THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 44, Issue of November 1, pp. 41624–41630, 2002
Printed in U.S.A.

Caspase-11 Gene Expression in Response to Lipopolysaccharide and Interferon-γ Requires Nuclear Factor-κB and Signal Transducer and Activator of Transcription (STAT) 1*

Received for publication, August 2, 2002, and in revised form, August 26, 2002
Published, JBC Papers in Press, August 26, 2002, DOI 10.1074/jbc.M207852200

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Murine caspase-11, together with caspase-1, is essential for the production of IL-1β in response to lipopolysaccharide (LPS). In most cells, caspase-11 is only expressed upon induction with pro-inflammatory stimuli. To understand how caspase-11 expression is transcriptionally regulated, we isolated the caspase-11 gene promoter by genome walking and investigated the mechanisms regulating caspase-11 gene expression in macrophages that are treated with LPS and interferon-γ. Transient transfections with caspase-11 promoter-luciferase reporter constructs and deletion/mutation analysis revealed an essential role for NF-κB binding in the up-regulation of caspase-11 in response to LPS. In the case of interferon-γ stimulation, signal transducer and activator of transcription 1 binding to the caspase-11 promoter could be shown to be required for caspase-11 expression.

The inflammatory response is a reaction of the body to certain foreign molecules and pathogens. It requires the activation of different proteins and genes in different cell types (1, 2). Macrophages are one of the main mediators of the inflammatory response to remove invading pathogens. When challenged with lipopolysaccharide (LPS)[1] and/or interferon-γ (IFN-γ), they secrete high levels of pro-inflammatory cytokines *i.e.* interleukin (IL)-1, -6, -18 and tumor necrosis factor (3–5), which contribute to the development of a proper immune response against the invading pathogen. However, uncontrolled production of these cytokines may lead to side effects, including septic shock. Both IL-1 and -18 are made as inactive precursors, which need to be proteolytically activated by caspase-1 (6–9). The latter belongs to a family of aspartic acid-specific cysteine proteases that mediate cytokine maturation and/or apoptosis (10). Caspase-1-deficient mice no longer produce IL-1β in response to LPS and are resistant to LPS-induced septic shock (11, 12). Mechanisms regulating caspase-1 activity are still largely unknown. However, it has previously been shown that caspase-1 activation is dependent on another caspase, caspase-11 (13). Indeed, similar to caspase-1-deficient mice, caspase-11-deficient mice fail to produce IL-1β in response to LPS and are also resistant to LPS-induced septic shock. Caspase-11 does not process pro-IL-1β directly, but its expression is essential for the activation of caspase-1 (13). Under certain pathological conditions (*e.g.* middle cerebral artery occlusion in mouse brain), caspase-11 can also activate caspase-3, leading to apoptosis of neuronal and microglial cells (14).

In contrast to most other caspases, such as caspase-1 and -3, caspase-11 is not constitutively expressed in most cell types, but its expression can be induced by different pro-inflammatory stimuli. An increase in caspase-11 was seen in macrophages, lymphocytes, and hepatocytes upon stimulation with LPS or IFN-γ *in vitro* (15–17). Likewise, the expression of caspase-11 is undetectable in healthy mice but highly inducible upon injection of LPS (18). Different groups have recently reported that ischemia also is able to trigger the induction of caspase-11 expression in the brain of rodents (14, 19, 20). Although for most caspases the proteolytical processing of constitutively expressed proforms is the most important regulatory step in their activation (10, 21, 22), transcriptional control of the expression level appears to be more important in the case of caspase-11. Although a role for other proteases or activators cannot be excluded, increased expression of caspase-11 most likely leads to its auto-activation. So far, there are only a few reports available about the transcriptional regulation of caspase expression. Liu *et al.* (23) recently demonstrated a role for Sp1 elements in the regulation of basal caspase-3 promoter activity. Caspase-9 promoter activation in response to severe hypoxia has also been reported (24). To study the regulation of caspase-11 expression, we cloned 5′-flanking promoter regions of the caspase-11 coding sequence. Analysis of the cloned fragments using luciferase reporter gene vectors demonstrated various levels of inducible promoter activity in a mouse macrophage cell line. Both LPS and IFN-γ, conditions that are known to induce caspase-11 expression in macrophages, could activate the promoter sequences. Binding elements for various transcription factors were identified, and binding of NF-κB and STAT1 was shown to be necessary for, respectively, LPS- and IFN-γ-inducible expression of caspase-11.

* This work was supported by the Belgische Federatie voor de Studie tegen Kanker, the Interuniversitaire Attractiepolen, the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-technologisch Onderzoek in de Industrie, and the Fonds voor Wetenschappelijk Onderzoek-Vlaanderen. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF539416.

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[1] The abbreviations used are: LPS, lipopolysaccharide; IL, interleukin; NF-κB, nuclear factor-κB; WT, wild type; STAT, signal transducer and activator of transcription; IFN-γ, interferon-γ; nt, nucleotide(s); CDTA, 1,2-cyllohexanediaminetetraacetic acid.

EXPERIMENTAL PROCEDURES

Cloning of the Mouse Caspase-11 Gene Promoter and Derived Mutants—To clone the 5′-flanking region of the murine caspase-11 gene, we used a mouse genome walker kit (Clontech). The kit provides five sets of murine genomic libraries generated from mouse genomic DNA cut with different restriction enzymes (*Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Ssp*I) and ligated to short adaptor sequences. These ligated DNA pools were then used as templates for two rounds of PCR. Primary and nested PCRs were performed using forward primers complementary to the adaptor sequence (AP1, 5′-GTAATACGACTCACTATAGGGC-3′; AP2, 5′-ACTATAGGGCACGCGTGGT-3′) and reverse gene-specific primers (GSP1, 5′-TTCAGCCATGAGAAAAAGCCTCAGT-3′; GSP2, 5′-CCTCAGTTCTTTCGCACTGTGAAGA-3′) that were designed based on the 5′-end of the mouse caspase-11 cDNA sequence (GenBank™ accession number NM_007609; Ref. 25). Each PCR mixture consisted of 25 mM $Mg(OAc)_2$, dXTPs (2.5 mM each), 1 ×Tth PCR reaction buffer, primers (10 μM each), and 1.25 unit Tth Advantage genomic polymerase (Clontech) performed in a reaction volume of 50 μl. In the primary PCRs, we used 1 μl of each library as template. In the nested PCRs, 1 μl of primary PCR product was used (7 cycles at 94 °C, 2 s, 70 °C, 3 m; 33 cycles at 94 °C, 2 s, 65 °C, 3 m). 3′-A overhangs were blunted off the PCR products. DNA from four PCR-positive clones was isolated and subcloned in a dephosphorylated *Sma*I-linearized pGL-3 basic plasmid vector (Promega) using T4 DNA ligase (Roche Molecular Biochemicals) to generate pGL3casp11p and pGL3casp11pΔ1-Δ3 reporter constructs. Upon sequencing, cloned fragments were shown to contain 926, 408, 194, and 186 bp, respectively, of the 5′-upstream sequence relative to the transcription start site of the caspase-11 gene. Eight progressive deletion mutants of the caspase-11 promoter were made by PCR. The fragments that were generated contained between 201 and 48 bp of the caspase-11 promoter sequence. Forward primers used were 5′-CGACGCGTGCAGCCGCTTTAGTTGTGTTTGT-3′ (Del1, 201 bp); 5′-CGACGCGTTGTTTGTTCCCATGACGAAGTGA-3′ (Del2, 180 bp); 5′-CGACGCGTAATTCAGGAAAAGGGAGGCCAGTT-3′ (Del3, 154 bp); 5′-CGACGCGTGGCCAGTTTTATGAGTTACAGGGGAT-3′ (Del4, 138 bp); 5′-CGACGCGTTTCCCCACAGCAGTTGATACTTTCT-3′ (Del5, 112 bp); 5′-CGACGCGTAGCAGCTCTTTCAACATCTCCTGGA-3′ (Del6, 86 bp); 5′-CGACGCGTCTCTTTCAACATCTCCTGGAAGTCC-3′ (Del7, 81 bp); 5′-CGACGCGTCTATGCTAAGGAAATGCCCAGTAAC-3′ (Del8, 48 bp). As a reverse primer 5′-CATGCCATGGCCTCAGTTCTTTCGCACTGTGAAGA-3′ was used. PCR was done in the same conditions as described above using Tth genomic polymerase (Clontech) for polymerization and pGL3casp11p as template. The amplified DNA fragments were unidirectionally cloned between the *Mlu*I and *Nco*I sites of the pGL3-basic plasmid by standard procedures. Nucleotide (nt) sequences of the cloned DNA fragments were confirmed in each case by sequencing with an ABI prism 377 DNA sequencer. To construct a mutant caspase-11 promoter in which the binding site for NF-κB is destroyed, two primers containing a double point mutation (underlined), were synthesized (Invitrogen): 5′-AGCTTACAGGGGATGGCCCCACAG-3′ (NFMutFW) and 5′-AGCTCTGTGGGGCCATCCCCTGTA-3′ (NFMutRev). The mutant construct pGL3casp11pMutNF was made by overlap PCR using two pairs of primers. The first round of PCR generated two mutant promoter fragments. The 5′-PCR fragment containing the upstream sequence to the mutation site was made using pGL3casp11p as a template and AP2 (see above) and NFMutRev as primers. The 3′-PCR fragment from the mutated site to the transcription start site was generated using pGL3casp11p as template and NFMutRev and GSP2 (see above) as primers. In the second round of PCR, a mixture of the 5′- and 3′-PCR fragments mentioned above was used as template and AP2 and GSP2 were used as primers. Conditions for PCR were: 9 cycles at 94 °C, 30 s, 66 °C, 1 m; 20 cycles at 94 °C, 2 s, 63 °C, 1 m with a similar reaction mixture as described above. The product of the secondary PCR was a complete caspase-11 promoter with a mutation that abolishes NF-κB binding. A similar PCR-based approach was used to introduce a mutation in the STAT binding site. Therefore we used the following primers: 5′-AGCTCAACATCTCCTGTTAGT-3′ (STMutFW) and 5′-AGCTACTAACAGGAGATGTTG-3′ (STMutRev), as well as AP2 and GSP2 (see above).

Cell Culture, Transfection and Luciferase Assay—Mf4/4 macrophage cells (26) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 0.03% L-Gln (Merck), 1 mM sodium pyruvate, 100 units/ml penicillin, 100 mg/ml streptomycin, and 50 μM β-mercaptoethanol at 37 °C in a humified 5% CO_2 atmosphere. For transfection, 2×10^6 cells were resuspended in 500 μl of RPMI 1640 without serum and antibiotics and transiently transfected with 5 μg of pGL3 reporter plasmid and 5 μg of pEF6/*Myc*-His/*LacZ* (Invitrogen) via electroporation (a single pulse of 300 V, 1200 μF, and an infinite resistance; Equibio, EasyjecT Plus). After electroporation, the cells were resuspended in MCDB 131 medium (Invitrogen) with 10% fetal calf serum and seeded into 24-well plates. After 12 h, the medium was replaced by the usual RPMI 1640 medium. Forty-eight hours after transfection, cells were treated with LPS (*Salmonella abortus equi*; Sigma), 1 μg/ml and/or IFN-γ (250 international units/ml), for 6 h. Cells were then harvested and lysed in lysis buffer (25 mM Tris-HCl (pH 7.8), 2 mM dithiothreitol, 2 mM CDTA, 10% glycerol, 0.5% Triton X-100 supplemented with 1 mM leupeptin, 0.3 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors) and analyzed for luciferase activity using a Topcount luminometer (Packard). For measuring β-galactosidase activity, the lysates were incubated with CPRG substrate buffer (Roche Molecular Biochemicals), and absorbance was measured with a Benchmark microplate reader (Bio-Rad).

Nuclear Extract Preparations—Mf4/4 cells (2×10^6) were stimulated with LPS and/or IFN-γ for various times. After washing in phosphate-buffered saline, cells were resuspended in a buffer containing 10 mM Hepes (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2 mM Pefabloc, and 0.3 mM aprotinin and incubated for 15 m at 4 °C. 50 μl of 10% Nonidet P-40 were then added, and the cells were vortexed and centrifuged at 20000 × *g* for 15 m. The pellet was resuspended in a buffer containing 20 mM Hepes (pH 7.5), 1% Nonidet P-40, 1 mM $MgCl_2$, 400 mM NaCl, 10 mM KCl, 20% glycerol, 0.5 mM EGTA, 0.1 mM EDTA, 0.5 mM dithiothreitol, 2 mM Pefabloc, and 0.3 mM aprotinin. After an additional incubation for 20 m on ice, the suspension was centrifuged again at 20000 × *g* for 5 m, and the supernatant was stored at −70 °C.

Electrophoretic Mobility Shift Assay—DNA binding was analyzed by incubating 8 μg of nuclear proteins for 30 m at room temperature with a specific ^{32}P-labeled oligonucleotide probe. Binding buffer consisted of 20 mM HEPES (pH 7.5), 60 mM KCl, 4% Ficoll 400, 2 mM dithiothreitol, 100 mg/ml poly[d(I·C)], and 1 mg/ml bovine serum albumin. Samples were analyzed by electrophoresis on a 5% native polyacrylamide gel that was run for 1.5 h at 100 V in 0.5 ×TBE buffer, pH 8.0. Gels were dried, and bands were visualized by autoradiography. In supershift experiments, 1 μg of anti-c-Rel polyclonal antibody (Santa Cruz Biotechnology) or 1 μg of anti-phospho-STAT1 polyclonal antibody (Upstate Biotechnology) was added to 8 μg of nuclear extract and incubated for 15 m before adding the ^{32}P-labeled oligonucleotide probe. The oligonucleotides used were based on the putative NF-κB and STAT binding sites in the caspase-11 promoter, as predicted by the MatInspector Professional (genomatix.gsf.de/cgi-bin/mat fam/mat fam.pl; Ref. 27) and Biobase Transfac (www.biobase.de/cgi-bin/biobase/transfac/start.cgi; Ref. 28) transcription factor search programs. NF-κB-specific wild type (WT) and mutant (underlined) oligonucleotides were: 5′-agctTACAGGGGATTTCCCCACAG-3′ (NF-κBWT) and 5′-agctTACAGGGGATGGCCCCACAG-3′ (NF-κBmut). STAT-specific WT and mutant (underlined) oligos were: 5′-agctTCAACATCTCCTGGAAGT-3′ (STAT-1WT) and 5′-agctTCAACATCTCCTGCCAGT-3′ (STAT-1Mut). Double-stranded oligonucleotides were obtained by mixing the single-stranded oligos with their complements in a molar ratio of 1:1, incubating them for 10 m at 95 °C, and cooling down slowly to 4 °C.

RESULTS

Isolation of the Mouse Caspase-11 Promoter Region—The 5′-flanking region of the mouse caspase-11 gene was cloned using the mouse genome walker kit (Clontech). Primers were designed based on the 5′-end of the caspase-11 cDNA (GenBank™ accession numbers NM_007609 and U59463). We isolated four caspase-11 promoter fragments of the five genomic DNA pools, with lengths ranging from 951 to 211 bp (Fig. 1*A*). The fragments were subcloned into the pGL3-basic reporter plasmid (Promega) to generate pGL3casp11p and pGL3casp11pΔ1-Δ3. After sequencing, we determined whether the DNA fragments contained upstream sequences of the caspase-11 gene. Therefore sequence overlap was analyzed with the BCM ClustalW multiple sequence alignment tool (searchlauncher.bcm.tmc.edu/multi-align/multi-align.html; Ref. 29). As expected, all four fragments contained overlapping parts of the caspase-11 promoter sequence and have at their 3′-end the first 25 bp of the caspase-11 cDNA (Fig. 1*A*). This confirms that the genomic sequences isolated by genomic walking correspond to the proximal 5′-flanking regions of caspase-

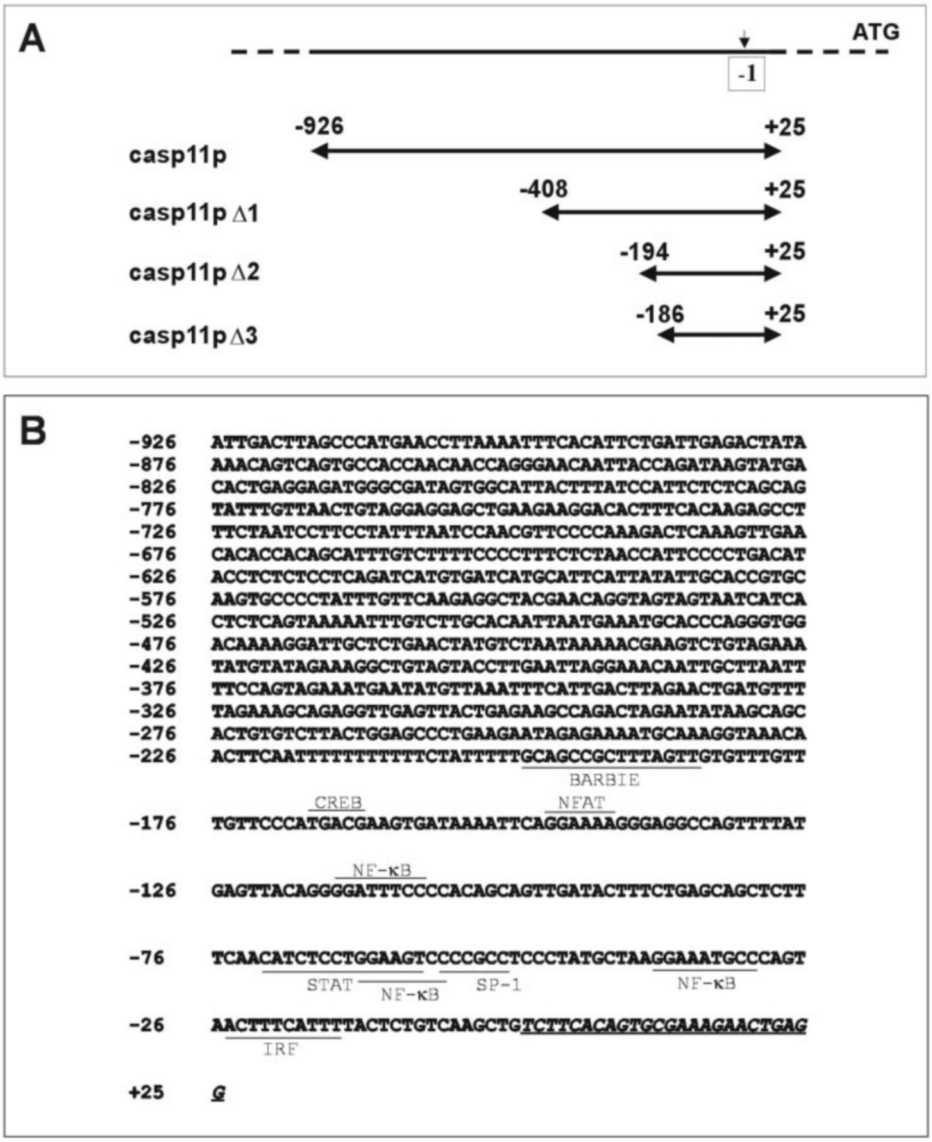

FIG. 1. **Overview of the cloned caspase-11 promoter fragments and the caspase-11 promoter sequence.** *A*, schematic representation of the size and overlap of the cloned 5′-upstream sequences of the caspase-11 gene. Four caspase-11 promoter fragments ranging from 951 to 211 bp were generated through PCR on the genome walker libraries. Each fragment contains 25 bp of the cDNA and overlapping parts of the upstream promoter sequence ranging from 926 to 186 bp. An *arrow* indicates the start of the mRNA (−1). *B*, nt sequence of the caspase-11 gene promoter. The 5′-flanking region of the caspase-11 gene was isolated via genomic walking, subcloned in the pGL3-basic vector, and sequenced. Numbering is relative to the start of the mRNA (first 25 nt of the cDNA are shown in *italics* and are *underlined)*. Binding sites for transcription factors were predicted using the MatInspector program. Only a selection of putative binding sites is shown.

11. The determined sequence of the caspase-11 promoter fragment was deposited to GenBank™ under accession number AF539416 (Fig. 1*B*). Blast analysis of the cloned sequence against the mouse genome data base confirmed that the cloned sequence was directly 5′-upstream of the transcription start site of caspase-11. Analysis of this 5′-flanking region using PromoterInspector software (Genomatix Software GmbH, Munich) did not result in the prediction of a core promoter region. No TATA-box and no GC-box were found in the environment of the transcription start site. Despite the lack of these important elements, we could identify several putative transcription factor binding sites in the caspase-11 promoter region (partially shown in Fig. 1*A*; MatInspector professional, Genomatix Software GmbH, Munich; with a perfect core similarity (= 1) and an optimized matrix similarity).

Functional Characterization of the Caspase-11 5′-Flanking Region in Macrophages—To test whether the cloned fragments contained promoter activity, we transiently transfected the pGL3casp11p and pGL3casp11pΔ1-Δ3 constructs, encoding the modified firefly luciferase preceded by putative caspase-11 promoter fragments into the Mf4/4 macrophage cell line (Fig. 2). These cells were previously shown to up-regulate caspase-11 expression upon stimulation with LPS (17). Mf4/4 cells were stimulated for 6 h with either 1 μg/ml LPS or 250 international units/ml IFN-γ, or both. Background levels of luciferase expression from all reporter constructs were comparable and most likely result from stress-induced activation of the caspase-11 promoter due to the transfection procedure. However, LPS as well as IFN-γ led to a 3- to 4-fold increase in luciferase expression from the pGL3casp11p construct. In some experiments, up to 10-fold inductions could be obtained (data not shown). A combination of both stimuli resulted in a synergistic effect on caspase-11 promoter-driven luciferase expression. Because the shortest promoter fragment, casp11pΔ3 (-186/+25), is still as active as the longest casp11p, (-926/+25), we can conclude that the most important caspase-11 promoter regions that confer

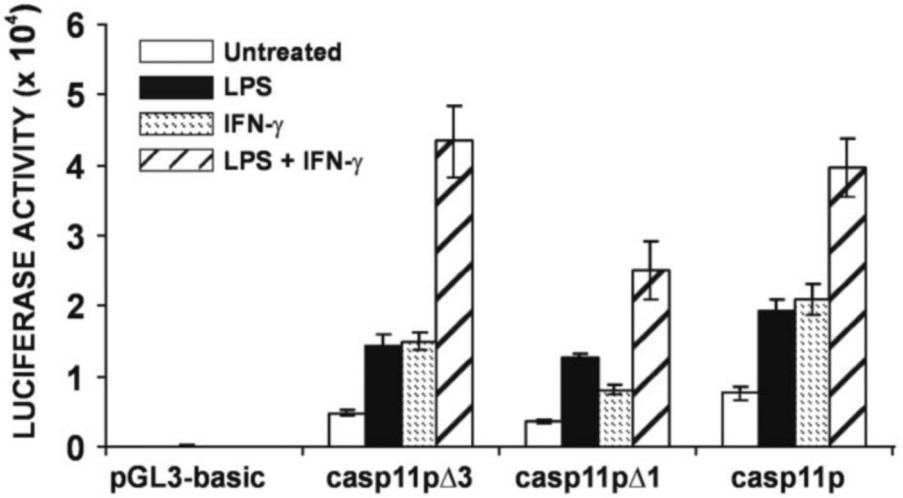

FIG. 2. **Effect of LPS and IFN-γ on caspase-11 promoter activity.** Three WT reporter constructs (pGL3casp11p (−926/+25), pGL3casp11pΔ1 (−408/+25), and pGL3casp11pΔ3 (−186/+25)) were cotransfected with pEF6/*Myc*-His/*LacZ* into Mf4/4 macrophage cells by electroporation. Luciferase activity was assayed after 6 h of stimulation with LPS (1 μg/ml) and/or IFN-γ (250 units/ml). The ratio of firefly luciferase and β-galactosidase activity was calculated. Results are represented as mean values ± S.D. of a representative experiment done in triplicate.

responsiveness to LPS and IFN-γ are located within the first 186 bp upstream of the transcription start site.

Role for NF-κB and STAT Binding Sites in Regulation of the Caspase-11 Promoter—To identify the segments of the caspase-11 promoter that are responsible for the LPS- and IFN-γ-inducible expression of caspase-11, we generated eight additional 5′-progressive deletion mutants of the cloned promoter sequence by PCR. Amplified DNA fragments (schematically represented in Fig. 3*A*) containing caspase-11 promoter regions that vary in length from 201 to 49 bp were again cloned into the pGL3-basic vector and transfected into Mf4/4 macrophages. Firefly luciferase and β-galactosidase activity were measured after 6 h of stimulation with LPS and/or IFN-γ. The −201/+25 and −181/+25 fragments showed a similar activation pattern as the casp11pΔ3 construct (−186/+25 fragment) (Fig. 3*B*). Deletion of a region between nt −181 and −155 led to a small drop in LPS inducibility, whereas IFN-γ-dependent induction was still unchanged. LPS inducibility disappeared completely upon deletion of a region between nt −139 and −113, suggesting the presence of an essential transcription factor binding site in this region. Although the IFN-γ response also decreased upon deletion of this region, complete loss of IFN-γ responsiveness only occurred upon deletion of a region between nt −82 and −49. A search with the MatInspector software (Genomatix) revealed a putative κB site between nt −119 and −109 (core similarity = 1, matrix similarity = 1), and a putative STAT binding site between nt −71 and −59 (core similarity = 1, matrix similarity = 0.83), suggesting a role for NF-κB and STAT transcription factors in the regulation of caspase-11 promoter activity in response to LPS and IFN-γ, respectively. To confirm the role of NF-κB and STAT, two mutant pGL3casp11pΔ3 reporter constructs were made that contain a double point mutation in the core of the binding sites for NF-κB and STAT, respectively (Fig. 4*A*). Design of the mutant primers was based on the transcription factor consensus binding sequences found in the Transfac data base (Biobase). To alter the κB site, two T nt were replaced by two Gs. In the case of the STAT site, a G and an A were replaced by two T nt. The goal of these point mutations was to abolish binding of the transcription factors to their target DNA. WT pGL3casp11pΔ3 and mutant reporter constructs were transfected in Mf4/4 cells, and luciferase activity was assayed after stimulation for 6 h with LPS and/or IFN-γ. Results demonstrated that a double point mutation in the κB site completely abrogated the ability of the caspase-11 promoter to respond to LPS, whereas IFN-γ inducibility was retained (Fig. 4*B*). This indicates that the NF-κB site is indeed essential for LPS-induced caspase-11 promoter activity. As already indicated by the results obtained with the deletion constructs, mutation of the STAT binding site results in a complete loss of IFN-γ responsiveness of the caspase-11 promoter. Mutation of the STAT binding site also abolished the induction by LPS, suggesting that this region is also involved in the regulation of LPS responsiveness. Most likely this reflects the involvement of a second NF-κB site, which partially overlaps with the STAT binding site (see Fig. 1*B*), in the LPS responsiveness of the caspase-11 promoter.

NF-κB and STAT Factors Bind to the Caspase-11 Promoter DNA—To further confirm the binding specificity of NF-κB and STAT transcription factors to the predicted κB and STAT binding elements, nuclear extracts of LPS- and/or IFN-γ-treated Mf4/4 macrophages were analyzed in a gel shift experiment with ^{32}P-labeled double-stranded oligonucleotides corresponding to WT or mutated κB and STAT regions of the caspase-11 promoter. The mutations were similar to those that were shown to abolish LPS- or IFN-γ-induced caspase-11 promoter activation in transient transfection studies described above. LPS induced a rapid and sustained binding to the WT κB probe (Fig. 5*A*) but not to the mutant κB probe (Fig. 5*B*). In contrast, IFN-γ failed to induce binding to the κB probe, which is in agreement with our observation that mutation of the κB site does not abrogate the IFN-γ responsiveness of the caspase-11 promoter. Because the MatInspector software revealed that the NF-κB family member c-Rel had the highest probability to bind the identified κB site, we also performed a gel shift assay in which we preincubated the nuclear extracts with anti-c-Rel-specific antibodies before incubating them with the radioactively labeled WT κB probe. Indeed, a supershift of the κB-specific band confirmed the presence of c-Rel in the complex that is bound in an LPS-responsive way to the κB element in the caspase-11 promoter (Fig. 5*C*, *last lane*). Because the supershift was only partial, it is likely that other NF-κB family members are also able to bind the κB element in the caspase-11 promoter.

Binding to the STAT site was shown in a similar way. Only IFN-γ was able to induce a gel shift with the STAT-specific probe (Fig. 5*D*). No binding of NF-κB to this probe was detected, because only part of the NF-κB binding site was present here. Mutation of the STAT site abrogated the binding of STAT proteins (Fig. 5*E*). A supershift of the STAT band with anti-STAT-1-specific antibodies confirmed the IFN-γ-induced binding of STAT-1 to this region of the caspase-11 promoter (Fig. 5*F*, *last lane*). In conclusion, our results show that LPS and IFN-γ can induce caspase-11 expression by activating the bind-

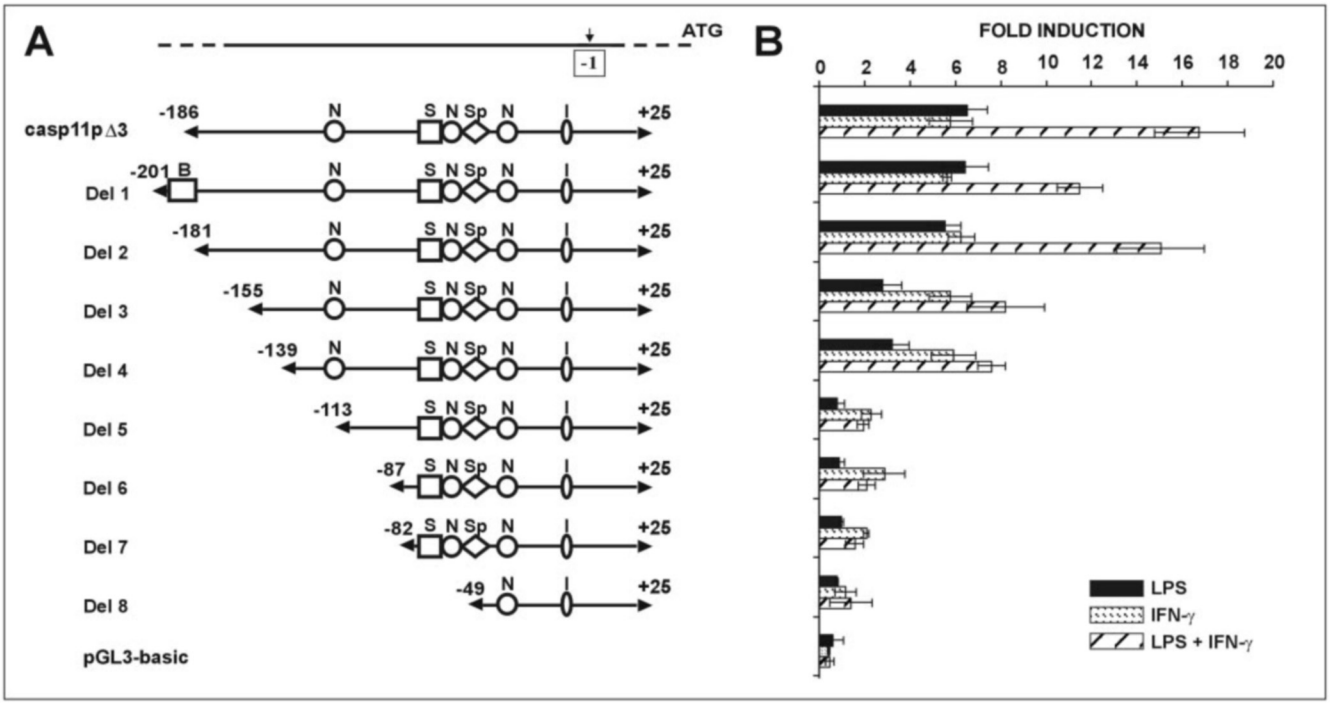

FIG. 3. **Deletion analysis of caspase-11 promoter activity in Mf4/4 cells.** *A,* schematic representation of 5′-deletion constructs of the caspase-11 promoter. Eight 5′-progressive deletion constructs, containing between 201 and 49 bp of the 5′-upstream sequence of the caspase-11 gene and 25 bp of the cDNA, were generated by PCR and cloned into the promoterless pGL-3 basic vector. A selection of putative transcription factor binding sites is shown: *B*, BARBIE (barbiturate inducible element); *N*, NF-κB; *S*, STAT; *Sp*, stimulating protein 1; *I*, interferon regulatory factor. *B*, Each reporter construct was cotransfected with pEF6/*Myc*-His/*LacZ* into Mf4/4 macrophage cells, and luciferase and β-galactosidase activity were measured after 6 h stimulation with LPS (1 μg/ml) and/or IFN-γ (250 units/ml). The activity ratio of unstimulated cells was arbitrarily set at 1 for each construct. Fold induction of the luciferase activity in stimulated conditions is shown. Experiments were repeated several times, and results of a representative experiment are given as means ± S.D. ($n = 3$).

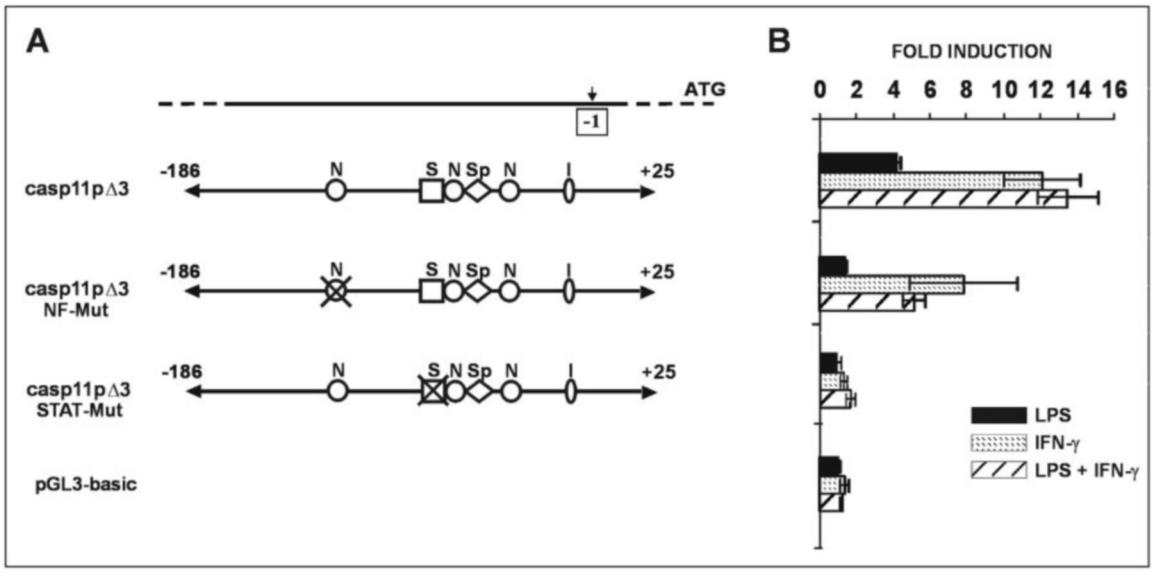

FIG. 4. **Site-specific mutational analysis of the caspase-11 promoter.** *A,* specific double point mutations were introduced in the caspase-11 reporter constructs by PCR. Mutated sites are indicated by *crossed symbols*. *B*, each WT or mutant reporter construct was cotransfected with pEF6/*Myc*-His/*LacZ* into Mf4/4 macrophage cells, and luciferase and β-galactosidase activity were assayed after 6 h of stimulation with LPS (1 μg/ml) and/or IFN-γ (250 units/ml). The ratio of firefly luciferase and β-galactosidase activity was calculated. The activity ratio of unstimulated cells was arbitrarily set at 1 for each construct. Fold induction of the luciferase activity in stimulated conditions is shown. Results are given as mean values ± S.D. of a representative experiment done in triplicate.

ing of NF-κB and STAT proteins, respectively, to the caspase-11 promoter.

DISCUSSION

The expression of caspase-11 is the most stringently regulated among all the caspases identified so far. Whereas most of the caspases can be detected in unstimulated cells, caspase-11 expression is below the detection limit in most tissues or cells. Most interestingly, its expression is highly inducible upon injection of mice with LPS (18). Apart from LPS, a variety of other stimuli, including ischemic brain injury, systemic inflammation, and head trauma (14, 19, 20), also induce the produc-

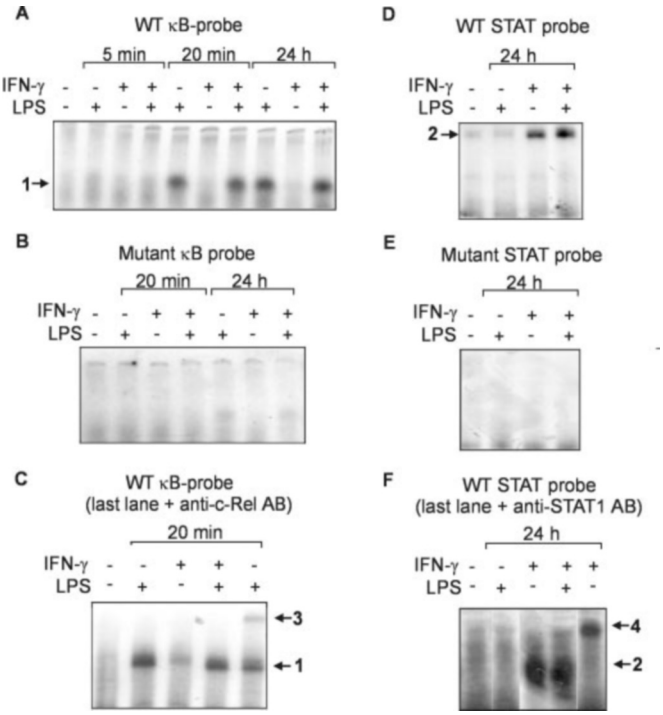

FIG. 5. **NF-κB and STAT1 bind to the κB and STAT binding sites in the caspase-11 promoter.** Mf4/4 cells were stimulated for the indicated time course, and nuclear extracts were prepared. Bandshift analysis was performed using fragments of the isolated WT or mutant κB and STAT-containing promoter as probes. Binding of c-Rel NF-κB and STAT1 to the corresponding probe was demonstrated by a supershift upon preincubation of nuclear extract with anti-c-Rel NF-κB- (*C*, *last lane*) or anti-STAT1- (*D*, *last lane*) specific antibodies. *Arrows* indicate the location of the shifted and supershifted NF-κB (1, 3) and STAT (2, 4) complexes, respectively.

tion of caspase-11. Although caspase-11 can be proteolytically activated, the cleavage may not be essential for its activation. Most likely, elevated expression of caspase-11 protein concentration in stimulated cells is sufficient for its autoactivation. This is also suggested by the observation that, although caspase-11 is essential for LPS-induced production of IL-1β in mice, its cleavage into active p20 and p10 subunits cannot be observed in any tissue studied so far (14, 18). Similarly, it is now generally accepted that other long prodomain caspases, such as caspase-9, can be activated without cleavage (30).

Because caspase-11 is a dual activator of caspase-1 and -3 under pathological conditions, including endotoxic shock, multiple sclerosis, and brain ischemia (19, 31), it is critical to identify the mechanisms that regulate its transcriptional activation. The present work demonstrates that LPS- and IFN-γ-induced expression of caspase-11 in macrophages is mediated by elements that are situated within the first 200 bp upstream of the transcription initiation site. More specifically, site-specific mutagenesis and gel shift experiments revealed the essential binding of NF-κB and STAT transcription factors to specific regions in the caspase-11 gene promoter in response to LPS and IFN-γ, respectively. We previously showed (17) that LPS-induced activation of NF-κB-dependent gene expression in Mf4/4 macrophages is p38 mitogen-activated protein (MAP) kinase-dependent. Recently, LPS-induced expression of caspase-11 in astrocytes was found to depend on p38 MAP kinase (32). Taken together, these observations suggest a crucial role for the p38 MAP kinase and NF-κB pathway in the LPS-induced regulation of caspase-11 expression. In contrast, NF-κB does not seem to play a role in the IFN-γ responsiveness of the caspase-11 promoter. However, in the case of IFN-γ a role for members of the STAT family could be demonstrated. This is in line with the observations of Lee *et al*. (16), who found that basal caspase-11 expression in lymphocytes is abolished in STAT1 and IFN-γ receptor-deficient mice. Mutation of the STAT binding site also leads to mutation of a second NF-κB site, which partially overlaps with the STAT site. This probably explains why the LPS response also is abrogated in the mutant STAT reporter constructs. NF-κB and STAT transcription factors consist of a whole family of related proteins. In many cases, several family members can bind to a single binding site, and it is still largely unclear what determines specificity within a single family of transcription factors. In the case of NF-κB, which binds as a homo- or heterodimeric complex to a specific recognition sequence in the promoter, we were able to demonstrate the LPS-inducible binding of c-Rel to the κB site starting 119 bp upstream of the transcription initiation site. Because not all NF-κB complexes could be supershifted with a c-Rel-specific antibody in a gel shift experiment, we suspect that other NF-κB family members are also involved. Similarly, it has been well established that several effects of IFNs are mediated by STAT1 and STAT3 (33, 34). However, both transcription factors have a very similar consensus DNA binding sequence, making it impossible to clearly differentiate between these two *in silico*. Supershift experiments showed that at least STAT1 is able to bind the caspase-11 gene promoter at a putative STAT-binding site situated between −71 and −59 bp upstream of the transcription initiation site. Our reporter gene experiments also showed a synergistic increase following stimulation of Mf4/4 cells with both LPS and IFN-γ. This is consistent with findings showing that stimulation of this macrophage cell line with both LPS and IFN-γ synergistically increases the protein levels of caspase-11 (data not shown). Transfection of reporter plasmids with mutated promoter fragments revealed that mutation of either the NF-κB or the STAT site abrogated the synergistic effect of LPS and IFN-γ, showing that both transcription factors are involved in the synergistic effect. Gel shift experiments did not reveal an increased binding of NF-κB or STAT in extracts from Mf4/4 cells stimulated with both LPS and IFN-γ, as compared with stimulation with either agent alone. It should be noted that these gel shift experiments were performed with fragments that contained either the NF-κB or the STAT site. Therefore, we cannot exclude that the synergistic effect of LPS and IFN-γ, as observed with reporter plasmids containing the whole promoter, involves the cooperative binding of NF-κB and STAT or any other unknown transcription factors.

We could identify several putative consensus binding sequences for other transcription factors as well, including IFN regulatory factor (IRF)-1 and -2 at positions −665/−653, −658/−646, −251/−239, −238/−226, and −24/−12) and activator protein 1 at −555/−545. Lee *et al*. (16) already showed that IRF-1 is not involved in IFN-γ- and LPS-induced caspase-11 expression. Our results with deletion mutants demonstrate that Activator Protein 1 also is not essential for caspase-11 expression in response to LPS or IFN-γ. Recent reports revealed that active caspase-11 can also be found in ischemic brain (14, 19, 20). In such conditions, hypoxia-inducible factor 1α (HIF1α) has been shown to be a very important transcription factor (35), suggesting a possible role for HIF1α in caspase-11 expression in ischemic brain. However, we could not identify a putative HIF1α binding sequence within the first 926 bp upstream of the transcription initiation site. This indicates that there are probably other regulatory elements farther upstream of the cloned promoter fragments that are important for hypoxia-inducible expression of caspase-11.

The identity of the human ortholog of murine caspase-11 is still unclear. Caspase-11 shares the highest homology with

human caspase-4 and -5. However, caspase-4 expression appears to be present in unstimulated cells (36). In contrast, human caspase-5 has been shown to be LPS-inducible in THP1 monocytes (37), making it a more likely candidate for the human ortholog of caspase-11. Nevertheless, cloning of the human caspase-5 gene promoter did not reveal any obvious homology with the caspase-11 promoter fragment that is described in the present study (data not shown), suggesting that both caspases might be regulated differently. Future studies will have to focus on how caspase-11 is expressed upon other stimuli, as well as on how it is activated once it is expressed. Because knockout studies have already revealed the essential role of caspase-11 in several diseases, identification of the underlying mechanisms will be important for the development of novel therapeutic strategies.

Acknowledgments—We thank A. Meeus and W. Burm for assistance with cell culture work.

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