

Identification of Conserved Domains in *Salmonella muenchen* Flagellin That Are Essential for Its Ability to Activate TLR5 and to Induce an Inflammatory Response *in Vitro**

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The bacterial surface protein flagellin is widely distributed and well conserved among distant bacterial species. We and other investigators have reported recently that purified flagellin from *Salmonella dublin* or recombinant flagellin of *Salmonella muenchen* origin binds to the eukaryotic toll receptor TLR5 and activates the nuclear translocation of NF- κ B and mitogen-activated protein kinase, resulting in the release of a host of pro-inflammatory mediators *in vitro* and *in vivo*. The amino acid sequence alignment of flagellins from various Gram-negative bacteria shows that the C and N termini are well conserved. It is possible that sequences within the N and C termini or both may regulate the pro-inflammatory activity of flagellin. Here we set out to map more precisely the regions in both termini that are required for TLR5 activation and pro-inflammatory signaling. Systematic deletion of amino acids from either terminus progressively reduced eukaryotic pro-inflammatory activation. However, deletion of amino acids 95–108 (motif N) in the N terminus and 441–449 (motif C) in the C terminus abolished pro-inflammatory activity completely. Site-directed mutagenesis analysis provided further evidence for the importance of motifs N and C. We also present evidence for the functional role of motifs N and C with the TLR5 receptor using a reporter assay system. Taken together, our results demonstrate that the pro-inflammatory activity of flagellin results from the interaction of motif N with the TLR5 receptor on the cell surface.

The whiplike flagellum of Gram-negative bacterium is implicated in microbial pathogenicity, serving as a means of propulsion and attachment and invasion of host epithelium. Recently it has been shown that the monomeric constituent of flagella, the 55-kDa protein flagellin, is a potent activator of pro-inflammatory eukaryotic cell signaling via its interaction with membrane-bound TLR5. Flagellin-TLR5 binding induces NF- κ B nuclear translocation and activation of mitogen-activated protein kinase, resulting in the up-regulated expression of cytokines, such as tumor necrosis factor- α and IL-1-6, chemokines, includ-

ing IL-8, and pro-inflammatory free radical synthesizing enzymes, such as the inducible nitric-oxide synthase (1–6). Because many Gram-negative pathogens express flagellin, it is conceivable that flagella contribute to the host inflammatory response to infection. Evidence in support of this role comes from experimental studies in which we have shown that administration of flagellin engenders systemic tissue injury, characterized by exudative lung inflammation, liver necrosis, and circulatory shock. Therefore, flagellin presents all the features of pathogen-associated molecular patterns (PAMPs) and can be regarded as another PAMP.

Therapeutic opportunities to interrupt the pathologic effects of flagellin will be based upon a precise delineation of its interaction with TLR5 that induces pro-inflammatory signaling pathways. Biochemical analysis of *Salmonella* flagellin reveals that both conserved domains within both termini are important in inducing pro-inflammatory responses in cultured intestinal epithelial cells (6). Recent studies also suggest that the hypervariable domain is not involved in pro-inflammatory activation. A recombinantly expressed flagellin mutant, in which the central hypervariable domain was deleted, was shown not to detract from its ability to induce NF- κ B signaling, suggesting that the highly conserved N and C termini are sufficient for TLR5 activation (6). Further definition of the pro-inflammatory flagellin sequences has been provided by evidence that neutralization of the N-terminal “RINSA” domain (amino acid 31–52) by monoclonal antibodies blocks the pro-inflammatory activity of recombinant flagellins of different bacterial origin (5).

Recent studies (7, 8) have also delineated the extramembranous region of TLR5 responsible for flagellin binding. The crystal structure of flagellin resembles an aircraft, with two wings and a central rod-shaped body. The core of the latter is derived from the α -helices of both the N and C termini, whereas the outer surface of the rod is constituted by the hypervariable domain (9).

Recently, interest has been focused on *Salmonella* flagellin and its role in the induction of host pro-inflammatory responses. Although recombinant flagellin or flagella from other Gram-negative organisms, such as *Serratia*, *Proteus*, *Pseudomonas*, *Escherichia*, and *Yersinia*, stimulate pro-inflammatory responses, recombinant flagellin or flagella from *Salmonella* strains are generally the most potent inducers (2, 3, 5, 10). LPS and flagellin induce similar pro-inflammatory responses, but

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¹ The abbreviations used are: IL, interleukin; NO, nitric oxide; PAMPs, pathogen-associated molecular patterns; TLR, Toll-like receptors; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine

serum; IFN- γ , interferon- γ ; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assays; CHO, Chinese hamster ovary; LPS, lipopolysaccharide.

flagellin is much more potent and induces widespread oxidative stress in most organs (11). Previously, it was thought that pro-inflammatory activity of flagellin might be secondary to contaminating LPS, but extensive studies have confirmed the intrinsic pro-inflammatory properties of flagellin.

It is now clear that Toll-like receptors (TLR) are involved in the signal transduction pathways of mammalian, insect, and plant PAMPs. TLRs are an evolutionarily conserved family of receptors that function in innate immunity. These proteins consist of an extracellular domain with a leucine-rich repeat, one or two cysteine-rich regions, and a cytoplasmic region called the Toll/IL-1 receptor domain. The mechanism of TLR signaling is quite similar to that of the IL-1 receptor family, because both receptor families possess Toll/IL-1 receptor domains. Recently, it has been shown that TLR5 mediates flagellin-dependent signaling in transfected mammalian cells (12). Activation of NF- κ B-mediated gene expression in the TLR5 signal transduction pathway involves a signaling complex that includes MyD88 and IL-1 receptor-associated kinase (12–14). Further studies also demonstrated that TLR5 is expressed on the basolateral surface of intestine epithelial cells, and flagellin translocates across epithelial monolayers from the luminal domain to the basolateral membrane domain (15, 16). In a recent study, it was established that TLR5 forms heterodimeric complexes with TLR4 as well as homomeric complexes and that these TLR5-TLR4 complexes are involved in the induction of nitric-oxide synthase expression by flagellin (17).

The purpose of the present investigation was to further define the regions in the N and C termini that contribute to the eukaryotic pro-inflammatory activity of flagellin. By using deletion and site-directed mutagenic methods, we have mapped the active site to a relatively short region in the N- and C-terminal domains. Additional evidence is provided on the specific region of flagellin responsible for functional activation of TLR-5.

MATERIALS AND METHODS

Cell Culture and NO Production Assay—Monolayers of Chinese hamster ovary (CHO)-K1 fibroblast cells (ATCC, American Type Culture Collection, Manassas, VA) grown in RPMI 1650 (Invitrogen) were supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 units/ml penicillin and streptomycin. DLD-1 cells (ATCC) grown at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) were supplemented with 10% FBS, 4 mM glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 1 mM sodium pyruvate, and antibiotics. Cells, between passages 5 and 15, were seeded at a density of 50,000 cells/cm² in 96-well plates and allowed to grow 72–96 h to confluence before use. Growth medium was changed the day before use. Cells were washed once with DMEM without FBS but containing antibiotics before the addition of flagellin proteins diluted in DMEM containing IFN- γ and antibiotics. After incubating cells with flagellin for 20 h, culture supernatants were collected. The combined concentration of nitrite and nitrate, the degradation products of NO in the culture medium, was determined by the Griess reaction after nitrate reduction, as described previously (18).

IL-8 Secretion—Human alveolar epithelial cells (A549), an adenocarcinoma cell line with the alveolar type 2 phenotype, were grown in 96-well plates in RPMI 1650 medium containing 10% FBS. Confluent cells were stimulated with recombinant flagellin for 24 h. The conditioned medium (in 96-well plates) was aspirated, centrifuged, and assayed for the production of IL-8 by commercial ELISA (R&D Systems, Minneapolis, MN).

Construction of Wild-type and Mutant Flagellin Plasmid DNAs—The construction of a bacterial expression plasmid pET30-Fla containing *S. muenchen* flagellin-coding sequences has been described previously (5). This plasmid contains an N-terminal His₆ fusion under control of a T7 promoter and was used as the base for subsequent mutagenesis. To construct truncation, deletion, and chimeric mutant plasmids, appropriate oligonucleotides with restriction sites were used to PCR-amplify flagellin sequences. PCR was performed on 10 ng of template DNA in a 50- μ l volume using PCR SuperMix (Invitrogen) and 0.5 μ M concentrations of each primer for 30 cycles of 95 °C for 30 s, 62 °C for 30 s, and

72 °C for 1 min. PCR products were digested with appropriate restriction enzymes, agarose gel-purified, and ligated into a pET-30C (Novagen, Madison, WI) plasmid after the plasmid had been cut with corresponding enzymes and gel-purified. To construct NC and its deletion derivatives (e.g. mNC8, mNC6, and mNC4) without the hypervariable region, two separate PCR products of N- and C-terminal domains were inserted into an expression plasmid simultaneously. Point mutations in the flagellin gene were created by site-directed mutagenesis (Clontech, Palo Alto, CA). The recombinant plasmids were then introduced into *Escherichia coli* BL21 (DE3) (Novagen) by transformation and selected in the presence of kanamycin (50 μ g/ml). All mutants were confirmed by DNA sequence analysis.

Cloning and Expression of Human TLR5—Full-length human TLR5 cDNA was cloned from a human fetal lung library. A mammalian expression vector containing FLAG epitope sequences fused to the N-terminal end of TLR5 was generated by inserting TLR5 coding sequences into a eukaryotic expression vector pFLAG-CMV3 (Sigma). Expression levels of TLR5 protein in CHO cells were monitored by transient transfection followed by immunoblotting with anti-FLAG (M2 monoclonal, Sigma) and anti-TLR5 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

GFP Reporter Plasmid Construction—IL-8 promoter sequences were amplified by PCR using a recombinant plasmid DNA (a kind gift from Dr. Gary D. Wu, University of Pennsylvania, Philadelphia) as the template. The following primers with restriction sites (underlined) were used: forward IL-8 (135), 5'-GCCATCGATGAAGTGTGATGACTCAGG; IL-8 (+46), 5'-CCGGAATTCGAAGCTTGTGTGCTCTGC. PCR-generated DNA was digested with ClaI plus EcoRI and subcloned into the ClaI/EcoRI sites of pGLOW-TOPO vector (Invitrogen) yielding the reporter construct WT-GFP.

Purification of Recombinant Proteins—A single colony of *E. coli* containing the desired plasmid was grown at 37 °C in Luria broth containing 50 μ g/ml kanamycin to an A₆₀₀ of 0.5 and then induced for 3 h with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside. Following induction, bacteria were harvested and washed with phosphate-buffered saline (PBS, pH 7.2). Cell-free lysates were prepared in 6 M guanidine chloride containing 5 mM imidazole and 0.1% Nonidet P-40 (binding buffer). After removing the insoluble material by centrifugation, the lysate was applied to a nickel-nitrotri-acetic acid-agarose (Qiagen, Valencia, CA) column, washed extensively with binding buffer, and then eluted with binding buffer containing 200 mM imidazole. The purified proteins were extensively dialyzed against PBS, and protein concentrations were determined by the Bradford method. The final proteins were checked by 10% SDS-PAGE and visualized with Coomassie Blue staining to assess protein purity, integrity, and concentration (Fig. 1B).

Stable Transfections—CHO-K1 cells (2×10^6) were plated onto 10-cm dishes and transfected the following day with 5 μ g of WT-GFP DNA. All transfections were performed using LipofectAMINE plus reagent according to the manufacturer's instructions (Invitrogen). After 4 h of incubation, medium was removed, and cells were supplemented with fresh medium containing 10% FBS and allowed to grow for 20 h. Cells were then trypsinized, seeded at a lower density in 15-cm plates, and allowed to grow in a medium containing 400 μ g/ml G418, and the medium was replaced twice a week for 3–4 weeks. G418-resistant clones were transferred individually to small plates and propagated in G418 medium before analysis. Similarly, the G418-resistant clones were retransfected with the blasticidin-resistant vector (pEF6, Invitrogen) encoding full-length TLR5, and double stable transfectants were selected in the presence of 400 μ g/ml G418 and 2 μ g/ml blasticidin. The clones were analyzed by immunoblotting for proper phenotype.

Preparation of Whole Cell and Nuclear Extracts—Whole cell or nuclear extracts were prepared as described previously (19). To prepare whole cell extracts, cells were removed from the plate by scraping into PBS, harvested, and washed twice in PBS. Cell pellets were resuspended in extraction buffer (20 mM Tris-HCl, pH 8.0, 0.4 M NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 25 mM β -glycerophosphate, 25 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1 mM PMSF, 10 μ g/ml aprotinin, 10 mM *p*-nitrophenyl phosphate, and 10% glycerol) and incubated on ice for 20 min. After removing the insoluble material by centrifugation, supernatants were collected and stored at -70 °C in aliquots. To prepare nuclear extracts, cell pellets were suspended in 400 μ l of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF). After 10 min of incubation at 4 °C, 25 μ l of 10% Nonidet P-40 (Sigma) was added, and the tubes were vortexed gently for 10 s. Nuclei were collected by centrifugation at 10,000 \times g for 2 min, and the pellets were suspended in 50 μ l of ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF). The tubes were vigorously vortexed and rocked at 4 °C for

15 min. Nuclear protein extracts were obtained by centrifugation (10,000 × *g* for 5 min), and aliquots of the supernatant were stored at -70 °C.

Immunoblotting—Whole cell extracts (50 µg) were mixed with equal volumes of 2× SDS protein gel loading buffer, boiled, and fractionated on a 10% SDS-polyacrylamide gel. Electrophoretically separated proteins were blotted onto a polyvinylidene difluoride membrane. Membranes were blocked with 3% bovine serum albumin in 20 mM Tris, pH 7.4, 500 mM NaCl, and 0.1% Tween 20 for 16 h at 4 °C and then with rabbit polyclonal anti-FLAG or anti-GFP (Sigma) for 3 h. Blots were washed and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody. The antigen-antibody complexes were detected by chemiluminescence (Amersham Biosciences).

Electrophoretic Mobility Shift Assays—Standard DNA binding assays were performed using 0.5 ng of labeled probe. NF-κB oligonucleotide was purchased from Promega (Madison, WI). For the EMSA analysis, 5 µg of nuclear proteins were incubated with binding buffer (20 mM HEPES, pH 7.9, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, pH 8, and 10% glycerol) containing 1 µg of poly(dI-dC) and γ-³²P-end-labeled probe for 30 min at 4 °C. Protein-DNA complexes were resolved in low ionic strength 5% native polyacrylamide gels with 0.5× TBE as the running buffer. Electrophoresis was performed at room temperature until the dye reached the bottom of the gel. Gels were transferred to Whatman 3M paper, dried, and exposed to photographic film at -70 °C. For supershift assays, specific antibodies (p50, p65, and Stat3 from Santa Cruz Biotechnology) were added to the reaction mixture as indicated and incubated at 4 °C for an additional 10 min.

RESULTS

To test the functional significance of the conserved regions of flagellin, mutations were introduced into both terminal domains (Fig. 1), as described under “Materials and Methods.” *S. muenchen* flagellin coding sequences in a plasmid were used as a template to amplify mutant sequences by PCR with appropriate primers. After digesting PCR products with appropriate restriction enzymes, DNA fragments were cloned into an expression vector with His tag sequences. Recombinant proteins were expressed in *E. coli* and purified to near-homogeneity under denaturing conditions in order to minimize the contamination of other bacterial proteins and endotoxins that weakly interact with recombinant flagellin, by using affinity chromatography as shown in Fig. 1B. These proteins were tested at various concentrations for biological activity by incubating with IFN-γ-primed DLD-1 cells. After incubating for 20 h, the concentration of NO (nitrite and nitrate, stable breakdown products of NO) in culture supernatant was measured.

First, we made truncated recombinantly expressed proteins with deletions in the N terminus (HC, 173–505) or C terminus (NH, 1–417) or hypervariable region (NC, 1–172 + 418–505). Full-length recombinant protein, when tested at four different concentrations, elicited a significant pro-inflammatory response in DLD-1 cells at a concentration of 0.5 ng/ml, reaching a maximum response at 12.5 ng/ml. As shown in Fig. 2A, recombinant flagellin mutants lacking either terminal region failed to induce NO production. However, the recombinant NC mutant lacking the hypervariable domain induced a significant dose-dependent accumulation of NO comparable with that of wild-type recombinant flagellin. From several independent experiments, we consistently observed that the NC mutant was ~2-fold less active at lower concentrations in comparison to the full-length recombinant protein. There was also no increase or change in the activity of the NC mutant when maltose-binding protein sequences of similar length to that of the flagellin hypervariable region were inserted between the N and C domains (data not shown). Taken together, these results indicate that the conserved N and C domains are functionally important for pro-inflammatory signaling in eukaryotic cells and that the hypervariable domain, in contrast, is not functionally relevant to this process. The dispensability of the hypervariable domain was expected, because this region varies profoundly in length and sequence from species to species.

By having established that both terminal domains of flagellin are required to stimulate a pro-inflammatory response, we next sought to determine the specific regions within each domain that are required for pro-inflammatory activity. Expression plasmids were created lacking stretches of amino acids within the N terminus (N1, 53–505; N2, 81–505; N3, 95–505 and N4, 109–505) or C terminus (C1, 1–481; C2, 1–471; C3, 1–460, C4, 1–450; and C5, 1–440). These recombinant proteins were then tested for their ability to elicit biological activity, as measured by the level of NO production, in IFN-γ-primed DLD-1 cells. Terminal truncation of flagellin diminished pro-inflammatory activity, in proportion to the extent of the deletion, up to a maximum deletion of 95 amino acids in the N terminus (N1, N2, and N3) and beyond 450 amino acids in the C terminus (C1, C2, C3, and C4), (Fig. 2, A and B). Further deletions in either the N terminus (N4, 109–505) or the C terminus (C5, 1–440) abolished DLD-1 production of NO induced by flagellin. Taken together, these results suggest that both termini of flagellin contribute to its pro-inflammatory activity and that critical residues involved in this process are localized to a 14-amino acid region in the N terminus (motif N, 95–108) and a 9-amino acid region in the C terminus (motif C, 441–449). Interestingly, the most highly conserved region of flagellins across Gram-negative pathogens, the so-called RIN-SKA region (31–47) in the N terminus, was not essential for the full induction of NO production in DLD-1 cells.

Pro-inflammatory activity of flagellin is readily assayed by measuring not only NO production but also by measuring the epithelial production of pro-inflammatory cytokines, including tumor necrosis factor-α, IL-6, IL-8, and IL-12. Previously, it has been shown that epithelial cells produce IL-8 in response to flagellin (4, 20–22). The extent of eukaryotic pro-inflammatory gene expression in response to flagellin varies according to cell type. Human intestinal epithelial (DLD-1) cells are less responsive compared with human lung epithelial (A549) cells, which have been reported to induced IL-8 expression in response to concentrations of flagellin as low as 1 pg/ml (20).

Mutant and wild-type recombinant *S. muenchen* flagellins, described in Fig. 2, were incubated with A549 cells for 20 h at the indicated concentrations, and IL-8 release was quantified by ELISA (Fig. 3). The results obtained with A549 cells paralleled those obtained in DLD-1 cells (compare Fig. 2 and Fig. 3), clearly demonstrating that the N-terminal motif (95–108) and the C-terminal motif (441–449) are both essential for pro-inflammatory activity. Other regions in the conserved N and C termini also contributed to cytokine expression in flagellin-stimulated A549 cells but were not essential for pro-inflammatory induction.

Because the findings above raised the possibility that certain deletions within the N terminus (1–94) and C terminus (451–505) have a limited effect on the induction of pro-inflammatory signaling in eukaryotic cells, we first constructed and analyzed a recombinant mutant deleting both of these regions (mtFLA, 95–450). As shown in Fig. 4A, these double deletions resulted in a near total inhibition of pro-inflammatory activity. This finding is surprising because single deletions either in the N terminus (N3, 95–505) or C terminus (1–450) have no effect on flagellin activity. We next examined these deletions in an NC construct, in which the hypervariable region was totally removed. For this purpose we made three constructs deleting part of the N terminus (mNC2, 95–172 + 418–505), C terminus (mNC3, 1–172 + 418–450), or both (mNC1, 95–172 + 418–450). All three recombinant proteins (mNC1, mNC2, and mNC3) proved inactive in inducing NO production in IFN-γ-primed DLD-1 cells. To investigate these unexpected findings further, we tested four additional recombinant NC mutants,

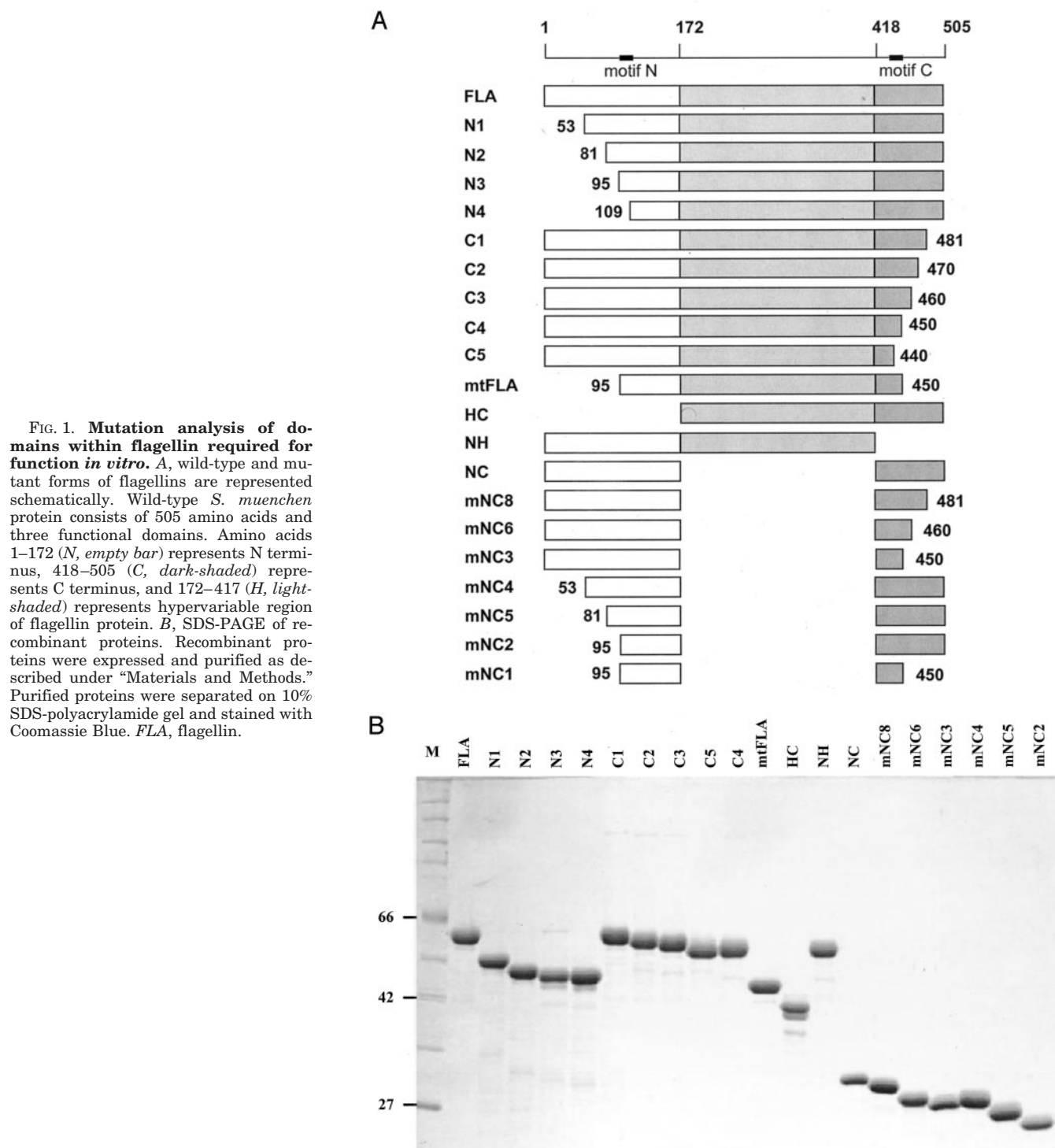


FIG. 1. Mutation analysis of domains within flagellin required for function *in vitro*. *A*, wild-type and mutant forms of flagellins are represented schematically. Wild-type *S. muenchen* protein consists of 505 amino acids and three functional domains. Amino acids 1–172 (*N*, empty bar) represents N terminus, 418–505 (*C*, dark-shaded) represents C terminus, and 172–417 (*H*, light-shaded) represents hypervariable region of flagellin protein. *B*, SDS-PAGE of recombinant proteins. Recombinant proteins were expressed and purified as described under “Materials and Methods.” Purified proteins were separated on 10% SDS-polyacrylamide gel and stained with Coomassie Blue. *FLA*, flagellin.

two with deletions in the N terminus and two with deletions located in the C terminus (Fig. 4B). Sequential deletion of amino acids 1–80 from the NC construct (mNC4, 53–172 + 418–505; and mNC5, 81–172 + 418–505) had only a slight effect on pro-inflammatory activity, whereas any deletion from the C terminus (mNC6, 1–172 + 418–481; and mNC7, 1–172 + 418–460) resulted in a sharp drop in pro-inflammatory activity. These results demonstrate that the N and C motifs are important for full pro-inflammatory activity and suggest that the remaining sequences in both domains play critical roles in maintaining proper secondary structure.

The above results encouraged us to further investigate the

behavior of recombinant flagellins in which mutations were introduced into several motif N and motif C mutational derivatives. In the previous experiments (Figs. 2 and 3), the loss in pro-inflammatory activity of mutants N4 and C5 may have resulted from a destabilization of flagellin structure caused by deletion of a larger portion of conserved N- and C-terminal domains. To eliminate this possibility, we constructed mutants in which all amino acids in motif N (N14) and motif C (C12) were changed to different amino acids simultaneously by site-directed mutagenesis. The amino acid and nucleotide sequences of wild-type and mutant *S. muenchen* flagellin are shown in Fig. 5A. Restriction sites were introduced during

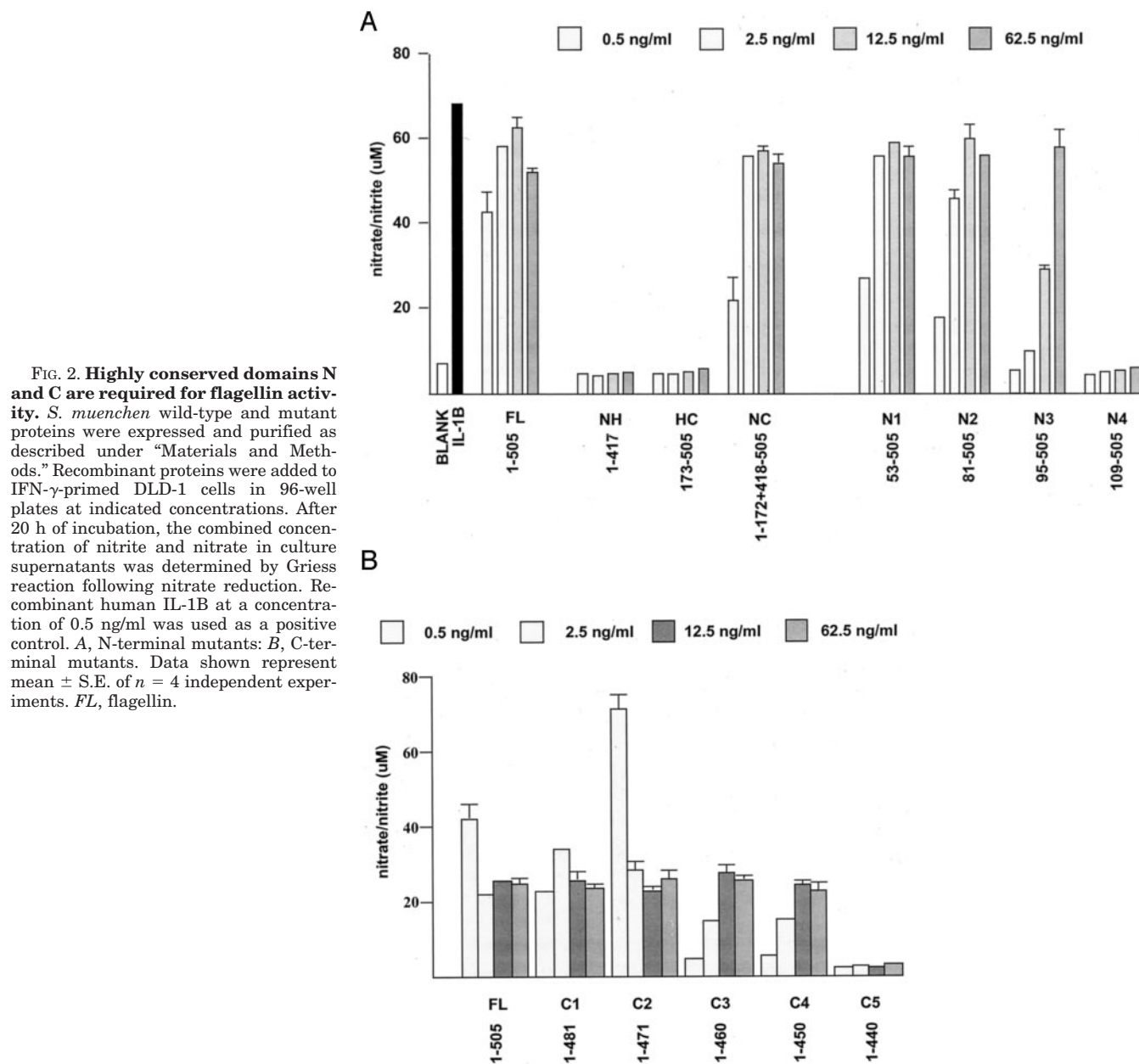


FIG. 2. Highly conserved domains N and C are required for flagellin activity. *S. muenchen* wild-type and mutant proteins were expressed and purified as described under "Materials and Methods." Recombinant proteins were added to IFN- γ -primed DLD-1 cells in 96-well plates at indicated concentrations. After 20 h of incubation, the combined concentration of nitrite and nitrate in culture supernatants was determined by Griess reaction following nitrate reduction. Recombinant human IL-1B at a concentration of 0.5 ng/ml was used as a positive control. **A**, N-terminal mutants; **B**, C-terminal mutants. Data shown represent mean \pm S.E. of $n = 4$ independent experiments. *FL*, flagellin.

cloning at the beginning and end of motifs N and C to facilitate in the deletion of the entire motif. In the next step we deleted the entire motif N (Δ N14) or motif C (Δ C12) or both.

As shown in Fig. 5B, point mutations (N14) or internal deletions (Δ N14) completely inhibited flagellin activity. Point mutations in motif C (C12) also reduced pro-inflammatory activity significantly, whereas deletion of the entire motif C (Δ C14) totally abolished pro-inflammatory activity. These results suggest that flagellin contains two widely separated motifs, neither of which is independently able to induce a pro-inflammatory response. Consistent with this hypothesis, simultaneous mutations (N14C12) or deletions (Δ N14 Δ C12) in motifs N and C abolished pro-inflammatory activity, confirming that sequences in both motifs are necessary to bring about an efficient stimulation.

By having identified those sequences in the N-terminal domain that are important for the pro-inflammatory activity of flagellin, we next sought to define the individual amino acids necessary to induce a pro-inflammatory response. We created three additional point mutations in motif N whereby (a) aspartic acid (Asp-108) was changed to alanine (D108A) or glycine

(D108G) and (b) asparagine (Asn-101) was changed to alanine (N101A). These two sites (Asp-108 and Asn-101) were selected based upon the crystal structure of flagellin, which directly reveals possible interactions between Asp-69 and Asn-100 and Asn-132 and Asp-100 of the upper and lower subunits of *S. typhimurium* flagellin, respectively (9). The NO inducing activity of recombinant flagellins with these three point mutations is shown in Fig. 6. Strikingly, the pro-inflammatory activity of all three single point mutants was similar to that of wild-type flagellin.

Because NF- κ B is known to mediate transcriptional induction of many cytokine-responsive genes (including the human nitric-oxide synthase gene), we used electrophoretic mobility shift assays (EMSA) to study flagellin interactions with IFN- γ -primed DLD-1 cells. Nuclear extracts were prepared from DLD-1 cells treated with wild-type or mutant flagellin and then assayed for NF- κ B activity. As shown in Fig. 7, the addition of wild-type flagellin or the N1 mutant induced a strong gel shift complex for NF- κ B, whereas mutants N4, C3, and N14 exhibited activity similar to that of untreated cells. With the mutant flagellin C1 or C12, induction of NF- κ B binding was reduced to

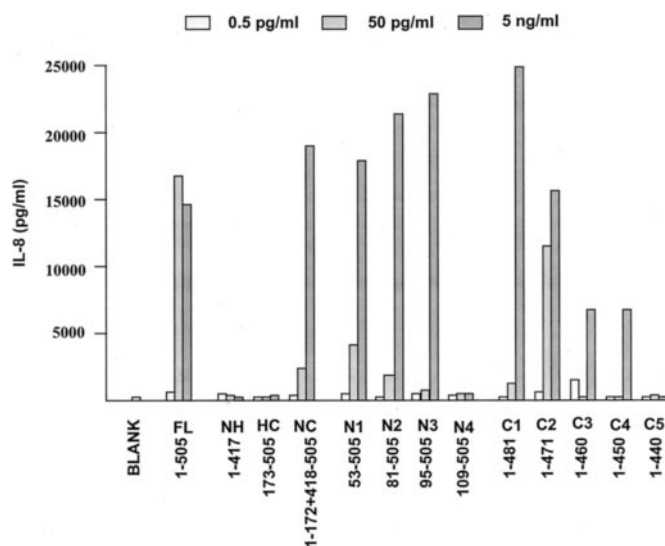


FIG. 3. IL-8 release from human alveolar epithelial cells (A549) in response to flagellin. Serial dilutions of recombinant proteins (used in previous experiment) were made in RPMI 1650 with antibiotics and were incubated with cells for 24 h. The amount of IL-8 released into the medium was measured by ELISA. Data show mean values from $n = 4$ wells obtained on one single experimental day. Similar results were obtained in three independent experiments. FL, flagellin.

a significant extent but not completely, consistent with previous results showing their incomplete suppression of NO or IL-8 production (compare with C1 and C12 activity in Fig. 2B, Fig. 3, and Fig. 5B). However, treatment with recombinant flagellin mutant proteins NH and HC showed no NF- κ B binding. In the same EMSA, supershift analysis utilizing antibodies directed against the p50 and p65 catalytic subunits confirmed the shifted complex as heterodimers of p50-p65, a well known transcriptional activator. However, the addition of a nonspecific antibody (Stat3 monoclonal antibody) had no effect on the formation of the NF- κ B complex (Fig. 7). These findings support our earlier conclusion that the pro-inflammatory activity of flagellin is located in motifs N and C.

In the preceding experiments, we presented evidence for possible involvement of motifs N and C in the pro-inflammatory activity of flagellin. However, the data presented did not show directly that these motifs in flagellin activate the putative eukaryotic receptor for flagellin, TLR5. It is now well established that the pro-inflammatory activity of flagellin is mediated via the TLR5 receptor (7, 12, 16). CHO cells expressing human TLR5 and a luciferase-linked reporter have been exploited to screen for PAMPs recognized by this receptor. TLR5 does not respond to any of the PAMPs known to stimulate TLR pathways, such as LPS, lipopeptide, yeast cell wall, or peptidoglycan but is activated by flagellin. Flagellin binds to TLR5, and the TLR5-flagellin interaction induces NF- κ B activation, which is required for the transcriptional induction of many pro-inflammatory cytokines (12). Although a number of studies have shown the importance of TLR5 in flagellin-mediated signal transduction, there is little information about the interacting domains of flagellin and TLR5.

We have taken the following approach to study functional activation of TLR5 by flagellin. We constructed CHO stable cell lines expressing human TLR5 protein and GFP reporter gene under the control of an NF- κ B-responsive promoter. Wild-type CHO cells express very low levels of TLR5 activity. Stable expression of human TLR5 in CHO cells resulted in significant NF- κ B activity in response to incubation with flagellin (Fig. 8A). Exposure of these engineered CHO/GFP/TLR5 cells to purified *S. muenchen* flagellin increased GFP

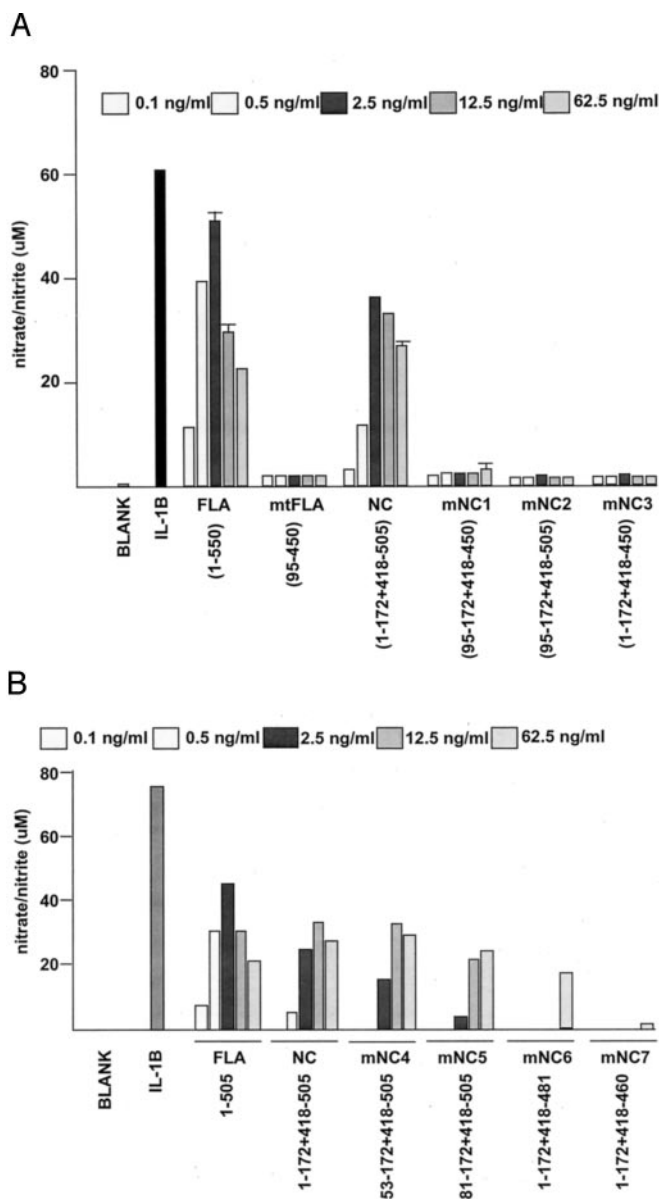


FIG. 4. Both N and C domains are required for proper folding of flagellin molecule and its activity. Deletions were made from the N or C terminus (A) or both ends (B) in flagellin NC mutant. The resultant recombinant proteins were tested for their ability to induce NO production in DLD-1 cells. There were three to five determinations per experimental group. FLA, flagellin.

expression, reaching a maximum approximately after 8 h of incubation (Fig. 8B). In order to identify the region of flagellin critical for its activation of TLR5, we utilized a series of mutant flagellin(s) employed in previous experiments and monitored for the expression of GFP in immunoblot assays (Fig. 8). These studies revealed that the mutations in N1 and C1 had no effect on TLR5-mediated NF- κ B activation and GFP expression. However, further deletion of the N and C terminus sequences of flagellin (N4, C3, NH, and HC) blocked GFP expression. Similarly, treatment with mutants N14 and C12, in which all the wild-type amino acids in motifs N and C are substituted, also failed to activate the reporter gene, such that GFP expression level was comparable with that of untreated cells. Taken together, these results indicate that the regions in flagellin required for pro-inflammatory activity and NF- κ B activation are also required for its functional activation of the TLR5 receptor.

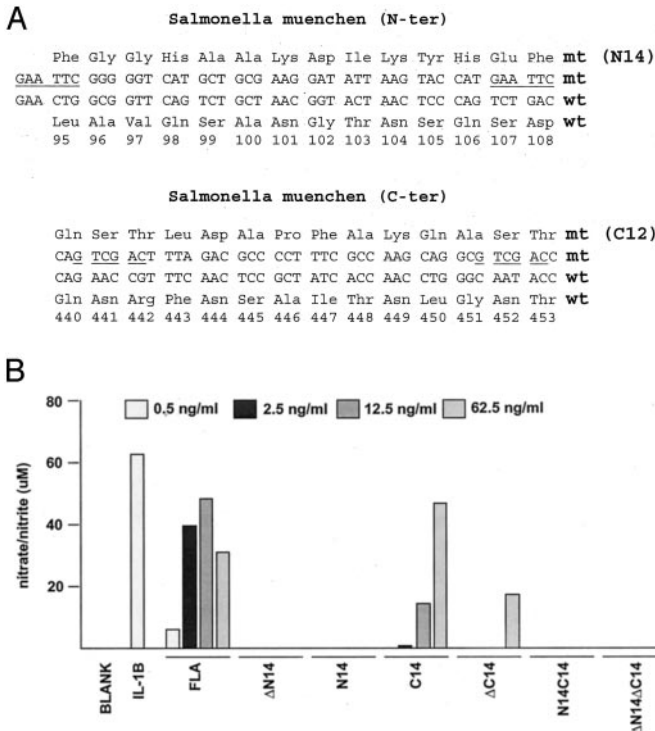


FIG. 5. Proinflammatory activity of flagellin is confined to 12–14-amino acid regions within N and C termini domains. A, nucleotide and amino acid sequences of wild-type and mutant flagellin. Amino acid sequences in the N- and C-terminal domains were changed by site-directed mutagenesis as described under “Materials and Methods.” Restriction sites were introduced in the beginning and end to facilitate in the construction of deletion mutants. B, mutations in the 12–14-amino acid region abolished flagellin activity. Mutant and wild-type proteins were assayed for their ability to induce NO production in DLD-1 cells. Data are expressed as mean \pm S.E. *FLA*, flagellin.

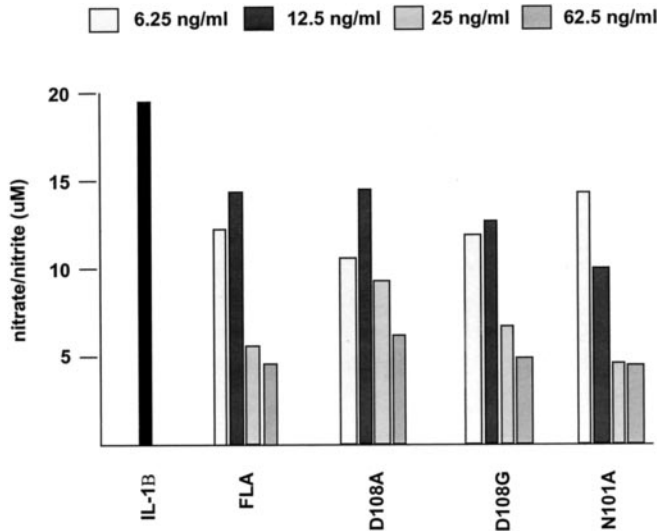


FIG. 6. Changes in single amino acid do not have any effect on flagellin activity. Point mutations in the N-terminal domain were made by site-directed mutagenesis. Incubating with DLD-1 cells and measuring NO concentration in culture supernatants tested the proinflammatory activity of these proteins. There were four samples per experimental group, and data are expressed as mean \pm S.E. *FLA*, flagellin.

DISCUSSION

Flagellin is the monomeric protein subunit of flagella, a common prokaryotic appendage traditionally posited as a means of propulsion or attachment to host cells, but is now

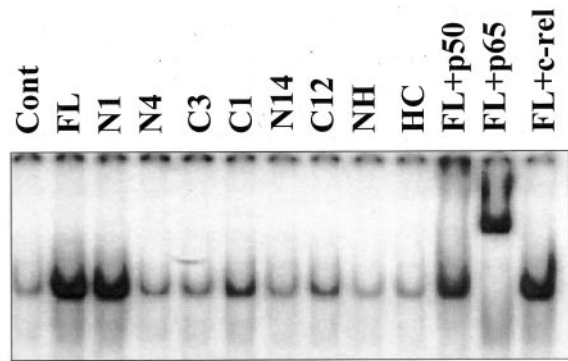


FIG. 7. Mutant flagellins containing only motif N and motif C regions activate NF- κ B. Nuclear extracts were prepared from DLD-1 cells treated with 100 ng/ml purified recombinant flagellin or flagellin mutant for 60 min. For the EMSA analysis, 5 μ g of nuclear proteins were incubated with radiolabeled consensus NF- κ B oligonucleotides in EMSA buffer for 10 min at room temperature. Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel. Gels were transferred to Whatman 3M paper, dried, and then exposed to photographic film at -70°C with an intensifying screen for 1 h. Supershift experiments were carried out with antibodies specific to NF- κ B p50 and p65 subunits and Stat3. *Cont*, control; *FLA*, flagellin.

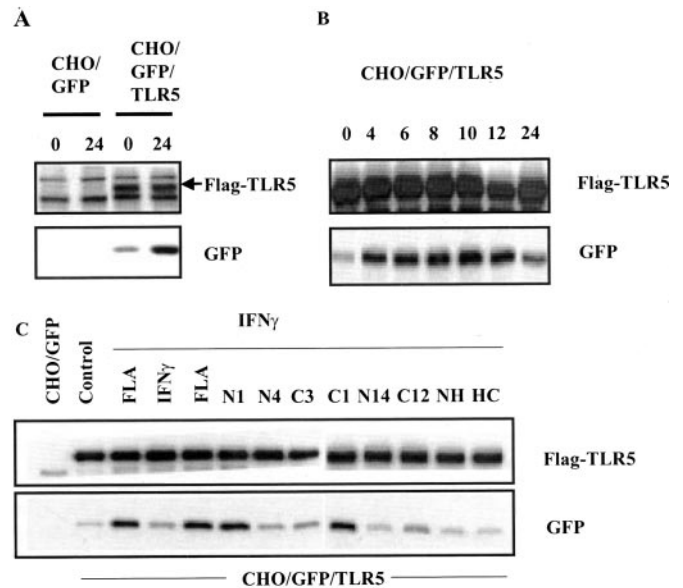
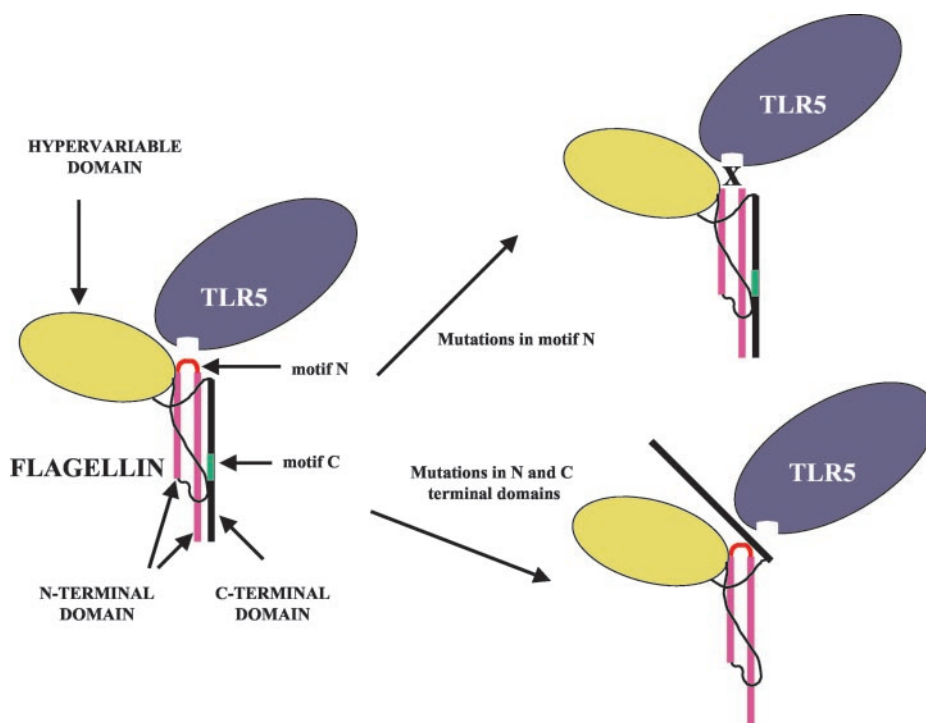


FIG. 8. TLR5 confers the ability to activate NF- κ B in response to flagellin. Stable CHO cell lines containing reporter gene (CHO/GFP) and expressing human TLR5 (CHO/GFP/TLR5) were analyzed for GFP expression. A, whole cell lysates of CHO/GFP or CHO/GFP/TLR5 cells treated with 100 ng/ml flagellin for 0 or 24 h were analyzed for TLR5 (upper) and GFP (lower) expression in immunoblots. Blots were probed with anti-FLAG antibody or anti-GFP antibody. B, the reporter gene, GFP, was induced in a time-dependent manner in CHO/GFP/TLR5 cells stimulated with *S. muenchen* recombinant flagellin. Confluent cells were exposed to 100 ng/ml flagellin for 0, 4, 6, 8, 10, 12, or 24 h; extracted total protein and equal amounts of protein were subjected to SDS-PAGE. Immunoblot analysis was performed as described above. C, flagellins exert its activity through motif N and motif C. Stable (CHO/GFP/TLR5) cells were treated with a variety of mutant flagellin proteins (100 ng/ml) described in previous experiments. Whole cell lysates were isolated after 8 h of treatment and then analyzed for TLR5 and GFP expression in immunoblots.

recently implicated as a powerful stimulus of eukaryotic proinflammatory gene expression. At low nanomolar concentrations, flagellin activates eukaryotic cells, such as macrophages, monocytes, and intestinal and pulmonary epithelial cells, to release a broad range of pro-inflammatory mediators *in vitro* and *in vivo*, including tumor necrosis factor- α , MIP-1 α , IL-6,

FIG. 9. Schematic diagram of changes in flagellin structure with respect to various mutations and possible interactions with TLR5. These structures are predicted based on the crystal structure of *Salmonella* flagellin. α -Helices in conserved terminal domains are represented by solid bars. The presumed locations of the motif N and motif C are marked.



IL-12p40, IL-10, as well as inducible NO synthase-derived NO (2–5, 12, 15, 16, 23–25).

The purpose of the present investigation was to delineate the regions and the specific amino acid residues within flagellin that contribute to its activation of pro-inflammatory gene expression. This effort was launched in response to conflicting data from other laboratories, claiming that a central hypervariable domain is essential for the induction of pro-inflammatory activity (2) or conversely stating that pro-inflammatory activation was dependent upon the highly conserved terminal regions (6, 26).

The precise residues within the N and C termini that mediate the pro-inflammatory host response to flagellin are currently unknown. To address this issue, we have utilized a mutational analysis of recombinant flagellin in order to conclusively identify those residues that are crucial for pro-inflammatory eukaryotic gene expression. In combination with the known crystal structure of flagellin, our results provide a structural functional correlation that permits a new model by which flagellin activates its host membrane receptor, TLR5.

Current knowledge of the structure of flagellin is based upon crystal structure analysis by Samatey *et al.* (9). This group has reported that the N-terminal domain of wild-type flagellin forms two α -helices separated by a small hinge, which is equivalent to our motif N. The first α -helix, located in the N-terminal end, is slightly larger than the one in the C-terminal end. The C-terminal domain contains only one α -helix, similar in length to the first α -helix in the N-terminal domain and runs parallel to it. These three α -helices are held together and together form the central axis, whereas the species-specific hypervariable region forms the outer surface (9).

Based upon our functional data and the crystal structure data from Samatey *et al.* (9), we have now constructed a model to account for the effect of various mutations on the tertiary structure of monomeric flagellin and to its functional activation of TLR5 (Fig. 9). Our model proposes several critical regions of flagellin that are obligatory for full activation of TLR5.

First, we have identified a 14-amino acid region (designated “motif N”) located within the N-terminal domain (95–108) that forms a hairpin loop. As expected, removal of amino acids 1–95

in the N-terminal domain showed negligible effect on pro-inflammatory activity induced by flagellin. However, deletion of motif N abolishes pro-inflammatory gene expression. Site-specific mutagenesis of all 14 amino acids likewise abolished pro-inflammatory activity. Mutation of individual residues had no effect, suggesting the numerous points of attachment within the motif are required for functional activation of TLR5. The relevance of these findings is strengthened by a recent report (8), based on conformation analysis, that predicted that the amino acids 88–97 of flagellin are involved in binding with amino acids 552–561 of the TLR5 receptor. Overall, our proposed binding site sequences (motif N) overlaps with these predicted sequences.

Second, we have identified a 9-amino acid region (441–449), termed motif C, located within the conserved C-terminal domain. Motif C is obligatory for functional activation of TLR5. Deletions or simultaneous point mutations of all amino acids in motif C reduced the pro-inflammatory activity, but not completely.

Third, we have identified sequences within the terminal domains (N and C) that may be involved in charge-polar interactions and are necessary for flagellin activity. We observed that single deletions in either the N terminus (N3, 1–94) or the C terminus (C4, 451–505) have limited effect on pro-inflammatory activity induced by flagellin. However, double deletions (mtFLA, 95–450) completely inhibited pro-inflammatory activity. Because there are many potential points of attachment between the N and C domains, which are aligned in parallel according to our model, our results suggest that modest deletions in either the N or C termini do not disrupt this arrangement. More extensive deletions, and deletions in both termini, however, are presumably incapable of maintaining the parallel alignment of the two termini, resulting in a loss of pro-inflammatory activity.

Fourth, the role of the hypervariable domain in the functional activation of flagellin has been a matter of dispute in the literature. To address this matter directly, we have prepared recombinant constructs of flagellin in which the hypervariable domain has been internally deleted, leaving the N and C termini intact. We have confirmed, in agreement with a previous

report (6), that truncated flagellin mutants deleting the N or C termini lack pro-inflammatory activity. In contrast, the NC mutant lacking the hypervariable domain dose-dependently induced NO production in DLD-1 cells comparable with that of wild-type flagellin (Fig. 2). These results imply that the conserved N and C domains are required for pro-inflammatory activity, and the hypervariable domain is dispensable and contributes little if anything to the induction of pro-inflammatory gene expression (6). Presumably, the NC flagellin mutant still maintains the rod-shaped structure proposed in our model, even in the absence of the hypervariable region.

Because the hypervariable region bridges the N and C termini, it occurred to us that it might stabilize the tertiary structure of flagellin, even though the NC mutant (which lacked the hypervariable domain) retained full pro-inflammatory activity. In order to address this issue, we tested the activity of N- or C-terminal deletions in the NC mutant. We observed that certain C-terminal deletions (mNC6 and mNC7) in recombinant NC constructs showed lesser pro-inflammatory activity than the same deletions in wild-type flagellin. Taken together, these results suggest that the hypervariable domain does play a role in maintaining a functionally active conformation of the N and C termini. Surprisingly, a similar effect was not observed when N-terminal deletions were made in the NC construct. This suggested that the C terminus may be relatively more critical for maintaining conformation than the N terminus.

The effect of various mutations in the above regions of flagellin on pro-inflammatory activation and tertiary conformation is no doubt a complex area, and much further work will be required to fully elucidate the role of individual residues. Because we have utilized functional activation of TLR5 as a proxy for the maintenance of wild-type flagellin structure, our claims regarding conformation are inferential. Specifically, we have not prepared crystal structures of the various recombinant mutants and cannot therefore rigorously describe their structure, nor can we definitely state that these mutations have altered the tertiary structure. A clue to this possibility is obtained from electrophoretic studies we have performed showing that recombinant mutant flagellins of similar mass (and presumably charge) migrate quite differently on SDS-PAGE gel.² For example, although mutant N14, mutant ΔN14, and wild-type flagellin are similar in size and amino acid composition, wild-type flagellin migrates faster by SDS-PAGE gel. In addition, there was no correlation between migration and molecular size of other flagellin proteins illustrated in Fig. 1. These observations provide a further hint that the functional alterations in pro-inflammatory activity involved significant alterations in the tertiary structure, which would affect migration through a gel in an electric field.

Recent reports have increased our understanding of the mechanisms by which flagellin induces pro-inflammatory gene expression. First, it has been established that the mammalian TLR5 receptor recognizes bacterial flagellin derived from both Gram-positive and Gram-negative bacteria. The TLR5-flagellin interaction culminates in the activation of NF-κB, which is required for the transcriptional induction of many pro-inflammatory cytokines. MyD88 appears to be an essential signal transducer of TLR5 for signaling (7, 12, 16, 17, 27–29). Our data in DLD-1 and A549 cells do not establish that all of the effects observed with various mutants were transmitted through TLR5. To address these questions, we used CHO cells stably transfected with human TLR5 and GFP-linked reporter constructed under the IL-8 promoter. Consistent with previous

results, flagellin mutants that failed to induce a pro-inflammatory response in epithelial cells also failed to stimulate GFP expression in TLR5-expressing CHO cells.

Overall, our findings on the ability of the flagellin mutants to induce NO or IL-8 production paralleled the ability of the mutants to induce TLR5 activation. These findings are consistent with the notion that TLR5 is the principal receptor for flagellin-induced signal transduction and inflammatory response. Recent studies have confirmed the potential pathogenic role of the flagellin-TLR5 axis in intestinal inflammation (15, 16, 21, 30), in systemic inflammation and shock (5, 11), and in various forms of pneumonia, pulmonary inflammation, and adult respiratory distress syndrome (1, 20).

Active or passive immunization strategies directed against flagellin may offer protection against pro-inflammatory processes induced by flagellated organisms and may offer new anti-inflammatory and anti-shock strategies in severe forms of clinical Gram-negative infections. The mapping of the pro-inflammatory activity of flagellin described in the current study may aid future studies in this direction.

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