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Identification of the Critical Residues Involved in Peptidoglycan Detection by Nod1

Stephen E. Girardin, Muguette Jéhanno, Dominique Mengin-Lecreulx, Philippe J. Sansonetti, Pedro M. Alzari, and Dana J. Philpott

Nod1 is an intracellular pattern recognition molecule activated following bacterial infection, which senses a specific muropeptide (L-Ala-D-Glu-meso-DAP) from peptidoglycan. Here we investigated the molecular basis of peptidoglycan sensing by human (h) Nod1. Our results identified the domain responsible for peptidoglycan detection in the center of the concave surface of hNod1 leucine-rich repeat domain. Amino acid residues critical for sensing define a contiguous surface patch that is largely conserved in Nod1 proteins from different species. Accordingly, the distinct specificities of human versus murine Nod1 toward muropeptide detection were also found to lie in this central cleft. Several splicing variants of Nod1 lacking repeats 7–9 have been characterized recently, the relative balance of which is thought to correlate with the onset of asthma or inflammatory bowel disease. We demonstrated that these isoforms failed to transduce NF-κB activation upon muropeptide stimulation. This study provided insights into the molecular mechanisms responsible for the detection of bacterial peptidoglycan by Nod1 and suggested that defects in Nod1-dependent peptidoglycan sensing may contribute to eliciting certain inflammatory disorders.

The innate immune system has evolved means to mediate recognition of microbes through the specific detection of highly conserved structures. Such conserved microbial motifs are generally molecules from the cell wall or nucleic acids, and in the case of bacteria, these include lipopolysaccharide, peptidoglycan, lipoproteins, lipoteichoic acid, flagellin, and CpG DNA. Recently, studies focusing on the innate immune responses to peptidoglycan have gained substantial attention due to the importance of peptidoglycan in bacterial cell wall structure and function. Peptidoglycan appears to represent a common property (2). However, although clear evidence exists for triggering innate immune responses, the function of mammalian PGRPs remains elusive. In addition to PGRPs, recent evidence has shown that the Nod proteins, Nod1 and Nod2, are two members of the growing family of Nod-like receptors (NLRs) that recognize peptidoglycan (3–5). Within the peptidoglycan polymer, Nod1 and Nod2 detect highly specific substructures, such as MurNAc-L-Ala-D-Glu-meso-DAP (M-TriDAP) and MurNAc-L-Ala-d-Glu (muramyl dipeptide or MDP), respectively (6–10).

Upon activation, Nod proteins trigger the activation of NF-κB, c-Jun N-terminal kinase/stress-activated protein kinase, and caspase pathways, which in turn governs some of the host responses to bacterial infection. Nod proteins have been shown to play a key role in host response to a variety of bacterial infections, including Shigella flexneri, enteroinvasive Escherichia coli, Helicobacter pylori, Listeria monocytogenes, Chlamydia pneumoniae, Streptococcus pneumoniae, and Pseudomonas aeruginosa (11–17). In addition, the key role of Nod proteins in the modulation of inflammatory processes is highlighted by the recent identification of the genetic association between mutations in Nod1 and several inflammatory disorders, including Crohn’s disease (18, 19), Blau syndrome (20), and early onset sarcoidosis (21). More recently, studies have also identified polymorphisms in Nod1 associated with genetic predisposition to inflammatory bowel disease (22), atopic eczema (23), and asthma (24). However, in the latter case, a link between the genetic observation and any functional significance remains to be clearly addressed, because the polymorphisms found lie within the ninth intron of the Nod1 gene. It has been proposed by the authors of the two studies that these mutations might affect the relative abundance of specific Nod1 splice variants.

It is becoming clear that Nod proteins, via the specific detection of muropeptide motifs, are key molecules involved in innate immunity and inflammation. However, the molecular basis of peptidoglycan detection by Nod proteins remains largely undefined. First, it is still not known whether Nod proteins interact directly with peptidoglycan fragments or whether sensing involves additional protein intermediates. Also, crystal structures of Nod LRR domains are still lacking, which restricts our current knowledge of structure-function for these pattern-recognition molecules (PRMs). In the case of Toll-like receptors (TLRs), the most widely studied family of mammalian pattern-recognition molecules, detection of microbial patterns also occurs through the TLR ectodomain. Still, a decade of intensive investigation on TLRs did not lead to substantial understanding of microbial detection by TLRs at a molecular level. However, with the recent characterization of the first structure of the...
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LRR domain from TLR3 (25), one can now expect that some of these questions will be solved in the near future.

In this context, we have undertaken an approach based on mutagenesis (either deletions or site-directed point mutations) to gain more insight into the molecular basis of peptidoglycan detection by Nod1. We mapped the region responsible for Tridap sensing within Nod1 LRR to a contiguous patch of amino acid residues in the center of the inner concave surface of the LRR. Because recent evidence has identified the existence of several Nod1 splicing variants and their correlation with an increased risk for asthma and inflammatory bowel disease (22, 24), we analyzed these variants for their ability to sense peptidoglycan and muropeptides. These isoforms all contained only a part of the Tridap sensing domain, and consequently, we observed that only the full-length molecule could transduce NF-κB activation upon stimulation. Together, this study illustrates the importance of defining with precision the molecular determinants responsible for detection of peptidoglycan by Nod1 and suggests a link between defective Nod signaling and the onset of inflammatory disorders.

MATERIALS AND METHODS

Muramyl Peptides—The experimental procedures relative to the synthesis of the muramyl peptides used in this study have been described elsewhere (8).

Reagents—Endotoxin-free fetal calf serum was from Hyclone (Logan, UT) and was used after heat inactivation 56 °C for 30 min. All cell culture reagents and antibiotics were from Invitrogen.

Muramyl dipeptide (MDP-LD) was from Calbiochem and reported to be 98% pure by TLC. Synthetic FK156 was obtained from Fujisawa Inc. (Japan).

Limon Amebocyte Assay—All reagents used in this study tested negative for lipopolysaccharide contamination by the Limulus amebocyte assay, according to manufacturer’s recommendations (QCL-1000, BioWhittaker, Verviers, Belgium). These reagents include M-TriDAP, MDp, and FK156.

Expression Plasmids and Transient Transfections—The expression plasmid for human Nod1 was from Gabriel Nunez (Ann Arbor, MI). The expression plasmid for human Nod2 was from Jurg Tschopp and Fabio Martinon (ISREC, Lausanne, Switzerland). The expression plasmid for human IPAF was from Jurg Tschopp and Fabio Martinon (ISREC, Lausanne, Switzerland). The expression plasmid for human Nod1 was from Gabriel Nunez (Ann Arbor, MI).

Materials and Methods

Site-directed Mutagenesis—The 30 mutations introduced into the LRR domain of hNod1 were obtained individually by PCR, using standard procedures. Briefly, pairs of complementary oligonucleotides (see the list below) were designed to carry the mutation of interest. These oligonucleotides were used as primers for circular PCR, to amplify the whole plc-hNod1 plasmid. Amplified plasmids were selected using the DpnI restriction enzyme. For each mutant, the HindIII-NotI domain spanning the LRR domain of hNod1 was then subcloned into a plc-hNod1 vector that had not been subjected to PCR and had been fully sequenced previously. This subcloning step was performed to avoid possible mutations in the rest of the hNod1 cDNA sequence. Following subcloning, the whole HindIII-NotI region was fully sequenced (Millgen, France) to ensure that the mutation of interest was present and that no additional mutation was introduced by the PCR.

Generation of Nod1 Isoforms Δ10, Δ10–11, and Δ10–12—The three Nod1 constructs Δ10, Δ10–11, and Δ10–12 were generated by PCR according to standard methods. The oligonucleotides used are listed below. Briefly, couples of primers 1F/2R, 1F/2Rbis, 1F/2Rter, 3F/6R, 4F/6R, and 5F/6R were used to amplify regions of hNod1 by using full-length hNod1 expressing vector as a template, leading to the generation of PCR products A, B, C, D, E, and F, respectively. PCR products A–F were excised from gel and purified, and A/D, B/E, and C/F were annealed and used together with primers 1F and 6R for a second round of PCR, therefore amplifying regions corresponding to constructs of interest D10, D10–11, and D10–12, respectively. Finally, PCR products were digested by using HindIII-Not1 restriction enzymes, and the fragment obtained was used to exchange the HindIII-Not1 fragment from a new (non-PCR-amplified) expression vector encoding for hNod1. Following subcloning, sequencing was performed (Millgen, France) to ensure that the right constructs were generated.

Oligonucleotides Used for Site-directed Mutagenesis—The following oligonucleotides were used: 1For GGGGACATCGGGCCAACTCCCTCCCTTTACGTC and 1Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC; 2For GCCGCAACTCATCCACGTCGTTCACTGCTACGGCTCTG and 2Rev GCAGGCTGTCGAGTCGAGTGTTGCTGCTAGTTGAGCC; 3For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 3Rev GCAGGCTGTCGAGTCGAGTGTTGCTGCTAGTTGAGCC; 4For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 4Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC; 5For GCAGGCTGTCGAGTCGAGTGTTGCTGCTAGTTGAGCC; 6For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 6Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC; 7For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 7Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC; 8For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 8Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC; 9For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 9Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC; 10For CCAATTACCTCCACGTCGTTCACTGCTACGGCTCTG and 10Rev CAGCTTTTGGAGGTGGCAGGCGAGTTGAGCC.

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13 For CCTGGATGAATGCAAAGGCCCTCACGTCTCTTAATCTGGA and 13 Rev TCCCAGTTTAAGAGACGTGAGGCCTTTGCATTC; 14 For GAATGCAAAGGCCTCACGCATCTTACGGGAAA and 14 Rev TTTCAGAGGTAAAGGTGCGTGAGGCCTTTGCATTC; 15 For GGCCTCACGCATCTTAATCTGCAAAACAAAATAACAAGTGAAGGAGGG and 15 Rev CCCTCTACTTGTTATTTTGTTTTTTGACAGTTTATAAGATGCGTGAGGC; 16 For GCAAATCAATCTCTTCGGTTGGGATGTGGGGCATCAAGTTGGG and 16 Rev CCCAACTTGATTGCCCGACATCCCAACC; 17 For GGTTCGATGTGGGGCATCAAGTTGGG and 17 Rev CCTTCATCCCCAACTTGATTGCCCGACATCCCAACC; 18 For GGTTGGGATGTGGGGCATCAAGTTGGG and 18 Rev CCTTCATCCCCAACTTGATTGCCCGACATCCCAACC; 19 For GCTTGACCGCCCTGAGTCTTGCGTCCAACGG and 19 Rev CCGTTGGACGCAAGACCCAGGGTGCC; 20 For GACCACCCTGGGTCTTGCGTCCAACGGCATC and 20 Rev GATGCCGTTGGACGCAAGACCCAGGGTGCC; 21 For GCTTGACCACCCTGAGTCTTTCGTCCAACGG and 21 Rev CCGTTGGACGCAAGCAGGGTGCC; 22 For GCAGCAGAACACGTCTCTAGAAGCACTGTGGCTGCC and 22 Rev GGGTCAGCCACAGTGCTTCTAGAGACGTGTTCTGCTGC; 23 For GTCTCTAGAATCTTCTTGTGCAGGCAACCCAGGGTGCC.

FIGURE 1. The LRR domain is fully responsible for the sensing specificity of hNod1. A, schematic representation of the domain organization of hNod1, hNod2, and hIPAF. The upper numbers represent the amino acid positions for the boundaries of each domain, plus the first and last amino acid of each protein. Below each representation is indicated the position selected for the domain swaps between hNod1 LRR and hNod2 LRR (creation of the chimeric molecule hNod1-LRR hNod2) or between hNod1 LRR and hIPAF LRR (creation of the chimeric molecule hNod1-LRR hIPAF). B, human HEK293 epithelial cells were co-transfected with several muramyl peptides (MDP LD, MDP LL, or TriDAP; all at 250 nM) in the presence of expression vectors for empty PCDNA3 vector (Vector), hNod1 (Nod1), hNod2 (Nod2), or the chimeric molecules (Nod1-LRR hNod2 or Nod1-LRR hIPAF), and the activity of an NF-κB-driven luciferase reporter gene was measured. RLU, relative light units. Data show the mean ± S.E. of duplicate experiments. MDP LD is the natural product from bacterial cell wall. MDP LL is a biologically inactive enantiomer derivative of MDP.

FIGURE 2. Deletions in the LRR domain of hNod1 hamper sensing of TriDAP. A, schematic representation of the domain organization of wild-type hNod1 (1) and of the four deletion constructs in the LRR domain (depicted as 2–5). B, human HEK293 epithelial cells were co-transfected with several muramyl peptides (MDP LD, MDP LL, or TriDAP; all at 250 nM) in the presence of expression vectors for hNod1 (Nod1 WT), Nod1 ΔLRR, Nod1 LRR1–4, Nod1 LRR1–8, or Nod1 LRR8–10, in the presence or the absence of TriDAP (as indicated on the figure; 20 nM), and the activity of a NF-κB-driven luciferase reporter gene was measured. Data show the mean ± S.E. of duplicates.

13 For CCTGGATGAATGCAAAGGCCCTCACGTCTCTTAAACTGGA and 13 Rev TCCCAGTTTAAGAGACGTGAGGCCTTTGCATTC; 14 For GAATGCAAAGGCCTCACGCATCTTACGGGAAA and 14 Rev TTTCAGAGGTAAAGGTGCGTGAGGCCTTTGCATTC; 15 For GGCCTCACGCATCTTAATCTGCAAAACAAAATAACAAGTGAAGGAGGG and 15 Rev CCCTCTACTTGTTATTTTGTTTTTTGACAGTTTATAAGATGCGTGAGGC; 16 For GCAAATCAATCTCTTCGGTTGGGATGTGGGGCATCAAGTTGGG and 16 Rev CCCAACTTGATTGCCCGACATCCCAACC; 17 For GGTTCGATGTGGGGCATCAAGTTGGG and 17 Rev CCTTCATCCCCAACTTGATTGCCCGACATCCCAACC; 18 For GGTTGGGATGTGGGGCATCAAGTTGGG and 18 Rev CCTTCATCCCCAACTTGATTGCCCGACATCCCAACC; 19 For GCTTGACCGCCCTGAGTCTTGCGTCCAACGG and 19 Rev CCGTTGGACGCAAGACTCAGGGTGCC; 20 For GACCACCCTGGGTCTTGCGTCCAACGGCATC and 20 Rev GATGCCGTTGGACGCAAGACCCAGGGTGCC; 21 For GCTTGACCGCCCTGAGTCTTGCGTCCAACGGCATC and 21 Rev CCGTTGGACGCAAGACTCAGGGTGCC; 22 For GACCACCCTGGGTCTTGCGTCCAACGGCATC and 22 Rev GATGCCGTTGGACGCAAGACCCAGGGTGCC; 23 For GTCTCTAGAATCTTCTTGTGCAGGCAACCCAGGGTGCC.

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AAATGAACTC and 23Rev GAGTTCATTTTGGGTCAGCGACAGTATTTCTAGAGAC; 24For GTCTCTAGAAATACTGTGGCTGGCCCAAAATGAACTCand 24Rev GAGTTCATTTTGGGCCAGCCACAGTATTTCTAGAGAC; 25For GTCAACCAGACGTTAAAGTCTTTATGGCTTATCCAGAATCAGATC and 25Rev GATCTGATCTGGATAAGCCATAAAGACTTTAACGTCTGGTTGA; 26For GTCAACCAGACGTTAAAGCATTTATCGCTTATCCAGAATCAGATC and 26Rev CTGATTCTGGATAAGCCATAAAGACTTTAACGTCTGGTTGA; 27For GTCAACCAGACGTTAAAGCATTTATCGCTTATCCAGAATCAGATC and 27Rev GATTCTGGCTAAGCCATAAAGACTTTAACGTCTGGTTGA; 28For GAGCAACACTGGCATAACATCGATTTGCCTAAATGGAAACCT and 28Rev CAGGTTTCCATTTAGGCAAATCGATGTTATGCCAGTGTTGCTC; 29For GGCAATAACAGAGATTCTTTTCTAGTTATGCCAGTGTTGCTC and 29Rev CTCTGGTTTTATCAGGTTTCCATTTAGGCAAATCGATGTTATGCC; and 30For GGCAATAACAGAGATTCTTTTCTAGTTATGCCAGTGTTGCTC and 30Rev GGTTTTATCAAGTTCGTTAAATCGAATCAGATC.

Oligonucleotides Used for the Generation of Nod1 Constructs

RESULTS

Early studies on Nod proteins Nod1 and Nod2 had demonstrated the key role of the LRR domain in achieving bacterial sensing (10, 19). However, it remains unclear whether the specificity for certain muropeptides (i.e. specific detection of MDP versus M-TriDAP) is carried exclusively by the LRR domain. To this end, we constructed chimeric molecules hNod1-LRRhNod2 and hNod1-LRRhIPAF in which the LRR domain of hNod1 was exchanged with the one for hNod2 and hIPAF, respectively (Fig. 1A). We observed that hNod1-LRRhNod2 could not detect TriDAP but rather MDP (Fig. 1B), thereby showing that the LRR domain is responsible for the specific detection of muropeptides by Nod molecules. As a negative control, swapping the LRR domains of Nod1 and IPAF (a molecule closely related to Nod1 and Nod2) resulted in a molecule (hNod1-LRRhIPAF) unable to detect both TriDAP and MDP (Fig. 1B), thereby showing that the LRR domain is responsible for the specific detection of muropeptides by Nod molecules. As a negative control, swapping the LRR domains of Nod1 and IPAF (a molecule closely related to Nod1 and Nod2) resulted in a molecule (hNod1-LRRhIPAF) unable to detect both TriDAP and MDP (Fig. 1B), thereby showing that the LRR domain is responsible for the specific detection of muropeptides by Nod molecules.

FIGURE 3. Choice of the amino acids for site-directed mutagenesis in the LRR domain of hNod1. A, homology model of hNod1 LRR. The arrows represent the β-strands from the C-terminal end of each repeat. B, amino acid sequence of hNod1 LRR domain showing the identity of the 30 mutations introduced (asterisks). The repeats are underlined and numbered, and the β-strands are represented by red arrows. C, alignment of the C-terminal extremity of the 10 repeats from the hNod1 LRR domain. All the amino acids selected for mutagenesis (3 per repeat) are highlighted by black rectangles. D, the nature of the amino acid change at each position is indicated. For each position, the replacement with either serine (S), alanine (A), threonine (T), or glycine (G) was designed in order to minimize the risk of altering the conformational organization of the LRR domain.

AAATGAACTC and 23Rev GAGTTCATTTTGGGTCAGCGACAGTATTTCTAGAGAC; 24For GTCTCTAGAAATACTGTGGCTGGCCCAAAATGAACTCand 24Rev GAGTTCATTTTGGGCCAGCCACAGTATTTCTAGAGAC; 25For GTCAACCAGACGTTAAAGTCTTTATGGCTTATCCAGAATCAGATC and 25Rev GATCTGATCTGGATAAGCCATAAAGACTTTAACGTCTGGTTGA; 26For GTCAACCAGACGTTAAAGCATTTATCGCTTATCCAGAATCAGATC and 26Rev CTGATTCTGGATAAGCCATAAAGACTTTAACGTCTGGTTGA; 27For GTCAACCAGACGTTAAAGCATTTATCGCTTATCCAGAATCAGATC and 27Rev GATTCTGGCTAAGCCATAAAGACTTTAACGTCTGGTTGA; 28For GAGCAACACTGGCATAACATCGATTTGCCTAAATGGAAACCT and 28Rev CAGGTTTCCATTTAGGCAAATCGATGTTATGCCAGTGTTGCTC; 29For GGCAATAACAGAGATTCTTTTCTAGTTATGCCAGTGTTGCTC and 29Rev CTCTGGTTTTATCAGTTCGTTTCCATTTAGGCAAATCGATGTTATGCC; and 29Rev CTCTGGTTTTATCAGTTCGTTTCCATTTAGGCAAATCGATGTTATGCC; and 30For GGCAATAACAGAGATTCTTTTCTAGTTATGCCAGTGTTGCTC and 30Rev GGTTTTATCAAGTTCGTTAAATCGAATCAGATC.

Oligonucleotides Used for the Generation of Nod1 Constructs

AAATGAACTC and 23Rev GAGTTCATTTTGGGTCAGCGACAGTATTTCTAGAGAC; 24For GTCTCTAGAAATACTGTGGCTGGCCCAAAATGAACTCand 24Rev GAGTTCATTTTGGGCCAGCCACAGTATTTCTAGAGAC; 25For GTCAACCAGACGTTAAAGTCTTTATGGCTTATCCAGAATCAGATC and 25Rev GATCTGATCTGGATAAGCCATAAAGACTTTAACGTCTGGTTGA; 26For GTCAACCAGACGTTAAAGCATTTATCGCTTATCCAGAATCAGATC and 26Rev CTGATTCTGGATAAGCCATAAAGACTTTAACGTCTGGTTGA; 27For GTCAACCAGACGTTAAAGCATTTATCGCTTATCCAGAATCAGATC and 27Rev GATTCTGGCTAAGCCATAAAGACTTTAACGTCTGGTTGA; 28For GAGCAACACTGGCATAACATCGATTTGCCTAAATGGAAACCT and 28Rev CAGGTTTCCATTTAGGCAAATCGATGTTATGCCAGTGTTGCTC; 29For GGCAATAACAGAGATTCTTTTCTAGTTATGCCAGTGTTGCTC and 29Rev CTCTGGTTTTATCAGTTCGTTTCCATTTAGGCAAATCGATGTTATGCC; and 29Rev CTCTGGTTTTATCAGTTCGTTTCCATTTAGGCAAATCGATGTTATGCC; and 30For GGCAATAACAGAGATTCTTTTCTAGTTATGCCAGTGTTGCTC and 30Rev GGTTTTATCAAGTTCGTTAAATCGAATCAGATC.
domains carry the global specificity for muropeptide detection, even at the stereoisomeric level.

In an attempt to narrow down the region responsible for TriDAP sensing in hNod1 LRR, several deletion mutants were generated (Fig. 2, A and B). However, this strategy was inappropriate because none of the mutants could retain any sensing of TriDAP (Fig. 2C), strongly suggesting that the structural integrity of the LRR domain is required to achieve full muropeptide detection. Next, an approach based on single amino acid mutagenesis was chosen. The LRR domain of hNod1 contains 10 repeats and is expected to fold into the right-handed, curved solenoid structure characteristic of these proteins (Fig. 3A). In most LRR domains, the long β-sheet forming the inner concave face is usually involved in protein-protein interactions (27). Therefore, we selected three positions at the center of the β-strand from each repeat, whose side chains are predicted to be accessible for ligand interactions and generated 30 mutants in hNod1 LRR (Fig. 3B). By the strategy used, all mutations could be aligned to corresponding positions within each repeat (Fig. 3C). In order to minimize the risk of generating mutant molecules that would be unstable or misfolded because of the amino acid exchange, the substitutions chosen were variable depending on the nature of the amino acid to replace (Fig. 3D). All these mutants were expressed and retained their basal capacity to activate the NF-κB pathway when overexpressed (Fig. 4A). It must be noted that most of the differences in NF-κB activation between the 30 mutants correlated with expression levels, as determined by Western blotting (Fig. 4B). Therefore, we concluded from these experiments that none of the mutants displayed any drastic artifactual loss of function because of defects of expression or stability. Next, each mutant was compared with the wild-type form of hNod1 for its capacity to detect TriDAP (Fig. 5A). We observed that mutations in the fifth and sixth repeats (mutants 13–18), as well as mutation 23 (W874S) in repeat 8, dramatically affected sensing, reducing TriDAP detection by more than 80%. Mapping the effect of these mutants into the concave surface of hNod1 LRR revealed that the amino acid residues critically affecting TriDAP sensing define a contiguous patch toward the C-terminal end of the middle β-strands (Fig. 5B, left), which closely matches the pattern of strictly conserved residues in available Nod1 sequences from different species (Fig. 5B, right).

We have demonstrated recently that human and murine forms of Nod1 do not detect the same muropeptide from bacterial peptidoglycan (28). Indeed, although the human form of Nod1 detected a tripeptide-containing muropeptide (MurNac-TriDAP or TriDAP), its murine ortholog needs a tetrapeptide structure for efficient sensing (MurNac-TetraDAP or TetraDAP). Therefore, the synthetic compound, FK156 (lactoyl-TetraDAP), represents an efficient agonist for mNod1 (28). We thus aimed to investigate whether the distinct agonist specificities of hNod1 and mNod1 also match the sensing pocket identified above. Taking advantage of our observation that a high concentration of FK156 (250 nM) can potentiate hNod1-dependent NF-κB activation ~7-fold (28), we screened the collection of 30 point mutants of hNod1 to investigate whether any of these would display better detection of FK156. Although for most of the mutants the level of residual activation by FK156 remained close to or below the detection limit of the test, two particular mutants (16 and 19) were activated significantly better than wild-type hNod1 (Fig. 6A). Most interestingly, mutant 16 (E816D) affects an amino acid position that is not conserved between hNod1 and mNod1. Both mutation 16 (E816S) and the mutation that directly mimics the amino acid difference between hNod1 and mNod1 (E816D) improved equally well the sensing of FK156 by hNod1 (data not shown). Mutation 19 (T844A) affects an amino acid position that, although conserved between hNod1 and mNod1, is spatially adjacent to Glu-816 and is surrounded by additional nonconserved residues between hNod1 and
mNod1 (Fig. 6B, right). Together, these results strongly suggested that we have mapped the region responsible for TriDAP sensing in hNod1 and identified a particular site (around amino acids 816 and 844) critical for the specific detection of DAP-containing tetrapeptide versus tripeptide muropeptides.

Several isoforms of Nod1 have been characterized recently as a result of alternative splicing, thus giving rise to Nod1 molecules lacking repeats 7, 7–8, or 7–8–9 (24). Two recent studies have suggested that the relative expression of Nod1 splice variants could correlate with the onset of asthma and inflammatory bowel disease (22, 24). These isoforms differ at the level of the LRR domain, and the splicing site is located at the junction between exons 10 and 11, in the heart of the TriDAP sensing pocket that we have identified (in the next vicinity to amino acids corresponding to mutations 16, 17, and 18). Therefore, we aimed to investigate how these isoforms detect TriDAP. We generated by PCR the three constructs, Nod1Δ10, Nod1Δ10–11, and Nod1Δ10–12, corresponding exactly to the alternative splice variants of Nod1 (Fig. 7A). These molecules were expressed and displayed the expected molecular weight as observed by Western blotting (Fig. 7B). In addition, the three splicing variants were able to activate the NF-κB pathway when overexpressed (Fig. 7C), thus showing that these molecules do not display gross defects in transducing downstream signals. However, none of the three splicing isoforms were able to activate the NF-κB pathway in response to TriDAP stimulation, even at high concentrations of the agonist. Indeed, 250 nM of TriDAP is a concentration ∼100 times higher than the minimal concentration activating full-length Nod1.
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(data not shown). Therefore, the three naturally occurring splicing variants of Nod1 were unable to induce NF-κB activation following stimulation with the peptidoglycan agonist. This correlates with our characterization of the critical residues involved in TriDAP detection; the splicing site at residue 819 (see Fig. 7A) is located in the heart of the sensing patch defined above. Therefore, it is likely that alternative splicing occurring within Nod1 LRR domain may represent a physiological means to drop off Nod1 signaling.

DISCUSSION

In this study, an analysis of the putative ligand-sensing domain of Nod1 was carried out in order to determine key regions necessary for ligand recognition. We took a systematic approach of site-directed mutagenesis focusing on the residues that lie within the concave portion of the leucine-rich repeat domain and are predicted to be accessible for ligand binding. Assuming a solenoid-like structure for the LRR domain of Nod1 similar to that of other homologous LRR proteins, conservative amino acid substitutions were thus carried out on three equivalent positions of each putative β-strand. By using this strategy, a peptidoglycan detection patch was identified within repeats 5–7, a region that is highly conserved in the Nod1 sequence from different species. Moreover, we were able to map what are likely to be the key residues involved in differential ligand sensing of mouse and human forms of Nod1. Finally, with this knowledge, the function of different naturally occurring isoforms of Nod1 that lack central LRRs within the interaction domain could then be postulated. Our speculation is that in certain disease states or perhaps in normal physiological conditions, expression of these isoforms would down-regulate Nod1 function thereby favoring the development of disease in some cases, although in others, this function may form an important regulatory loop to help terminate signals emanating from Nod molecules.

To date, studies on receptor-ligand interactions of PRMs and their microbial motif elicitors have been hampered by technical problems, and only in a few instances have studies been able to show direct interactions. Recently, Toll in Drosophila was shown to interact directly with its ligand, spatzle (29). Similarly, TLR5 has been shown to interact with its protein ligand flagellin (30). In the case of some PRMs, like TLR4 for example, demonstration of a direct interaction with the agonist, lipopolysaccharide, has not been possible because of the fact that coreceptors are required for this interaction. For NLRs, there are no data at the moment that implicate direct interaction between Nod1 or Nod2 and their specific muropeptide ligands. In the case of Nod1, the insolubility of the protein in overexpression systems and the uncertainty as to whether proper folding occurs in vitro has hampered any possibilities to test whether or not there is a direct interaction with TriDAP. Therefore, as an alternative approach, we embarked on a study to identify regions within the Nod1 molecule that are necessary for sensing and not necessarily for direct interaction because we cannot rule out that cofactor molecules could be involved. By using chimeric molecules swapping LRR domains between Nod1 and Nod2, and their specific muropeptide ligands. In the case of Nod1, the insolvability of the protein in overexpression systems and the uncertainty as to whether proper folding occurs in vitro has hampered any possibilities to test whether or not there is a direct interaction with TriDAP. Therefore, as an alternative approach, we embarked on a study to identify regions within the Nod1 molecule that are necessary for sensing and not necessarily for direct interaction because we cannot rule out that cofactor molecules could be involved. By using chimeric molecules swapping LRR domains between Nod1 and Nod2 and Nod1 and Ipaf, it was shown conclusively that the LRR domain carries the sensing specificity of Nod proteins toward their muropeptide agonist. Furthermore, a deletion and loss of function approach demonstrated that deletions within the LRR domain are likely to affect the tertiary structure of the molecule, thus ruling out the validity of this approach to map the interaction domain.

By using a random mutagenesis approach, Nuñez and co-authors (31) mapped a number of sites both in the convex and concave regions of Nod2 that appear to be important for the activity of the molecule (31). Many of these sites are located in the middle and C-terminal end of the concave portion of the LRR, although it is unclear whether some of the mutations also affect the basal activity of the molecule because of the random nature of the amino acid substitutions. Nevertheless, it is interesting to note that the most frequent mutation in Nod2 (hNod2 1007fs mutation), associated with Crohn disease in humans, also maps to the C-terminal portion of the LRR. This mutation results in a loss of sensing of the MDP agonist by Nod2 (7, 9). Taken together, these findings suggest that in both Nod1 and Nod2, the central to C-terminal regions of the LRR appear to be a “hot spot” in terms of muropeptide sensing.

Within this putative binding patch of the LRR of Nod1, we could also identify key residues that contribute to the differential agonist sensing between human and murine Nod1. Our recent data show (28) that human and murine forms of Nod1 strictly sense DAP-containing muropeptides, but murine Nod1 prefers muropeptides with four rather than three amino acids within the peptide chain. Human and murine forms of Nod1 therefore present very little overlap in the muropeptide agonist that they recognize, and our results demonstrate that this can be attributed, at least in part, to differences in a few amino acids within the LRR domain of Nod1.

Two recent studies have implicated polymorphisms in the gene encoding Nod1 in the development of asthma and inflammatory bowel disease. The polymorphisms lie within intron nine, and both studies

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suggest that this mutation may contribute to differences in expression levels of naturally occurring splice variants of Nod1. In normal tissue, isoforms of Nod1 are readily detected that lack either the 7th, 7th to 8th, or 7th to 9th repeats within the LRR domain. According to our studies, these isoforms should have the recognition site at least partially interrupted. Therefore, our goal was to examine these isoforms for their function in terms of muropeptide agonist sensing in comparison with full-length Nod1. Although they maintained the ability to activate the NF-κB pathway in overexpression studies, none of the truncated isoforms were able to sense TriDAP compared with the activity of full-length Nod1. These findings suggest that at physiological levels, Nod1 splicing variants fail to detect TriDAP.

In summary, our findings have defined a region within the LRR of Nod1 that appears to be critical for the sensing of TriDAP by this molecule. Within this region, there are key amino acid residues that contribute to the differential agonist sensing between human and murine Nod1. As it stands, much of these data support the idea that the interaction of Nod1 with its peptidoglycan agonist/ligand is direct, unlike the situation that is often the case in other PRMs, including plant NBS-LRR proteins, where cofactors are implicated in sensing (34). It is hoped that future studies will address this issue. Furthermore, our definition of a critical sensing region within the LRR of Nod1 led us to examine naturally occurring spliced variants of Nod1 that lack LRRs C-terminal to this domain. These isoforms display a clear defect in muropeptide sensing. Because different disease states may favor differential expression of these isoforms, the next goal will be to try to understand the mechanisms by which alteration of Nod1 sensing by these isoforms may contribute to the development of disease.

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