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Regulatory regions and critical residues of NOD2 involved in muramyl dipeptide recognition

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Multiple genetic variants of *CARD15/NOD2* have been associated with susceptibility to Crohn's disease and Blau syndrome. NOD2 recognizes muramyl dipeptide (MDP) derived from bacterial peptidoglycan (PGN), but the molecular basis of recognition remains elusive. We performed systematic mutational analysis to gain insights into the function of NOD2 and molecular mechanisms of disease susceptibility. Using an archive of 519 mutations covering ~50% of the amino-acid residues of NOD2, the essential regulatory domains and specific residues of NOD2 involved in recognition of MDP were identified. The analysis revealed distinct roles for N-terminal and C-terminal leucine-rich repeats (LRRs) in the modulation of NOD2 activation and bacterial recognition. Within the C-terminal LRRs, variable residues predicted to form the β-strand/βturn structure were found to be essential for the response to MDP. In addition, we analyzed NOD1, a NOD2-related protein, revealing conserved and nonconserved amino-acid residues involved in PGN recognition. These results provide new insights into the molecular function and regulation of NOD2 and related NOD family proteins.

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Introduction

The susceptibility to genetic diseases is largely dependent on the physiological consequence of genetic alterations. In certain disorders including neurofibromatosis and Crohn's disease (CD), the genetic alteration involves highly mutated disease-associated proteins (Rasmussen and Friedman, 2000; Hugot *et al*, 2001). In CD, a common chronic inflammatory disorder of the intestinal tract, nearly 40 variants of the *CARD15* gene have been detected (Hugot *et al*, 2001). NOD2, the product of *CARD15*, is a member of a growing family of proteins, called NODs (for nucleotide-binding oligomerization domains), that have been implicated in the regulation of immune responses and cell death in animals and plants (Girardin *et al*, 2002; Inohara and Nunez, 2003). NOD2 acts as a bacterial peptidoglycan (PGN) recognition molecule through specific detection of the conserved muramyl dipeptide (MDP) structure (Bonen *et al*, 2003; Chamaillard *et al*, 2003c; Girardin *et al*, 2003a; Inohara *et al*, 2003). Three major CD-associated NOD2 mutations, R702W, G908R and L1007fsinsC (Hugot *et al*, 2001; Ogura *et al*, 2001a), and multiple rare variants (Chamaillard *et al*, 2003c) have been found to be deficient in their ability to sense PGN and/or synthetic MDP (Girardin *et al*, 2003a; Inohara *et al*, 2003). Homozygosity and compound heterozygosity increase up to ~40-fold the genotype relative risk for CD compared to simple heterozygosity (~2–4-fold) (Hampe *et al*, 2001; Hugot *et al*, 2001; Ogura *et al*, 2001a).

The human NOD protein family contains approximately 20 members including NOD1, NOD2, Cryopyrin, Apaf-1 and CIITA (Inohara and Nunez, 2003). The great majority of NODs contain an N-terminal effector domain, a centrally located NOD and C-terminal leucine-rich repeats (LRRs) (Inohara and Nunez, 2003). NOD1 and NOD2 have one and two caspase-recruitment domains (CARDs), respectively, as N-terminal effector domains and activate NF-κB through interaction with the downstream factor RICK (also called RIP2 and CARDIAK) (Bertin *et al*, 1999; Inohara *et al*, 1999; Ogura *et al*, 2001b). Oligomerization of NOD1, and presumably of NOD2, promotes the proximity of RICK molecules and of the I-κB kinase (IKK) subunits, leading to IKK and NF-κB activation (Chamaillard *et al*, 2003a; Inohara and Nunez, 2003). In addition to CD, recurrent missense mutations (i.e. R334Q-W and L469F) in the NOD domain of NOD2 have been associated with Blau syndrome (BS), a monogenic, dominantly inherited disease characterized by early-onset granulomatous arthritis (Miceli-Richard *et al*, 2001). The BS-causing mutations exhibit enhanced basal NF-κB activity compared to wild-type NOD2 (Chamaillard *et al*, 2003c). Three other closely related autosomal-dominant diseases, familial cold autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and chronic infantile neurological cutaneous and articular syndrome (CINCA), are associated with missense mutations that localize to the NOD region of Cryopyrin (Chamaillard *et al*, 2003a; Inohara and Nunez,

2003). Moreover, mutations in the LRRs of CIITA are associated with type II bare lymphocyte syndrome, indicating an essential role for the LRRs in CIITA function (Chamaillard *et al*, 2003a; Inohara and Nunez, 2003). Similarly, plant NOD proteins are a class of disease-resistance (R) proteins that recognize pathogens through their LRRs and induce a signalling response against pathogens (Dangl and Jones, 2001; Meyers *et al*, 2003).

In the present study, we generated a comprehensive library of NOD2 and NOD1 variants to study the molecular basis of bacteria-induced NF-κB stimulation. In this study, 519 amino-acid residues of NOD2 were mutated. Functional analyses revealed mechanisms of NOD2 regulation as well as conserved and nonconserved mechanisms whereby NOD1 and NOD2 mediate the recognition of PGN.

Results

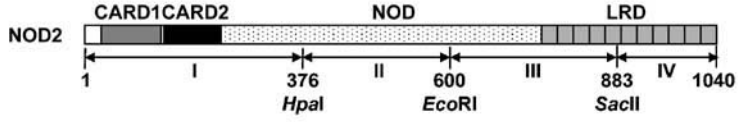
Construction of a library of NOD2 mutants

To define amino-acid residues that are important for NOD2 function and disease susceptibility, we designed an approach to introduce systematically point mutations in the entire coding region of *CARD15/NOD2* at a controlled rate by polymerase chain reactions (Shafikhani *et al*, 1997). The approach combines random mutagenesis and systematic functional analysis of each generated mutant clones. Because NOD2 is composed of 1040 amino acids, the mutants were constructed with four different cassettes separated by unique restriction sites (Figure 1A). Under our experimental conditions, the mutant library for each cassette contained an average of three nucleotide substitutions per 1000 base pairs (range 1–7) resulting in ~2 amino-acid substitutions per cassette (data not shown). In total, 806 nucleotide substitutions in the coding region were analyzed resulting in 519 amino-acid changes (~50% of the NOD2 residues; Figure 1B). Our present study involved 338 independent clones of which 148 contained single amino-acid substitutions and 190 contained multiple (range 2–5) amino-acid replacements in NOD2. A summary of all NOD2 mutant clones including the type of mutation, location of the mutation and *in vitro* functional activity is shown in Figure 1B. The ability of each mutant to induce MDP-dependent and -independent activation of NF-κB was determined using a luciferase reporter NF-κB assay (Inohara *et al*, 2001; Ogura *et al*, 2001b). Briefly, mutant and wild-type NOD2 clones were transiently expressed in HEK293T cells in the presence or absence of MDP. The analysis was primarily based on the 127 clones with single missense mutations and the 59 clones with nonsense and frameshift mutations. However, the results of all generated clones are provided in Supplementary Tables 1, 2 and 3.

CARDs of NOD2 in RICK-dependent NF-κB activation

We mutated ~57% (127/220) of the amino acids in the CARDs of NOD2 (Figure 1B and Supplementary Table 1). The analysis included 14 mutants with only one amino-acid substitution in the CARDs of NOD2, of which seven (Q31H, E69K, T91I, A106V, L145P, P177S and R180K) exhibited complete loss and/or greatly reduced activity in response to MDP (Supplementary Table 1 and Figure 2B), indicating that these CARD residues are essential for NF-κB activation. Representative results from a mutant clone with wild-type phenotype (M152L) and loss-of-function mutants with single amino-acid replacements in CARD1 (Q31H, E69K and A106V) or CARD2 (L145P and R180K) are shown in Figure 2B and/or in Supplementary Table 1. Immunoblotting analysis revealed that most of the loss-of-function mutants were expressed at levels similar to those of the wild-type protein (Figure 2C and Supplementary Table 1). However, some of the CARD point mutants including F161I, P177S and R180K were not expressed, suggesting that these mutations are associated

A



B

			CARD (*n* (%))	NOD (*n* (%))	LRD (*n* (%))	Total (*n* (%))
Changes	Nucleotide Amino acid		ND 127 (58)	ND 249 (48)	ND 143 (49)	806 (26) 519 (50)
Mutant clones single mutation (*n*=148)	Missense	WT	7 (3)	36 (7)	29 (10)	71 (7)
		Gain of function	0	2 (0)	0	2
		Loss of function	7 (3)	21 (4)	20 (7)	47 (5)
	Nonsense	Gain of function	—	—	—	7
		Loss of function	5 (2)	7 (2)	9 (3)	21 (2)
Mutant clones multiple mutations 2–5 (*n*=190)	Missense (529)	WT	36 (16)	84 (16)	40 (8)	171 (32)
		Gain of function	0 (0)	1 (0)	0 (0)	0 (0)
		Loss of function	72 (33)	86 (17)	32 (10)	190 (18)
		Not determined	87 (40)	201 (38)	62 (12)	358 (68)
	Nonsense (53)	WT	—	—	—	1 (2)
		Loss of function	6 (3)	14 (3)	4 (0)	24 (9)
		Not determined	0	6	0	6

Figure 1 Strategy for NOD2 mutagenesis and summary of studied NOD2 mutations. (**A**) Schematic representation of NOD2 including mutagenized cassettes (I–IV). The location of CARDs, NOD domain and leucine-rich repeat domain (LRD) are shown. Numbers represent the position of amino-acid residues. Restriction enzyme sites flanking each cassette are shown. (**B**) Summary of NOD2 mutations generated and analyzed. WT, wild-type; ND, not determined.

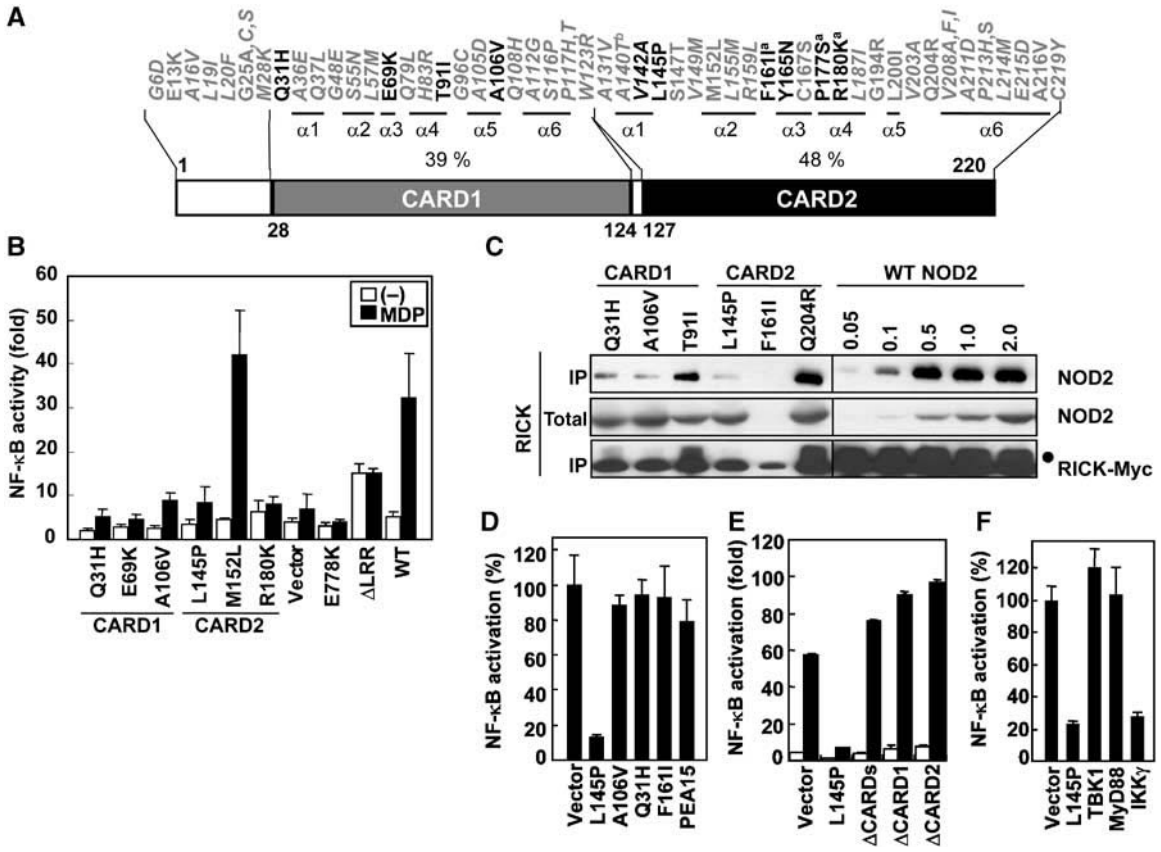


Figure 2 Mutational analyses of the CARD of NOD2. (**A**) Schematic representation of the N-terminus region of NOD2. Numbers indicate the position of amino-acid residues. Loss-of-function mutations are shown in black. Clones with wild-type activity are depicted in gray and mutations found in complex mutant clones (i.e. more than one amino-acid substitution) with wild-type activity are shown in gray italic. Mutations associated with reduced expression are labelled with the symbol [a]. Mutations found in Crohn's patients are labelled with the symbol [b]. (**B**) NF-κB activity of representative CARD mutants. E778K and ΔLRR are shown as controls. Values represent the mean of normalized data ± s.d. of triplicate cultures. (**C**) Interaction of NOD2 mutants with RICK. Extracts from HEK293T cells expressing indicated mutants were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-NOD2 antibody. IP, immunoprecipitation; total, immunoblotting of total lysates. A black dot denotes the mobility shift of the phosphorylated form of RICK. (**D**) Inhibition of RICK-induced NF-κB activation by CARD mutants and PEA15 control plasmid. Values represent the mean of normalized data ± s.d. of triplicate cultures. (**E**) Specific inhibition of MDP-induced NOD2-mediated NF-κB by the L145P mutant. An LPS preparation containing MDP-like activity (closed bars) was used in this assay. (**F**) Inhibition of MDP-induced NF-κB activation by the L145P mutant and dominant-negative form of IKKγ in HEK293T cells. DN denotes dominant-negative form.

with reduced protein stability (Figure 2C and Supplementary Table 1).

NOD2 associates with its downstream effector RICK through a homophilic CARD–CARD interaction to activate NF-κB (Ogura *et al*, 2001b). To determine whether the CARD mutants interact with RICK, we carried out immunoprecipitation experiments in which NOD2 and RICK proteins were transiently coexpressed in HEK293T cells. Immunoprecipitation analyses revealed that RICK interacted with T91I and Q204R, a control mutant with wild-type phenotype, as effectively as with wild-type NOD2 molecule (Figure 2E). In contrast, the loss-of-function NOD2 mutants, Q31H, A106V and L145P, exhibited reduced association with RICK (Figure 2C). As expected, the F161I mutant, which displayed greatly reduced expression, did not immunoprecipitate with RICK (Figure 2C). RICK has been found to be phosphorylated when overexpressed in mammalian cells (Ogura *et al*, 2001b), although the physiological role of phosphorylated RICK remains unclear. Notably, the NOD2 mutants with impaired ability to interact with RICK did not induce the mobility shift associated with RICK phosphorylation (Figure 2C), indicating that RICK phosphorylation correlates with the ability of NOD2 to interact with RICK and with the activation of NF-κB.

A dominant-negative mutant of NOD2 signalling

The interaction between NOD2 and RICK appears essential for NF-κB activation (Ogura *et al*, 2001b). Therefore, we tested if the loss-of-function CARD mutants could inhibit the activity induced through the NOD2 signalling pathway. The analysis revealed that L145P, a CARD2 mutant, but not A106V, Q31H nor F161I, inhibited the ability of RICK to activate NF-κB (Figure 2D). Moreover, L145P, but not mutants lacking one or two CARDs, inhibited the ability of wild-type NOD2 to activate NF-κB in the absence or presence of bacterial components (Figure 2E). HEK293T cells express a low level of endogenous NOD2 and activate NF-κB in response to a high concentration of MDP (5 μg/ml) (Supplementary Table 2). To determine whether the L145P mutant could inhibit the activity of endogenous NOD2, we transfected HEK293T cells with constructs expressing the L145P mutant or dominant-negative forms of TBK1 and

MyD88 (as negative controls) or IKKγ as a positive control and stimulated the cells with MDP. We found that the L145P mutant, like a dominant-negative form of IKKγ, exhibited a dominant-negative effect on NOD2-mediated signalling, whereas interfering mutants of TBK or MyD88 did not (Figure 2F). These results indicate that the dominant-negative effect of the mutation L145P is specific.

Critical residues in the NOD domain required for bacterial recognition

This work included missense mutants with substitutions in 249 amino-acid residues of the NOD domain (Figures 1B and 3A). The analysis of the generated mutant clones was primarily restricted to the 59 mutants with a single amino-acid substitution. Initial functional screening of these mutants using synthetic MDP revealed 21 residues in the NOD domain that are critical for NOD2 function (Figure 3A). These included mutation of the conserved Asp residue at position 379, D379A, in the Walker's B box (Figure 3B), which is essential for binding to the Mg^{2+}-substrate and nucleotide hydrolysis (Walker *et al*, 1982). The D379A NOD2 mutant did not respond to MDP (Supplementary Table 1), suggesting that nucleotide hydrolysis is essential for MDP-dependent NF-κB activation. The equivalent mutation of D284A in NOD1 as well as the K208R mutation in the A box of NOD1 resulted in loss of ligand-dependent NF-κB activation (Supplementary Table 2). Thus, the requirement of the B box for the response to bacterial components is conserved between NOD1 and NOD2. In addition, mutation of amino-acid residues A232, V295, F327, C333, S344, S396, T401, F408, A429, V492, L626, P639, K655, G680, L690, A691, S714 and H734, located within the NOD domain, resulted in loss of MDP-dependent NF-κB activation (Figure 3B). Similarly to D284A, most of these NOD mutants retained their ability to activate NF-κB when overexpressed in HEK293T cells (Figure 3B and Supplementary Table 1). Immunoblotting analysis showed appropriate expression of the mutants except for C333Y and A232P whose expression was undetectable (Figure 3B), suggesting that the latter residues are important for protein stability.

Distinct functional alteration of NOD2 variants associated with BS and CD

Several missense NOD2 mutants in NOD were identified that are found at a low frequency (i.e. less than 1%) in Western European populations (Hugot *et al*, 2001; Lesage *et al*, 2002). Seven of these natural mutations, A140T, R373C, S431L, V793M, N853S, V955I and G978E, were generated through random mutagenesis of NOD2. In addition, we engineered R334W and R334Q, two recurrent BS-causing mutations.

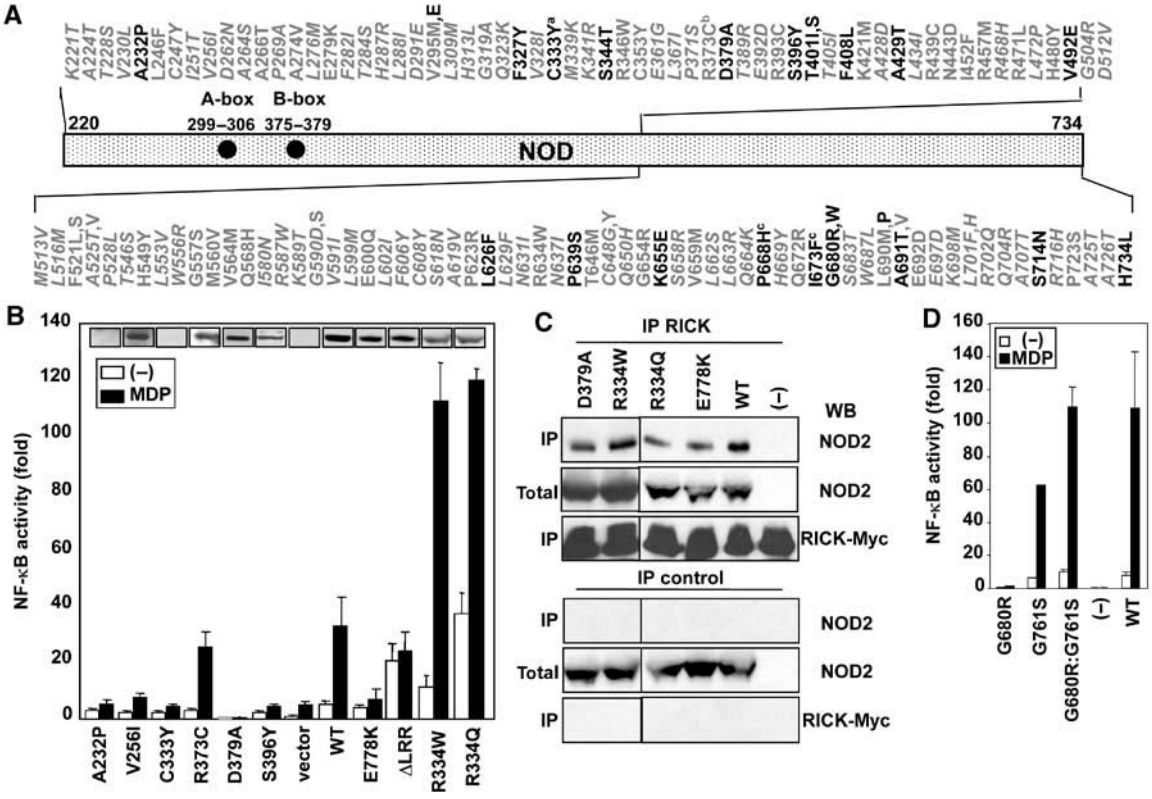


Figure 3 Mutational analyses of the NOD domain of NOD2. (**A**) Schematic representation of the NOD domain. The Walker's A and B boxes are depicted by solid dots. Numbers indicate the position of amino-acid residues. Symbols and color code are described in Figure 2. The gain-of-function mutations are labelled with the symbol [c]. (**B**) NF-κB activity of representative NOD domain mutants in the presence or absence of MDP. E778K and ΔLRR are shown as controls. Expression of NOD2 proteins is shown on top. (**C**) Interaction of NOD2 mutants with RICK. Extracts from HEK293T cells expressing indicated mutants were co-immunoprecipitated with anti-Myc antibody and immunoblotted with anti-NOD2 antibody. IP, immunoprecipitation; total, immunoblotting of total lysates. A black dot denotes phosphorylated form of RICK. (**D**) Evidence of intramolecular interaction between the C-terminal region of the NOD domain and proximal LRRs by functional complementation. NF-κB activity in the presence or absence of MDP is shown for NOD2 mutant and wild-type clones. Values represent the mean of normalized data ± s.d. of triplicate cultures.

Consistent with a recent report (Chamaillard *et al*, 2003c), both R334W and R334Q exhibited increased basal NF-κB activity, which was comparable to that observed with NOD2 lacking the LRRs (Figure 3B). Unlike the ΔLRR NOD2 mutant, the NF-κB activity of R334W and R334Q was further enhanced by addition of MDP (Figure 3B). Thus, the BS-associated R334W and R334Q variants function as hyper-responsive mutations. To determine whether the two BS-causing mutations (R334W and R334Q) and loss-of function mutants (D379A and E778K) could exhibit abnormal interaction with RICK, we coexpressed the NOD2 mutants or V295M (a mutant with wild-type phenotype) and Myc-tagged RICK and immunoprecipitated the cell lysates with anti-Myc antibody. Immunoprecipitation analyses showed that the activity of the mutants cannot be explained by an altered interaction with RICK (Figure 3C).

A novel regulatory region of NOD2

Multiple mutations (i.e. frameshift and nonsense mutations) resulted in premature C-terminal truncation of NOD2 (Figure 4). Truncation at the first or second CARD of NOD2 resulted in mutations that were unable to activate NF-κB (Figure 4). This is consistent with the observation that both CARDs of NOD2 are required for association with RICK, an essential downstream factor for NOD2 activity (Ogura *et al*, 2001b; Kobayashi *et al*, 2002). Truncation at the most C-terminal LRRs (residues 855-1040) of NOD2 resulted in mutants that were able to activate NF-κB at a level comparable to that of the wild-type protein, but were unresponsive to bacterial components (Figure 4 and Supplementary Table 1). Notably, sequential truncation from residue 664 to 854 resulted in NOD2 mutants with elevated basal NF-κB activity (Figures 4 and 5A and Supplementary Table 1). Additional truncation N-terminal to residue 664 or C-terminal to residue 855 (located in the fifth LRR) resulted in loss of the constitutive ability of NOD2 to activate NF-κB (Figures 4 and 5A). Immunoblotting analysis revealed that the expression levels of the truncated NOD2 mutants could not explain the observed differences in NF-κB activity (Figure 5B). These results indicate that the borders of the regulatory region are located between residues 664 and 854. Furthermore, the region from residue 855 to the C-terminus, which expands from the distal part of the fifth LRR to the 11th LRR, is sufficient to suppress the constitutive activation of NOD2. To test whether the mechanism of activation is conserved between NOD1 and NOD2, we generated a panel of NOD1 mutants and tested their ability to induce NF-κB in the presence or absence of a commercial lipopolysaccharide (LPS) preparation from *Escherichia coli* O55:B5, with traces of PGN-derived γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP), the moiety recognized by NOD1 (Chamaillard *et al*, 2003b; Girardin *et al*, 2003b). These experiments revealed that truncation of NOD1 from residues at positions 648 and 668 yielded mutants with enhanced basal NF-κB activity but unresponsive to the LPS preparation (Figure 5C and Supplementary Table 2). These results indicate that the distal LRRs of both NOD1 and NOD2 are essential for suppression of the constitutive activation of these proteins as well as for recognition of bacterial components (Figure 4 and below).

The analysis uncovered three loss-of-function mutants within the regulatory region that could be rescued in *cis* by mutations within the proximal LRRs. For example, G680W

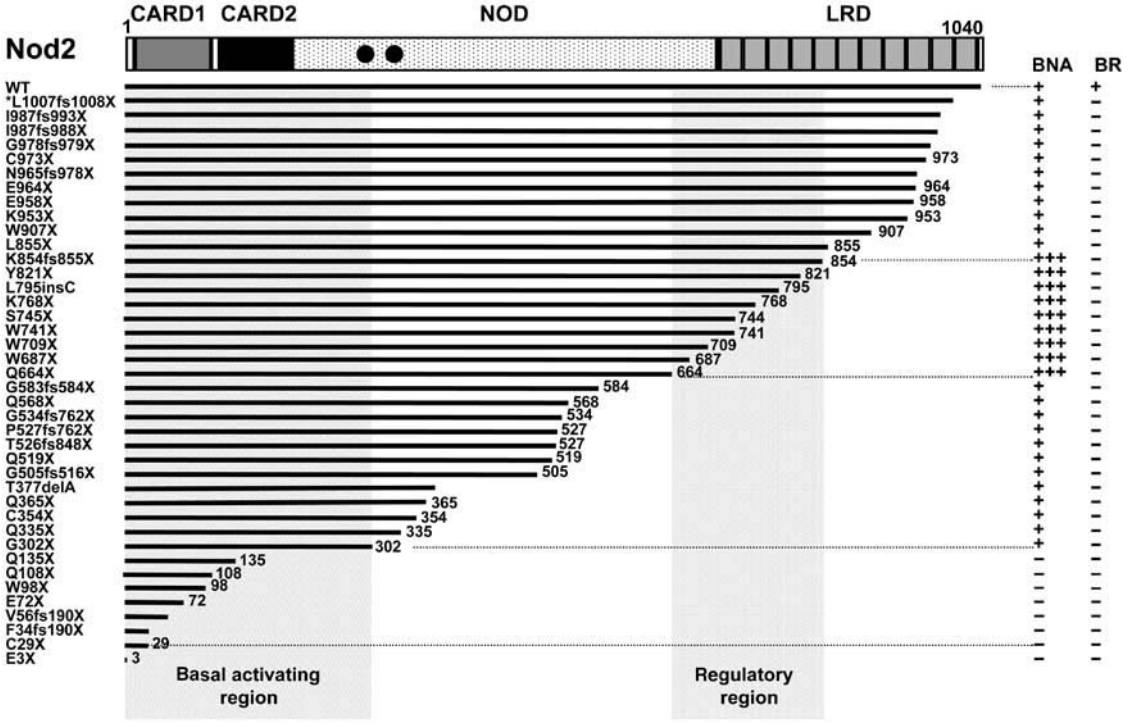


Figure 4 Analysis of truncated NOD2 mutants. The location of CARDs, NOD domain and LRD are shown. The Walker's A and B boxes in the NOD domain are depicted by solid dots. The basal NF-κB activity (BNA) and NF-κB response to bacterial components (BR) are shown. +, response similar to that obtained with the wild-type protein; −, no response above control plasmid; + + +, response at least three-fold greater than that obtained with wild-type NOD2. Representative results are shown in Figure 3. Numbers represent the position of amino-acid residues. The names of individual mutations are shown on the left. WT, wild-type clone.

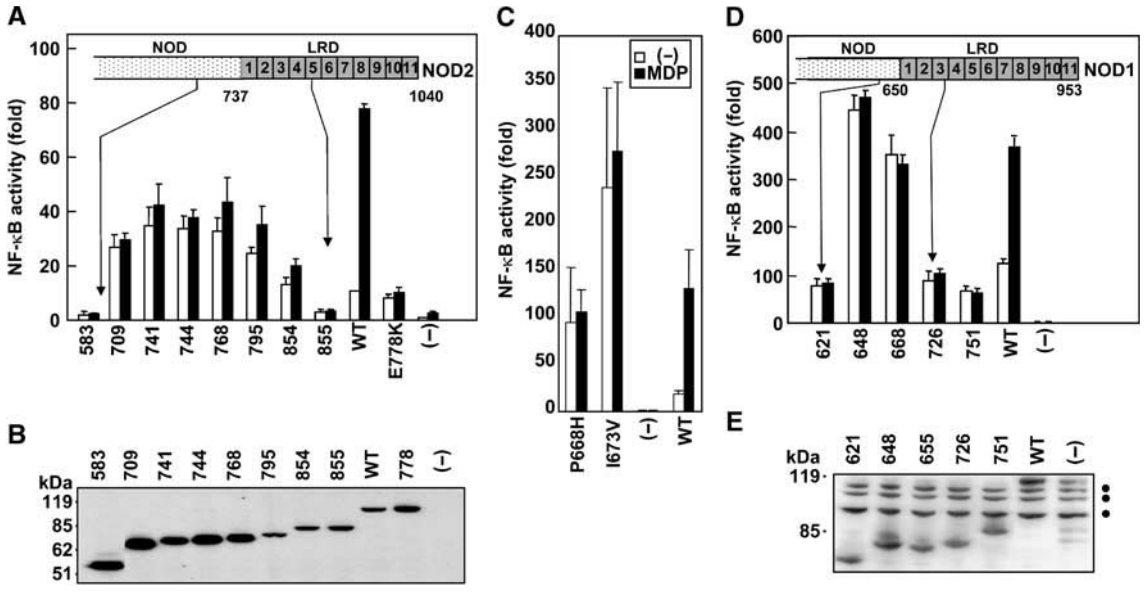


Figure 5 Analyses of truncated mutants reveal an inhibitory domain in NOD1 and NOD2. (**A**) NOD2 activity in the absence (open bars) and presence of LPS preparation (closed bars). A schematic diagram of part of NOD and LRD of NOD2 is shown. Numbers indicate the position (amino-acid residue) of C-terminal truncations. WT, wild-type. E778K represents a control loss-of-function mutant. (−) represents results obtained with control plasmid. (**B**) Immunoblotting analysis of NOD2 mutants. Analysis was performed with cell extracts from HEK293T cells transfected with indicated mutant or wild-type NOD2 plasmid or control plasmid (−) and immunoblotted with anti-FLAG antibody. (**C**) NF-κB activity of NOD domain gain-of-function mutants in the presence or absence of MDP. Wild-type (WT) and mock cells (−) are shown as controls. Values represent the mean of normalized data ± s.d. of triplicate cultures. (**D**) NOD1 activity in the absence (open bars) and presence of LPS preparation (closed bars). A schematic diagram of part of NOD and LRD of NOD1 is shown. Numbers indicate the position (amino-acid residue) of C-terminal truncation. WT, wild-type. (**E**) Immunoblotting analysis of NOD1 proteins. The analysis was performed with cell extracts from HEK293T cells transfected with indicated mutant and wild-type NOD1 plasmids or control plasmid (−) and immunoblotted with anti-NOD1 antibody.

was a loss-of function mutant in that it failed to respond to MDP (Figure 3D). Yet, the double G680W/G761S, as the single G761S, was fully competent to be activated by MDP (Figure 3D). Similarly, the point mutant G775D functioned as a loss-of-function mutant, whereas the A725T/A726T and G775D/A725T/A726T mutants responded normally to MDP (Supplementary Table 2). Similar complementation was observed with two additional loss-of-function mutants G680R and C779Y in that the triple G680R/C779Y/N872K exhibited a normal response to MDP (Supplementary Table 1). These results reveal functional interactions between residues located in the C-terminal region of the NOD domain and the proximal LRRs.

Missense mutations in the C-terminal region of the NOD domain can exhibit constitutive NF-κB activity

We identified several missense mutations within the inhibitory domain described above (P668H, I673F, G680R/C710Y, F719I/K731R and N637I/A725V) that exhibited constitutive NF-κB activity (Figure 5E and Supplementary Table 1). Unlike the BS-associated mutations, the increased basal activity of these mutants was not further enhanced by MDP (Figure 5C). The G680R mutation by itself is a loss-of-function mutation, but the G680R/C710Y mutant clone is a gain-of-function mutant, suggesting intramolecular interactions within the regulatory region (Supplementary Table 1). P668H and I673F represent mutants with single amino-acid substitutions. Significantly, we identified a point mutant, L620Q, in the corresponding region of NOD1 that also exhibited constitutive NF-κB activity (Supplementary Table 2), suggesting a conserved mechanism of NF-κB activation regulation. Amino-acid alignment of NOD2, Cryopyrin and related NOD family members revealed that I673 in NOD2 corresponds to amino-acid residue F573 in Cryopyrin (Supplementary Figure 1). Notably, an F573S mutation in Cryopyrin causes the autoinflammatory disorder CINCA (Hull *et al*, 2003). Thus, F573S may represent a constitutively active mutation of Cryopyrin leading to clinical disease.

Amino-acid residues in the LRR predicted to be solvent exposed are essential for bacterial recognition

The analysis included 50 LRR mutants involving single amino-acid residues (Figure 1B and Supplementary Table 1). Functional analysis revealed that 20 out of the 50 mutants were unable to respond to MDP (Figure 6 and Supplementary Table 1). In contrast, multiple LRR mutants including A759V, G761S, L762M, V793M, I805N, P812T, D824N, A860G, L876I, Q889P, L891M, A892V, L904M, D913V, A920T, A944V, L947M, L949M, M956T, Q968H, G978E, S984T, K986I, A1002S, T1012I/S, G1032C and L1038F exhibited a wild-type phenotype (Figure 6 and Supplementary Table 1). Three-dimensional molecular modelling using the structure of the placental ribonuclease inhibitor (Papageorgiou *et al*, 1997) revealed that the loss-of-function mutations E751K, G775D, E778K,C779Y, D829Y, G831D, T844I, V890A, T899A, D913E, G915E, G999R and N1010S are predicted to be located in the turn regions and α-helices of the LRRs (Figure 7C). In contrast, the loss-of-function mutations G879R, W907L, V935M, E959K, C961Y, K986I, K989E and S991F involve amino-acid residues that are predicted to form β-strands that constitute

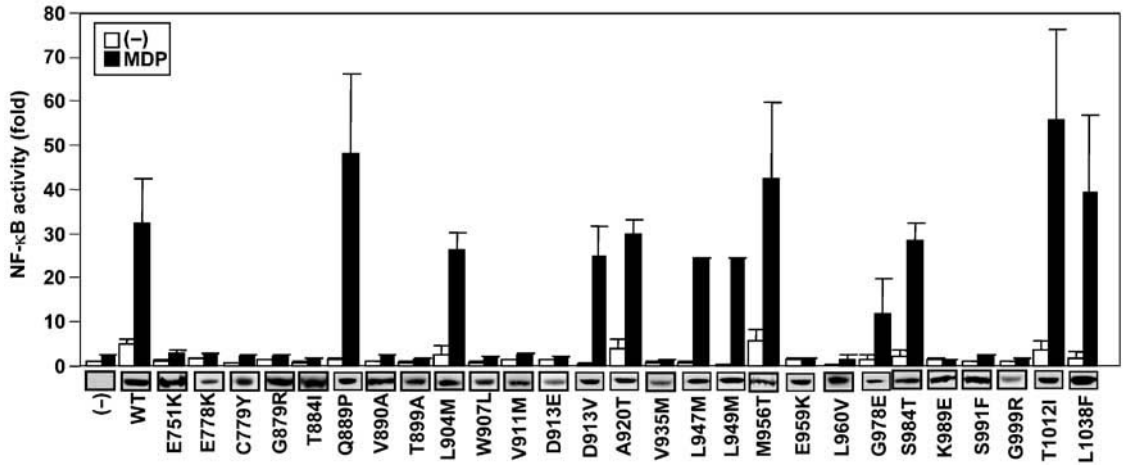


Figure 6 Mutational analyses of the LRRs of NOD2. NF-κB activity of LRR mutants in response to MDP. Values represent the mean of normalized data ± s.d. of triplicate cultures. Expression of NOD2 proteins is shown at the bottom.

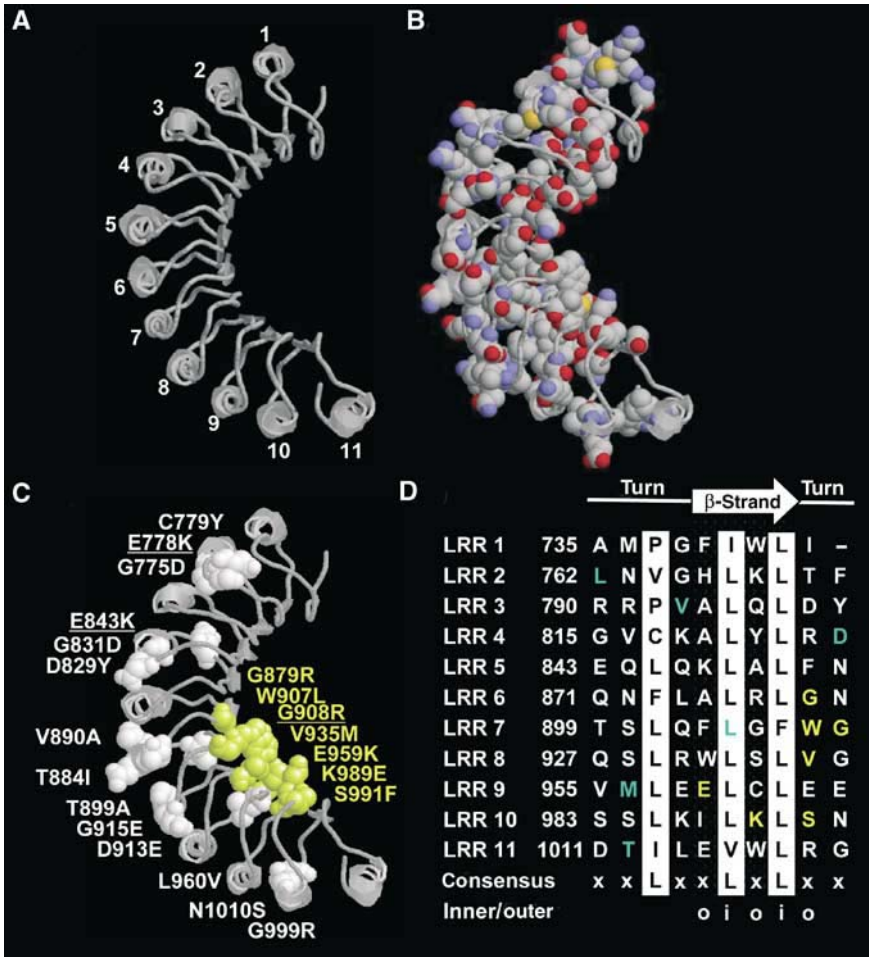


Figure 7 Computer-assisted molecular modelling of the LRRs of NOD2. (**A**) The predicted structure of the 11 LRRs of NOD2. (**B**) Location of amino-acid residues mutagenized. (**C**) Position of loss-of-function NOD2 mutants with single amino-acid substitutions in the LRRs. Mutations located in α-helix/turn regions and β-strands are depicted in white and yellow, respectively. The E778K, E843K and G908R mutants (underlined) have been found in CD patients. (**D**) Amino-acid alignment of the turn region and β-strands of LRRs of NOD2. Letters denote amino-acid residue. X denotes any amino acid. The locations of residues involved in amino-acid substitutions with wild-type and loss-of-function phenotype are shown in green and yellow, respectively. The specific amino-acid replacements are shown in Figure 6 and Supplementary Table 1.

the concave face of the LRRs (Figure 7D). Notably, all these amino-acid residues reside in LRR6–LRR11, indicating that the most C-terminal LRRs of NOD2 are critical for bacterial recognition. More specifically, these residues are confined to the β-strand/β-turn (xxLxLxx) motif (Kajava and Kobe, 2002). Amino acids G879, W907, V935, E959, K989 and S991 are variable in that they are not conserved in the corresponding regions of LRR proteins and are predicted to

face outward (Figure 7D). These nonconserved residues correspond to those known to mediate the interaction of other LRR proteins with their binding partners (Kobe and Deisenhofer, 1995; Papageorgiou *et al*, 1997; Price *et al*, 1998). These results suggest that the residues G879, W907, V935, E959, K989 and S991 might be involved in MDP binding and/or protein–protein interactions required for MDP recognition.

Comparison between the LRRs of NOD1 and NOD2

We generated 31 missense mutants by random mutagenesis involving the LRRs of NOD1. Eight residues were essential and 23 were dispensable for the response to the LPS preparation containing NOD1-stimulatoty activity (Supplementary Tables 2 and 3). To determine whether the residues required for recognition of bacterial components are conserved, we compared the activity of NOD1 and NOD2 mutants with homologous amino-acid substitutions at the same LRR positions. The comparison revealed that one pair of mutated residues exhibited impaired recognition of bacterial components (D691V and E778K in NOD1 and NOD2, respectively), four pairs had wild-type activity in both NOD1 and NOD2, and two pairs of homologous residues (G857R and A944V, and K989E and W902G, in NOD1 and NOD2, respectively) exhibited discordant phenotype in that the mutations impaired NOD2 but not NOD1 function. Notably, E778K is a loss-of-function NOD2 mutation found in CD patients (Hugot *et al*, 2001). These results suggest that conserved and nonconserved structures in the LRRs of NOD1 and NOD2 mediate the recognition of bacterial PGN motifs. As it was found in NOD2, several residues in the proximal LRRs of NOD1 (D691, L715 and N738) were essential for PGN recognition (Supplementary Table 3).

Discussion

In this report, we describe a systematic mutational strategy to get an insight into the molecular function of NOD2 and the mechanism of disease susceptibility. In the present study, we identified multiple residues in the LRRs that are essential for bacterial recognition. Several mutations deficient in MDP recognition (E751K, G775D, E778K, C779Y, D829Y, G831D, T884I, V890A, V911M, D913E, G915E, L960V, G999R and N1010S) involved residues predicted to be located in the α-helix/turn regions that form the convex face of the LRRs. The role of the α-helix/turn LRR residues in MDP recognition is unclear. They may be involved in protein folding or intramolecular interactions between the NOD and LRR domains or between the different LRRs rendering NOD2 competent for MDP recognition. Alternatively, these residues may be involved in interactions with MDP directly or indirectly through cellular factors. We favor the first two possibilities because structural and genetic analyses have implicated β-strands rather than the α-helical regions of the LRRs in ligand interactions (see below). Notably, the loss-of-function mutations E778K and E843K (Chamaillard *et al*, 2003c) are predicted to alter the α-helix/turn LRR structure (Figure 7) and have been found only in CD patients, suggesting a potential molecular mechanism leading to CD. Several amino acids located in the concave face of LRR7–LRR11 were found to be essential for MDP recognition. These amino acids, G879, T899, W907, V935, E959, K989 and S991, are predicted to form a β-strand/β-turn structure based on molecular comparisons of the LRRs of NOD2 with those of the human and porcine ribonuclease inhibitor proteins (Kajava and Kobe, 2002). Three-dimensional analyses of ribonuclease inhibitor proteins in complex with their targets revealed that LRR residues corresponding to G879, W907, G908, V935, E959, K989 and S991 within the LRRs of NOD2 are predicted to be exposed to solvent and mediate interactions with their protein partners (Kobe and Deisenhofer, 1995; Papageorgiou *et al*, 1997).

In plant NBS-LRR proteins, the LRR is the most variable region and is under diversifying selection driven by pathogens (Dangl and Jones, 2001). Within the LRRs of plant R proteins, most variation occurs within variable residues predicted to form the β-strand/β-turn structure of the LRRs (Dangl and Jones, 2001). Analyses of chimeric genes have revealed that the nonconserved residues confined to the β-strand/β-turn structure determine pathogen recognition and specificity of R genes (Dodds *et al*, 2001). Our studies suggest a shared molecular mechanism between animals and plants for intracellular pathogen recognition. A possible interpretation is that MDP interacts directly with the LRRs and more specifically with the β-strand/β-turn structure of the LRR6–11 of NOD2. Alternatively, NOD2 may require host cofactors to interact with MDP. It is also formally possible that these LRR residues are involved in some other function that is indirectly required for MDP recognition.

The present study has revealed a region encompassing the C-terminal region of the NOD domain and proximal LRRs that mediate inhibition of NOD2 activity. A similar inhibitory region has been identified in plant NBS-LRR proteins (Bendahmane *et al*, 2002; Shirano *et al*, 2002; Zhang *et al*, 2003). NOD1 also contains a similar region capable of repressing its basal NF-κB activity, suggesting a conserved downregulatory mechanism in plants and mammalian NOD family members. The regulatory function might be mediated through intramolecular interactions between the inhibitory region and sequences in the CARDs and/or the NOD domain. Such interaction(s) will be predicted to inhibit the activity of NOD2 in that the CARDs and NOD domain are essential for MDP-dependent NF-κB activation. Consistent with this model, there is evidence for intramolecular interactions including the LRR with the N-terminal region of the NOD domain in plant NBS-LRR proteins (Hwang *et al*, 2000; Moffett *et al*, 2002; Hwang and Williamson, 2003). Thus, the regulatory region of NOD2 may mediate an inhibitory function to retain the protein in an inert conformation in the absence of bacterial products. Alternatively, the regulatory region may mediate activation that is repressed by the C-terminal flanked LRRs. We identified functional complementation between amino-acid residues located in the first and second LRRs and the C-terminal region of the NOD domain. A possibility is that the proximal LRRs suppress NOD2 activation through the C-terminal region of the NOD domain. Upon exposure to MDP, the inhibition of NOD2 mediated by the proximal LRRs may be relieved leading to signalling through NOD oligomerization and recruitment of RICK.

We identified several point mutations located in the regulatory region and specifically within the C-terminal region of the NOD domain that exhibit constitutive activity. These NOD2 mutations appear to be related to signalling and/or regulation rather than recognition in that they activated

NF-κB in an MDP-independent manner. This C-terminal region of NOD corresponds to the previously described ARC subdomain (van der Biezen and Jones, 1998). Interestingly, similar gain-of-function mutations located in the ARC subdomain and N-terminal LRRs have been described in plant NBS-LRR proteins (Bendahmane *et al*, 2002; Shirano *et al*, 2002; Zhang *et al*, 2003). These results argue for conservation of regulatory mechanisms between plant and animal NOD proteins. Unlike the BS-associated mutations, located within the regulatory region and the N-terminal NB subdomain, the activity of these mutants including P668H and I673F was not further enhanced by MDP. Significantly, the corresponding residue at position 673 in human Cryopyrin is F573 whose Ser mutation causes the neonatal autoinflammatory disorder CINCA (Hull *et al*, 2003). Thus, Cryopyrin F573S might be a constitutively active mutant that is compatible with the dominant mode of transmission of the disease. These two types of activating mutations may explain, at least in part, differences in clinical presentation and severity observed between MWS/FCAS and CINCA. The molecular basis for the constitutive activation of NOD2 mutations located in the C-terminal region of the NOD domain is unclear. A possibility is that they disrupt the repressing function leading to deregulated activation. In such a model, these activating mutations may mimic recognition of MDP in that the inhibitory activity exerted by the proximal LRRs may be relieved by the mutation.

Numerous amino-acid variations have been found in *CARD15* of humans and mice (Hugot *et al*, 2001; Ogura *et al*, 2003). In humans, the great majority of NOD2 variants are rare in that they are found at a frequency of less than 1% in healthy and/or CD patients (Hugot *et al*, 2001; Lesage *et al*, 2002). Up to 20% of these rare alleles were retrieved in the present study. These results together with those of a recent study (Chamaillard *et al*, 2003c) indicate that rare and common loss-of-function NOD2 alleles are involved in disease pathogenesis. Consistent with previous findings (Chamaillard *et al*, 2003c), several of the rare CARD15/NOD2 variants found in CD patients, including A140T, R373C, V793M and G978E, exhibited wild-type function, suggesting that they likely represent neutral polymorphisms without clinical significance (Chamaillard *et al*, 2003a).

Several amino-acid residues in the CARDs of NOD2 were essential for NF-κB activation. The loss of activity of most of the CARD mutants could be explained, at least in part, by their decreased ability to associate with RICK. Three rare CD-associated NOD2 missense mutations were found to exhibit a loss-of function phenotype (Chamaillard *et al*, 2003c). The residues involved in these CD mutations are located in the α-helices of the CARDs, suggesting that these mutants may exhibit impaired binding to RICK. L145P, a loss-of-function CARD2 mutant, functioned as a dominant negative of NOD2 signalling. Three CD-associated NOD2 mutants were reported to act as dominant negative (Chamaillard *et al*, 2003c). The mechanism of inhibition mediated by L149P and the CD-associated mutations remains to be elucidated. These mutant proteins may act by interfering with the recruitment of active RICK complexes, NOD2 oligomerization and/or by sequestrating another factor required for downstream signalling.

The NOD2 variants associated with BS, R334W and R334Q exhibited increased basal NF-κB activity and enhanced response to bacterial components. This is in contrast to NOD2 mutations associated with CD, which display defective recognition of MDP (Bonen *et al*, 2003; Chamaillard *et al*, 2003c; Girardin *et al*, 2003a; Inohara *et al*, 2003). The centrally located NOD in plant/animal proteins corresponds to the NB-ARC domain that includes the NB and ARC subdomains (van der Biezen and Jones, 1998). These BS-associated mutants were located in the N-terminal region of the NOD domain or NB subdomain (van der Biezen and Jones, 1998). The difference in functional activity displayed by NOD2 mutations associated with CD and BS is consistent with the distinct mode of genetic transmission of these NOD2-associated diseases and could explain the clinical differences observed between these two disorders (Chamaillard *et al*, 2003a). Significantly, the R334W mutation in NOD2 corresponds to the R260W Cryopyrin mutation, which is known to cause the inflammatory disorders MWS and FCAS (Chamaillard *et al*, 2003c). Thus, BS and MWS/FCAS may share a common deregulated molecular mechanism.

Almost all residues whose mutation resulted in loss of function are conserved in both human and mouse NOD2 (Ogura *et al*, 2003) (Supplementary Table 1). However, substitution of Pro for Ser at position 177 in the CARDs of human NOD2 resulted in loss of function (Figure 4A). Notably, the corresponding residue at position 177 in mouse NOD2 is Ser (Ogura *et al*, 2003). Moreover, the W130R loss-of-function mutation is found in CD patients (Chamaillard *et al*, 2003c) but mouse NOD2 contains a Trp at the same position (Ogura *et al*, 2003). Similarly, P639S and K665E mutations result in loss of function in human NOD2, but puffer fish and zebrafish NOD2 carry Ser and Glu at these positions, respectively (Ogura *et al*, 2003). These observations reveal conserved and nonconserved structures in human NOD2 and suggest potential pitfalls when deciphering protein function based solely on conservation of amino-acid residues. The identification of residues acting in *cis* to alter the activity of mutant NOD2 highlights the importance of considering mutant combinations when performing functional and genetic analyses of disease-associated proteins.

The current work represents an exhaustive mutational analysis of NOD2, in that 519 out of the 1040 amino acids of NOD2 were mutated. Individual analysis of certain mutant proteins should be interpreted with caution in that some negative results are noninformative due to the potential misfolding of the mutant protein. In addition to providing insights into the molecular function of NOD2, up to 20% of the genetic variants found at rare frequency in CD patients were recovered in the current analysis. A similar approach could be used to analyze any disease-associated protein in the human population. Such approach could be facilitated by high-throughput functional analysis.

Materials and methods

Reagents

LPS from *E. coli* O55:B5 and MDP were obtained from Sigma-Aldrich (St Louis, MO). The source of LPS from *Campylobacter jejuni* was described (Inohara *et al*, 2001). iE-DAP was synthesized as described previously (Chamaillard *et al*, 2003b).

Mutagenesis of NOD1 and NOD2

The strategy to perform random mutagenesis of NOD2 is described in the Results section. Each cassette containing the coding region of

NOD2 was mutagenized using the GeneMorph PCR kit (Stratagene, La Jolla, CA), which is based on the use of an error-prone DNA polymerase and reaction conditions that allow the generation of mutations at a controlled rate (Shafikhani *et al*, 1997). Each primer was designed to contain restriction sites so that each PCR product was digested with appropriate restriction enzymes and inserted in-frame into the remainder of the coding region of NOD2 (see Figure 1B). A nucleotide cassette encoding the C-terminal region of NOD1 (amino-acid residues 605–953) was similarly mutagenized using the GeneMorph PCR kit. The entire coding sequence of NOD1 and NOD2 clones was verified by direct DNA sequencing. Expression plasmids producing E778K and BS-associated NOD2 mutants, R334W and R334Q, were generated by the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The authenticities of the constructs were confirmed by DNA sequencing.

Transfection and NF-κB activation assay

HEK293T cells were transfected with reporter plasmids (7.3 ng pBxIV-luc and 73 ng pEF-BOS-β-gal/well) plus control plasmid (−) or pcDNA3-NOD1-FLAG (0.3 ng/well) or pcDNA3-NOD2 (0.1 ng/well) as described previously (Inohara *et al*, 2001). Plasmids expressing dominant-negative forms of TBK1, MyD88 or IKKγ have been reported (Inohara *et al*, 2003). The NOD2 mutants, ΔCARD1, ΔCARD2 and ΔCARDs, have been described (Ogura *et al*, 2001b). NF-κB activation assays with HEK293T cells were performed as described (Inohara *et al*, 2003). LPS (1 μg/ml) and MDP (10 ng/ml) were added to the cultures 8 h post-transfection in the presence of calcium phosphate to allow their entry into the cells as reported (Inohara *et al*, 2003). All NOD2 mutant clones were tested for their response to synthetic MDP except truncation mutants (Figure 4), which were stimulated with an LPS preparation known to contain PGN fragments stimulatory of NOD2 (Bonen *et al*, 2003; Chamaillard *et al*, 2003c; Girardin *et al*, 2003a; Inohara *et al*, 2003).

To assess iE-DAP- and MDP-independent NF-κB activation, HEK293T cells were transfected as described above except that 100 ng of NOD1 or NOD2 plasmid, respectively, was used (referred as overexpression studies). At 24 h post-transfection, NF-κB-dependent transcription was determined (Inohara *et al*, 2003). Results were normalized for transfection efficiency with values obtained with pEF-BOS-β-gal.

Immunoprecipitations and immunoblotting

HEK293T cells were harvested 24 h following transfection and lysed in 0.2% Nonidet P-40 lysis buffer (Inohara *et al*, 2001). Immunoprecipitations were carried out using monoclonal anti-HA antibody (Roche, IN) to immunoprecipitate RICK protein complexes. The products were then resolved by 12% SDS–PAGE, and detected by immunoblotting with rabbit anti-NOD2 (Ogura *et al*, 2001b) or rabbit anti-HA antibody to detect RICK (Santa Cruz, CA). Total lysates were similarly analyzed by immunoblotting with rabbit anti-NOD1 or anti-NOD2. Polyclonal anti-NOD1 antibody was generated by immunization of rabbits with recombinant human NOD1 (amino acids 1–216). A reference extract from HEK293T cells transfected with the wild-type NOD2 cDNA was included in all immunoblotting experiments to compare the expression of mutant and wild-type proteins.

Bioinformatics

Alignment of LRRs of NOD2 was performed using the program O with U2A′ (PDB 1A9N) and coordinates of ribonuclease inhibitor (PDB 1DFJ) as templates. After slight manual adjustment, coordinates were energy minimized using CNS (Brunger *et al*, 1998).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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