag agarose at 4 °C overnight. The agarose beads were washed five times with lysis buffer, and the precipitated proteins were then fractionated on 12% SDS-PAGE and analyzed by Western analysis with anti-Flag antibody. The protein content of the supernatant was determined, and equal amounts of protein were separated by 12% SDS-PAGE and analyzed by Western analysis with Myc antibody. The expression of Myc-IL-1RI by Western analysis with Myc antibody. The expression pattern of the MyD88 protein was assessed by β-galactosidase expression filter assays. ++ indicates strong color development within 60 min of the assay, and + indicates no development of color within 24 h. Interaction with the empty vector (pETM116 and pGAD10) served as negative controls. Interaction of TNF-R1-ID with TRADD and Fas-ID with Fas-ID served as positive controls (data not shown).

MyD88 self-associates in yeast. Full-length MyD88 and MyD88 mutants are schematically represented. The black and gray rectangles represent the death and Toll domains, respectively. MyD88 containing a point mutation, F56N, in the death domain (represented by a white circle) is referred to as MyD88-lpr. Yeast CTY10–5d were co-transformed with expression vectors encoding the various GAL4 transcription activation domain fusion proteins. Interaction of the indicated proteins was assessed by β-galactosidase expression filter assays. ++ indicates strong color development within 60 min of the assay, and + indicates no development of color within 24 h. Interaction with the empty vector (pETM116 and pGAD10) served as negative controls. Interaction of TNF-R1-ID with TRADD and Fas-ID with Fas-ID served as positive controls (data not shown).

Table 2. MyD88 self-associates in yeast. Full-length MyD88 and MyD88 mutants are schematically represented. The black and gray rectangles represent the death and Toll domains, respectively. MyD88 containing a point mutation, F56N, in the death domain (represented by a white circle) is referred to as MyD88-lpr. Yeast CTY10–5d were co-transformed with expression vectors encoding the various GAL4 transcription activation domain fusion proteins. Interaction of the proteins was assessed by β-galactosidase expression filter assays. ++ indicates strong color development within 60 min of the assay, and + indicates no development of color within 24 h. Interaction with the empty vector (pETM116 and pGAD10) served as negative controls. Interaction of TNF-R1-ID with TRADD and Fas-ID with Fas-ID served as positive controls (data not shown).
in the ovary, adrenal gland, prostate, and thymus (Fig. 1C). However, in certain tissues (kidney, liver, and spleen), which have MyD88 transcript the protein was not detected, possibly as a result of post-transcriptional regulation in these tissues. MyD88 Forms Homodimers—Many adapter proteins involved in signal transduction form homo- and heterotypic interactions through like domains. As the death domain is a region that frequently promotes such interactions, MyD88 was analyzed for this capacity. Two-hybrid analysis, in vitro binding studies, and mammalian cell coimmunoprecipitations revealed that MyD88 forms homodimers (Figs. 2 and 3, A–C). To assess whether the DD mediates self-association, various point mutants and deletion mutants of MyD88 were generated. Phe56 was chosen for mutagenesis based on sequence alignment of the MyD88 DD with the DD of Fas (3). A mutation at this position corresponds to the lpr sign mutation (33) known to abolish cytotoxic signaling of Fas, probably by disrupting the conformation of the DD as revealed by recent NMR experiments (34). Mutation of Phe56 to Asn inhibited association of full-length MyD88 with a truncated form containing the DD (MyD88-DD), indicating that dimerization is mediated through the DD of MyD88-DD. Surprisingly, MyD88-lpr was still capable of interacting with MyD88 (Figs. 2 and 3A), suggesting the presence of a second domain involved in self-association. Two constructs encoding the Toll domain of MyD88 (MyD88-Toll and MyD88-ΔDD) were therefore tested for their ability to bind to MyD88 (Figs. 2 and 3, A and B). MyD88-Toll bound to full-length MyD88 and also to itself but not to MyD88-DD. However, MyD88-Toll did not dimerize with either Toll-N (containing the first half of the domain), Toll-C (containing the second half of the domain), or Toll-Δ282–296 (missing 15 amino acids at the C terminus), suggesting that the entire domain is required for dimerization. The peptide deleted in Toll-Δ282–296 is homologous to a peptide in the Toll domain of the IL-1RII within which are residues that have been shown to be critical for IL-1 signaling (35).

DD-DD or Toll-Toll interactions mediate self-association of MyD88, which, together, may result in stable association of MyD88 proteins. It was also possible that the presence of these two dimerization domains could allow the formation of higher homo-oligomeric structures through head to tail aggregation. To address this question, Flag-MyD88 expression constructs were transfected into 293T cells, and cellular extracts were fractionated by gel permeation chromatography. MyD88 eluted as a defined peak with an apparent molecular mass of approximately 60 kDa (corresponding to a 2.0-mer) suggesting that MyD88 forms homodimers in vivo (Fig. 3C).

Heterotypic interactions between DD-containing proteins are critical for linking adapter proteins in a number of signaling pathways. Given the properties of MyD88, the ideal candidate for binding to its DD was the Ser/Thr kinase, IRAK which also contains a N-terminal DD. We tested this possibility via the yeast two-hybrid system and in mammalian cell coimmunoprecipitation experiments using 293T cells overexpressing MyD88 and IRAK, but did not detect an interaction. However, while this manuscript was under revision, MyD88 was found to associate with underphosphorylated IRAK (36). Wesche et al. (36) also reported that overexpression of IRAK induces its spontaneous autophosphorylation, thus precluding its association with MyD88 in mammalian cell coimmunoprecipitation experiments. Using the two-hybrid system we also tested other...
known DD-containing receptor and adapter proteins (TNFR1, TRAMP, FAS, FADD, TRADD, RIP, and RAIDD) for their ability to interact with MyD88, but found no partners for MyD88 among this group of proteins.

**Signaling Activities of MyD88; Activation of JNK**

To learn more about the physiological activity(ies), we first tested whether overexpression of MyD88 could induce cytotoxicity like many of the DD-containing proteins. Overexpression of MyD88 and MyD88-DD, but not MyD88-lpr or MyD88-Toll induced apoptosis in 293T cells (293T cells were transfected with DNA of each expression vector. Total cell extracts were prepared 24 h after transfection, and 10-μg aliquots were combined with 32P-labeled NF-κB oligonucleotide probe. Supershift reactions were performed by adding 1 μl of preimmune serum or anti-p65 serum at the same time as the probe. Specific NF-κB complexes are indicated by arrows. B, activation of NF-κB by MyD88 deletion mutants. 293T cells were transfected with 1 μg of a NF-κB luciferase reporter plasmid (pNF-κBLuc), 0.5 μg of pCMV β-gal, and the indicated MyD88 constructs (1.5 μg of each). The expression levels of the various MyD88 constructs were examined by Western blot analysis with anti-Flag antibodies (lower panel). C, effect of increasing MyD88 expression on NF-κB activity. NF-κB assays were performed following cotransfection of 293T cells with 1 μg of pNF-κBLuc, 0.5 μg of pCMV β-gal, and the indicated amounts of MyD88 to give 3 μg of total DNA. D, the specificity of NF-κB activation. 293T cells (2 × 10⁵) were transfected with 1 μg of HIV promoter derived NF-κB luciferase reporter (HIV-Luc) or a reporter plasmid containing a point mutation in the NF-κB site (HIV-84 Luc) and with 0.5 μg of pCMV β-gal, 1.5 μg of MyD88 (dark shading); or 1.5 μg of empty vector (light shading). Luciferase activities in B–D were determined 24 h after transfection and normalized on the basis of β-galactosidase values. Values shown are averages for representative experiments in which each transfection was carried out in duplicate.

**MyD88 Mediates JNK and NF-κB Activation**

**FIG. 5.** NF-κB is activated by MyD88 overexpression. A, Electrophoretic mobility shift assay of NF-κB activation in 293T cells. 293 cells were transfected with 6 μg of each expression vector. Total cell extracts were prepared 24 h after transfection, and 10-μg aliquots were combined with the 32P-labeled NF-κB oligonucleotide probe. Supershift reactions were performed by adding 1 μl of preimmune serum or anti-p65 serum at the same time as the probe. Specific NF-κB complexes are indicated by arrows. B, activation of NF-κB by MyD88 deletion mutants. 293T cells were transfected with 1 μg of a NF-κB luciferase reporter plasmid (pNF-κBLuc), 0.5 μg of pCMV β-gal, and the indicated MyD88 constructs (1.5 μg of each). The expression levels of the various MyD88 constructs were examined by Western blot analysis with anti-Flag antibodies (lower panel). C, effect of increasing MyD88 expression on NF-κB activity. NF-κB assays were performed following cotransfection of 293T cells with 1 μg of pNF-κBLuc, 0.5 μg of pCMV β-gal, and the indicated amounts of MyD88 to give 3 μg of total DNA. D, the specificity of NF-κB activation. 293T cells (2 × 10⁵) were transfected with 1 μg of HIV promoter derived NF-κB luciferase reporter (HIV-Luc) or a reporter plasmid containing a point mutation in the NF-κB site (HIV-84 Luc) and with 0.5 μg of pCMV β-gal, 1.5 μg of MyD88 (dark shading); or 1.5 μg of empty vector (light shading). Luciferase activities in B–D were determined 24 h after transfection and normalized on the basis of β-galactosidase values. Values shown are averages for representative experiments in which each transfection was carried out in duplicate.

MyD88-induced cytotoxicity in 293T cells may be a secondary effect due to high levels of MyD88 in these cells.

Many signaling pathways which implicate DD-containing adapter proteins lead to activation of the JNK/stress-activated protein kinase pathway, which prototypically involves the sequential activation of MEKK1, SEK1, JNK, and c-Jun (37, 38). To test the possibility that MyD88 might activate this kinase cascade, 293T cells were cotransfected with MyD88 and Flag-JNK. Flag-JNK was immunoprecipitated from cell lysates and tested for its activity using GST c-Jun as a substrate. MyD88, but not MyD88-lpr induced JNK activation (Fig. 4A). Activation of JNK was also detected by Western analysis using an antibody that specifically recognizes the active, phosphorylated form of the kinase. MyD88 and MyD88N significantly induced activation of JNK (Fig. 4B). That neither MyD88-lpr nor MyD88-Toll activated JNK suggests that DD of MyD88 is critical for this activity.

**Activation of the Transcription Factor NF-κB**

Both the death and Toll domains represent motifs that are often involved in NF-κB activation. To test whether MyD88 could activate NF-κB electrophoretic mobility shift assays were carried out on cellular extracts from transfected 293 cells. Overexpression of MyD88 or MyD88-DD led to significant activation of NF-κB in the absence of exogenous stimuli (such as TNF-α or IL-1) (Fig. 5A), whereas in cells transfected with empty expression vector, MyD88-lpr or MyD88-DD specific NF-κB complexes were not detected, suggesting that the DD of MyD88 is responsible for this activation. Supershift experiments with p65 antibody demonstrated that p65 is a component of the activated NF-κB complex (Fig. 5A, right panel).
MyD88 Mediates JNK and NF-κB Activation

We also examined the ability of MyD88 to activate a NF-κB-dependent reporter plasmid (pNF-κB-Luc) by cotransfecting 293T cells with pNF-κB luc and the various MyD88 constructs (Fig. 5B). In agreement with the electrophoretic mobility shift assays, MyD88 and MyD88-DD strongly induced NF-κB activation in 293 cells, and the mutants MyD88-lpr and MyD88-DD were inactive. MyD88 induced the activity of pNF-κB-Luc in a dose-dependent manner (Fig. 5C) and failed to activate pHIV-84, a luciferase reporter plasmid containing a mutation of the NF-κB elements of the HIV promoter, confirming the specificity of this activation (Fig. 5D). NF-κB activation following transient transfection with the MyD88 expression vector was also observed in HeLa and McF7 cells (data not shown).

MyD88 Is an Adaptor Protein in the IL-1-Signaling Pathway—The observations that MyD88 efficiently induced NF-κB and JNK activation together with its dual domain structure hinted at a role for MyD88 in IL-1 signaling. TRAF6 is required for IL-1-induced NF-κB activation, whereas TRAF2 has been implicated in NF-κB activation signaled through TNF (39–41). Dominant negative versions of these TRAFs are known to block IL-1-induced NF-κB and JNK activation. When a cell line (293 Myc-IL-1RI), which overexpresses the IL-1RI and responses to IL-1 treatment by activating the NF-κB-dependent reporter plasmid, approximately 20-fold was transfected with MyD88-lpr; IL-1-dependent NF-κB activation was inhibited in a dose-dependent manner (Fig. 6B). Overexpression of MyD88-lpr did not affect NF-κB activation signaled by TNF or induced by coexpression of TRAMP, demonstrating that the dominant-negative effect of the MyD88 mutant to IL-1 signaling was specific (Fig. 6B). MyD88-Toll overexpression also blocked IL-1-mediated NF-κB activation (Fig. 6B). IL-1-induced JNK activation was similarly inhibited in 293 IL-1RI cells by overexpression of MyD88-lpr or -Toll (Fig. 6C).

The ability of MyD88-Toll to block these IL-1-induced signals suggested the possibility that MyD88 may directly associate with the IL-1RI and/or IRAK. We tested this possibility using the yeast two-hybrid system, but did not detect an association of MyD88 with either of the receptor chains. Also an association between MyD88 and one of the receptor chains alone was also not detected in coimmunoprecipitation experiments.
IL-1 is a potent cytokine that elicits multiple diverse effects on immunological and inflammatory processes. It exerts its various biological activities mainly through activation of the transcription factors NF-κB and activating protein 1, which regulate the expression of numerous genes involved in these processes. Signaling cascades leading to the activation of these transcription factors are initiated by IL-1-induced complex formation of the IL-1RI and the IL-1RAcP (15–17). This in turn leads to IRAK recruitment to the receptor complex where IRAK becomes highly phosphorylated (19). Phosphorylated IRAK is then believed to dissociate from the receptor complex and interact with TRAF6, and it becomes rapidly degraded via a proteasome-dependent pathway (39, 42). The results described above show that overexpression of MyD88 activates both NF-κB (in a pathway upstream of IRAK and TRAF6) and JNK (activating protein 1) and therefore mimics these two IL-1-induced cellular responses. Moreover, MyD88 associates with the IL-1RI complex in an IL-1-dependent manner and dominant-negative forms of MyD88 (MyD88-lpr and MyD88-Toll) block IL-1 signaling. Our findings therefore demonstrate that MyD88 has an important role in mediating the cellular responses to this cytokine.

How can our findings be incorporated into the current model of IL-1 signaling? Experiments with the dominant negative mutants of MyD88 (MyD88-lpr and MyD88-Toll) indicate the MyD88-Toll domain links MyD88 with upstream components of the IL-1 pathway. Indeed, the Toll domain of MyD88 has an important role in mediating the cellular responses to this cytokine. MyD88 associates with the receptor complex after IL-1 stimulation and thusrogenesis, interacts with TRAF6, and it becomes rapidly degraded via a proteasome-dependent pathway (39, 42). The results described above show that overexpression of MyD88 activates both NF-κB (in a pathway upstream of IRAK and TRAF6) and JNK (activating protein 1) and therefore mimics these two IL-1-induced cellular responses. Moreover, MyD88 associates with the IL-1RI complex in an IL-1-dependent manner and dominant-negative forms of MyD88 (MyD88-lpr and MyD88-Toll) block IL-1 signaling. Our findings therefore demonstrate that MyD88 has an important role in mediating the cellular responses to this cytokine.

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MyD88, an Adapter Protein Involved in Interleukin-1 Signaling
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