

The Molecular Functions of Nod Proteins and their Associated Diseases

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Abstract: Nod proteins are defined as proteins carrying nucleotide-oligomerization domains (NODs) and are involved in regulation of immune responses and apoptosis. The Nod protein family contains 23 human members including Nod1, Nod2, cryopyrin, Ipaf, Apaf-1 and CIITA, as well as thousands of plant proteins, which are involved in pathogen-specific defense responses. A Nod protein generally contains an amino-terminal domain for binding downstream effector molecules, a central NOD and a carboxyl-terminal ligand recognition domain (LRD). Nod1 and Nod2 are involved in host recognition of small molecules that are components of bacterial peptidoglycan and activate nuclear factor κ B (NF- κ B) in response to sensing these molecules. This NF- κ B activation occurs in a RICK- and IKK-dependent manner. The core ligand structure for Nod2 is muramyl dipeptide, a structural motif common in all bacteria, whereas the ligand for Nod1 is a dipeptide designated as iE-DAP, a motif found in only certain subgroups of bacteria. These molecules and their derivatives mediate host innate responses against bacteria and also function as immunostimulatory adjuvants through induction of cytokine secretion and co-stimulatory molecule expression. Although the mechanism is unknown, genetic and functional defects of Nod proteins are associated with several inflammatory diseases and immunodeficiency. These include susceptibility for Crohn's disease and Blau syndrome (Nod2), three related inflammatory diseases (cryopyrin) and type II bare lymphocyte syndrome (CIITA). Functional analyses of mutant Nod proteins suggest a common molecular basis for these diseases.

INTRODUCTION

Nod proteins are a group of key switching proteins that are involved in recognition of upstream signaling molecules and turn on the downstream events by activating effector molecules. The Nod protein family contains around 20 human members, as well as rodent, fish, and chordate orthologues and paralogues, and including thousands of plant proteins, which are involved in pathogen-specific host defense responses (Table 1) [for review, 1]. The initial Nod family members, including nematode Ced-4, human Apaf-1, CIITA, cryopyrin, Mater, NAIP, and several plant homologues, were identified independently [2-8]. These proteins were found as key regulators of programmed cell death, human disease-associated gene products, or in plant pathogen resistance. Soon, large numbers of their homologues were discovered by bioinformatic analysis [1, 9-24]. Due to the diversity of their actions, initial members were not analyzed to find common molecular functional features among them. However, further functional comparisons between several Nod family members revealed important functional features of the nucleotide-binding oligomerization domain (NOD) that all these family members have in common [1, 9-24]. The NOD found in Nod proteins is a member of a unique subgroup of ATP-binding cassette (ABC) domain superfamily and is distinguishable from millions of other ABC-containing proteins by an extended homology region designated as NACHT, NB-ARC, and/or death ATPase [25-27]. Although Nod proteins are

typically defined as NOD-containing proteins, some mammalian Nod proteins are also called CATERPILLER proteins or NALPs [22,23], according to other structural homologies or molecular functions.

MOLECULAR STRUCTURE AND FUNCTION OF NOD2

Typical Nod proteins have three functional domains: an amino-terminal effector molecule-binding domain (EBD), a central NOD, and a carboxyl-terminal ligand sensor domain (Table 1, Fig. 1). Nod proteins are involved in a number of diverse signaling pathways. The structure of the EBDs and ligand sensor domains included in a particular Nod protein determine the upstream ligand and downstream effector molecule specificities, respectively.

Many human Nod proteins have homophilic domains as EBDs, namely caspase-recruitment domains (CARDs) and pyrin domains (PDs), which bind downstream effector molecules. For example, Apaf-1 and its nematode homologue Ced-4 bind to pro-apoptotic cysteine proteases, (caspase-9 and Ced-3, respectively), through N-terminal CARD interactions [28, 29]. Nod1 and Nod2 bind to RICK through homophilic CARD interactions as well [10,11]. Cryopyrin and Ipaf bind to ASC by homophilic interactions through their PDs or CARDs, respectively [15,16,30]. These downstream effector molecules (or complexes) bound to the N-terminal EBDs of Nod proteins are all activated when brought into close proximity. The close proximity of several caspase-9 molecules induces conformational changes in caspase-9 resulting in an enzymatically active form [31,32]. The close proximity of RICK and ASC induces activation of a pro-inflammatory transcription factor, nuclear factor- κ B

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Table 1. Human Nod Proteins

Name	Synonyms	Locus	EBD	Sensor Expression ¹	Downstream domain	effectors	Ligand	Functions
Apaf-1		12q23	CARD	ubiquitous	WDRs	Caspase-9	cytochrome c	Caspase activation
Nod1	CARD4	7p14	CARD	ubiquitous	LRRs	RICK	iE-DAP	NF- B and caspase activation
Nod2	CARD15	16q12	CARD x2	APC	LRRs	RICK	MDP	NF- B and caspase activation
Ipaf	CARD12, CLAN	2p22	CARD		LRRs	ASC		NF- B and caspase activation
Cryopyrin	NALP3, PYPAF1, CIAS1	1q44	PD	APC	LRRs	ASC		NF- B and caspase activation
NALP2	PYPAF2	19q13	PD	ubiquitous	LRRs			
Nod12	PYPAF3, NALP7	19q13	PD		LRRs			
PAN2	PYPAF4, NALP4	19q13	PD	ubiquitous	LRRs			
PYPAF5	NALP6	11p15	PD	ubiquitous	LRRs			
Nod17	PYPAF6, NALP11	19q13	PD	ubiquitous	LRRs			
PYPAF7	Monach1, NALP12, RNO2	1219q13	PD	APC	LRRs			
Mater	PYPAF8, NALP5	19q13	PD	E	LRRs			
Nod16	NALP8	19q13	PD		LRRs			
Nod6	NALP9	19q13	PD	ubiquitous	LRRs			
PYNOD	NALP10, Nod8	11p15	PD	ubiquitous	None			
Nod14	NALP13	19q13	PD		LRRs			
Nod5	NALP14	11p15	PD	ubiquitous	LRRs			
Nod27		19q13	PD	APC	LRRs			
NAC	DEFCAP, NALP1, CARD7	17p13	PD, CARD ²	APC, B, T, NK	LRRs	ASC, Caspases ²		Caspase activation
CIITA		16p13	AD ³	APC, B, T, NK	LRRs	TFs		MHC-II Expression
NAIP	BIRC1	5q13	BIRx3	APC, m ⁴	LRRs			
Nod3		16p13	x	ubiquitous	LRRs			
Nod9		11q23	x	ubiquitous	LRRs			

Only the function and downstream effector molecules that have been reported by two or more groups are shown. ¹The tissues with the highest expression levels of each Nod protein in public human tissue microarray database SymAtlas v0.7.3 (<http://symatlas.gnf.org>) are shown. ²NAC is reported to bind ASC through N-terminal PD and bind caspase-4, 5 through C-terminal CARD to activate procaspase-1. ³Dendritic cells-specific CIITA also has an N-terminal CARD which enhances the ability to induce MHC-II expression. ⁴NAIP has been shown to be expressed in macrophages and confer resistance against *Legionella* [76]. Abbreviation: AD, activation domain; APC, antigen presenting cells and their precursors (BDCA4+, CD33+ or CD14+); B, CD19+ B cells; CARD, caspase-recruitment domain; CD71+ early erythroid cells; D, effector-binding domain; PD, pyrin domain; LRRs, leucine-rich repeats; m, macrophages; NK, CD56+ NK cells; PD, pyrin domain; T, T cells; TFs, transcription factors; WDRs, WD40 repeats; X, unclassified domain

(NF- B) through the actions of I B kinase (IKK), a general regulator of NF- B [33,34]. Because of this common molecular feature of downstream factors, it is thought that self-oligomerization of Nod proteins induce activation of the downstream molecules.

Biochemical analyses of Ced-4 and Apaf-1 suggest that self-oligomerization is regulated by upstream factors of the Bcl-2 family. Ced-9, a pro-survival Bcl-2 family member, directly binds to the NOD of Ced-4 and inhibits the oligomerization of Ced-4 and activation of an effector molecule Ced-3 by Ced-4 [35]. A pro-apoptotic Bcl-2

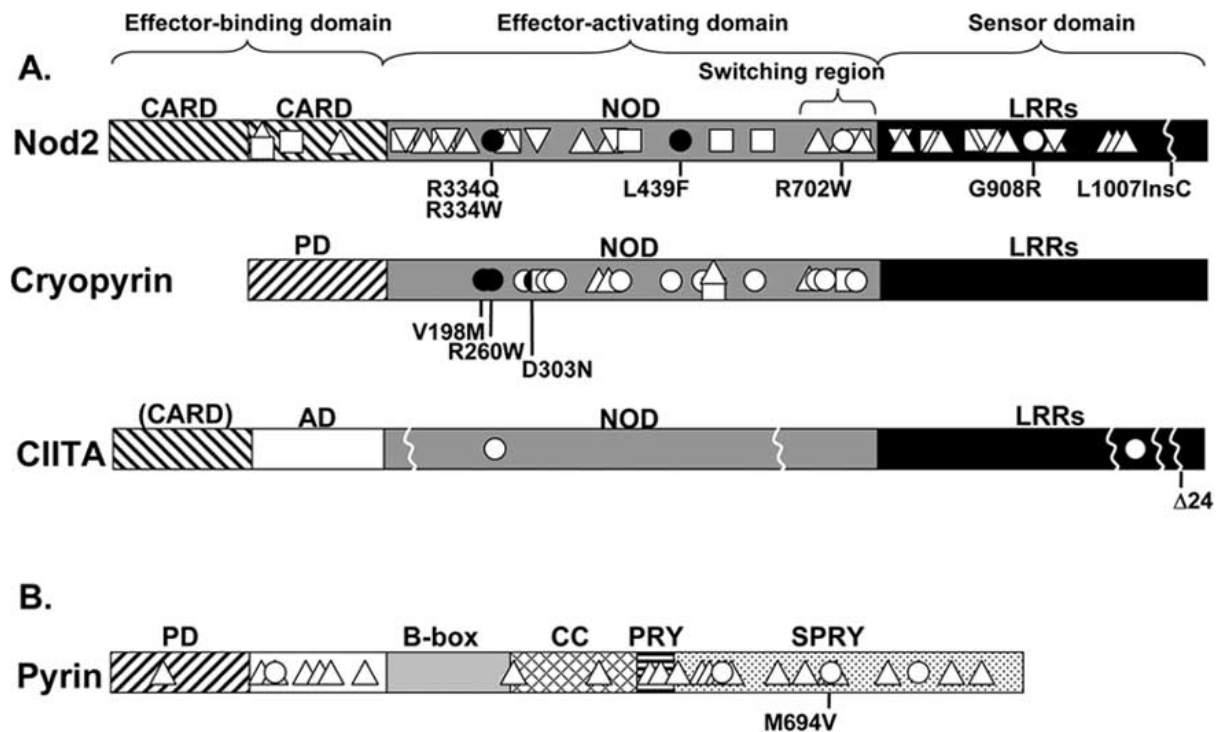


Fig. (1). Human disease-associated mutations in Nod proteins (A) and pyrin (B).

The positions of the three major Crohn's disease (CD)-associated Nod2 mutations R702W, G908R (open circles), L1007fsinsC (wavy line), minor CD-associated Nod2 mutations (boxes), Blau syndrome-associated mutations R334W, R334Q and L439F (closed circles), neutral polymorphisms (reverse triangles) and uncharacterized polymorphisms found in CD patients (triangles) were shown within the schematic structure of Nod2. The mutations associated with familial cold autoinflammatory syndrome (FCAS; boxes), Muckle-Wells syndrome (MWS; closed circles), chronic infantile neurological cutaneous and articular syndrome (CINCA; open circles) are shown within cryopyrin structure. The mutations associated with both FCAS and MWS are indicated by triangles. The point mutations (open circles) and truncated or frame-shift mutations (wavy lines) associated with type II bare lymphocyte syndrome are shown within CIITA structure. The dendritic cell-specific CIITA has a CARD. The five major (circles) and other minor (triangles) mutations found in FMF patients are shown within pyrin structure which contains PD, B-box type zinc finger, coiled-coil (CC), PRY and SPRY domains. For abbreviations, see text.

member, EGL-1, is induced during developmental programmed cell death and causes the release of Ced-9 from Ced-4, resulting in the activation of Ced-3 through Ced-4 oligomerization [36]. Ced-4 apparently does not need to have an additional ligand sensor domain at its C-terminus, because the NOD of Ced-4 functions as a sensor domain for upstream factor Ced-9. In contrast, human Apaf-1 has a C-terminal WD40 repeats-containing region (WDR), which prevents spontaneous self-oligomerization of Apaf-1 by blocking NOD/NOD interaction and also functions as a sensor domain for cytochrome c released from mitochondria [31,37,38]. Because Bcl-2 family members regulate cytochrome c release, they indirectly regulate Apaf-1 oligomerization and subsequent caspase activation.

Unlike Ced-4 and Apaf-1, many animal and plant Nod proteins have C-terminal regions composed of leucine-rich repeats (LRRs). In plants, Nod proteins are involved in host recognition of and resistance against specific pathogens [8,39]. Through deletional analysis of animal Nod family members it has been proposed that the C-terminal LRR domains inhibit the ability of the NOD and EBD to activate downstream factors [10,14,17, 34,40]. The results of these

studies suggest that the LRR domains of these Nod family members also function as a sensor domain of their upstream factors, like the WDR of Apaf-1. Indeed, the C-terminal LRRs of Nod1 and Nod2 are essential for response to their ligands [41-44]. Thus, Nod proteins function as key switching molecules in different signaling pathways using various N-terminal and C-terminal domains that interact with specific ligands (upstream factors) and downstream effector molecules.

THE ROLE OF NOD PROTEINS IN IMMUNE RESPONSES

Apaf-1 mediates caspase activation and apoptotic cell death of specific cells during developmental morphogenesis [44,45]. This apoptotic process results in the fragmentation of cells and degradation of cellular contents, while conserving membrane structure. This is important because phagocytic cells can remove the apoptotic cells without the leakage of intracellular contents, which include pro-inflammatory signaling molecules and potential autoantigens, and thereby minimize immune responses. Unlike the Apaf-1-mediated process, stimulation of signaling

pathways mediated by Nod1, Nod2, cryopyrin, and Ipaf result in transcriptional activation of genes by pro-inflammatory NF- κ B signaling [9,10,16,34,40], suggesting a potential role for these Nod proteins in inflammatory responses.

Indeed, Nod1 and Nod2 have been found to be involved in host recognition of bacterial components. Ectopic expression of Nod1 or Nod2 confers responsiveness of insensitive human cell lines to bacterial extracts [41]. By using cell lines ectopically expressing Nod1 or Nod2 and stimulating with purified chromatographic fractions of bacterial extracts or synthetic compounds, the core structures of Nod1 and Nod2 ligands were found to be a dipeptide designated as iE-DAP and muramyl dipeptide (MDP), respectively [42,43,46,47]. Both iE-DAP and MDP structures are found in bacterial peptidoglycan, its degradation products, and its synthesis intermediates [42,43,46-48]. Macrophages from mice lacking Nod1 or Nod2 show a loss of response to synthetic iE-DAP or MDP, respectively, suggesting that Nod1 and Nod2 are essential sensors of these molecules for macrophages [43,47,49]. MDP-containing molecules exist in all bacteria; however, iE-DAP-containing molecules are only found in certain subgroups of bacteria, such as most Gram-negative bacteria and several Gram-positive bacteria [50]. Therefore, Nod1 and Nod2 seem to be involved in host responses against different sets of bacteria. Plant Nod proteins are also known to mediate host response against specific pathogens [8].

Together with membrane-spanning pathogen-recognizing proteins, intracellular Nod proteins seem to be part of the most conserved host defense system against pathogens. Interestingly, both intracellular and membrane-spanning signaling molecules that are involved in host recognition of pathogens share common structures. Similar to Nod1 and Nod2, mammalian proteins known as Toll-like receptors (TLRs) have LRR domains that are essential for host recognition of their ligands from pathogens [51,52]. TLRs commonly have intracellular TIR domains and these domains are also found at the N-termini of many plant Nod proteins [8,51,52]. Several membrane-spanning pathogen recognizing proteins in plants have kinase domains, which are homologous to RICK, a downstream kinase of Nod1 and Nod2 [8]. These facts may suggest the basis of a conserved mechanism for host recognition of pathogens.

Beside NF- κ B activation, Nod1, Nod2, Ipaf, and cryopyrin also activate caspases and overexpression of these proteins promotes apoptosis [9,10,14,17,40]. However, the role of the caspase activation by these Nod proteins seems to be different from the role of caspase activation by Apaf-1, because these Nod proteins activate NF- κ B, which is known to function as an anti-apoptotic factor through induction of several anti-apoptotic proteins including A1 and c-FLIP [53,54]. The dual activation of NF- κ B and caspases is also induced by signaling pathways mediated by TLRs and TNF receptor families [55,56]. At physiological levels of stimulation of signaling pathways mediated by Nod and TLR proteins, the caspase activation may not be involved in apoptosis induction, but be involved in the processing of intracellular proteins including IL-1 and IL-18 [57]. Indeed, overexpression of Nod1, Ipaf and cryopyrin with caspase-1

induces IL-1 secretion [17,18,58] dependent on activation of both NF- κ B and inflammatory caspases [57,59]. Caspase activation in certain types of cells is also known to be important for their survival, which is independent from IL-1 signaling [57]. Nod1 and RICK are also reported to mediate activation of JNK and p38 [60-62]. Together with NF- κ B and caspases, activation of these non-classical MAP kinase pathways by Nod proteins might be involved in induction of several cytokines through transcription factors such as AP-1 and Elk-1 [51,52].

Although it still not yet known if all immune responses of MDP and iE-DAP related molecules are mediated by Nod1 and Nod2, both types of molecules are known to induce innate and acquired immune responses [50]. Administration of these ligands confers broad-specificity resistance against pathogens including Gram-negative, Gram-positive bacteria, even Fungi and viruses [63]. This gained resistance seems to be associated with induction of IL-1 and/or TNF, general cytokines, which mediate pathogen resistance [64-66]. MDP and iE-DAP molecules also function as immunostimulatory adjuvants to enhance immunization and delayed-type hypersensitivity [63], implying that MDP and iE-DAP can stimulate both CD4+ and CD8+ T cells through antigen-presenting cells. MDP and its derivatives have been shown to induce co-stimulatory molecules of T cell activation including B7 family members and intercellular adhesive molecules (CAMs) [67]. MDP conjugates facilitate class switching of immunoglobulin (IG) subclasses, especially IgG2a and IgG2b mediated by Th1 cytokines, and inhibit IgE production mediated by Th2 cytokines [68]. Therefore, MDP administration to mice can prevent IgE-mediated asthma-like allergic responses [69]. Because both MDP and iE-DAP derivatives are reported to be mitogenic for splenocytes [63,70], it is possible that these molecules stimulate T-cell independent B cell proliferation. All of these effects on immune responses are also reported for TLR ligands including lipopolysaccharide (LPS), TLR4 ligand, and this remarkable similarity of immunostimulatory activities of TLR and Nod ligands suggests the close relevance of TLR and Nod functions in immune responses. TLR and Nod proteins also seem to cooperate together for host recognition of pathogens. LPS induce gene expression of Nod2, RICK, cryopyrin and ASC [62,71-73], whereas MDP induce MyD88 expression [74]. MDP and LPS show synergistic enhancement in cytokine production [74]. Because commercial LPS preparations, which are used for previous studies, are often contaminated with Nod1 and Nod2 ligands [41-43], some previous findings on immunostimulatory activity of LPS may need to be re-evaluated using pure synthetic TLR4 and Nod ligands.

Two Nod family members, CIITA and NAIP, which have non-homophilic EBDs are also known to be involved in immunity, although it is unknown if these proteins recognize and are activated by ligands. CIITA does not induce NF- κ B activation but is critical for induction of MHC molecules, especially class II proteins, which mediate presentation of antigen peptide to T cells [4]. Previous studies suggest that CIITA does not bind DNA directly, but rather it interacts with several transcription factors including RFX complexes and NF-Y [75]. However, the precise mechanism by which CIITA activates MHC transcription remains unclear. The

“neuronal” apoptosis inhibitory protein, NAIP is actually expressed in macrophages and other leukocytes [76], also general microarray data of tissue expression in <http://symatlas.gnf.org>). Mouse NAIP is encoded by multiple genes located within Lgn1, the locus for the susceptibility of *Legionella pneumophila* found in mouse A/J strain [77]. Recent analysis suggests that *NAIP5*, one of *NAIP* genes, confers resistance to *Legionella* [78,79], although the molecular mechanism by this occurs remains unclear. The human genome encodes many Nod proteins which have unknown functions. Some of them including Pypaf5, Pypaf7, Pan2 and PYNOD are implied to function in immune responses [18-20, 24]. Further analysis will reveal novel mechanisms in which immune responses are regulated by these Nod proteins.

HUMAN DISEASES RELATED TO LOSS-OF-FUNCTION TYPE MUTATIONS OF NOD PROTEINS

Notably, mutations of several Nod proteins are associated with human immune diseases (Fig. 1). Mutations in Nod2 gene were found to associate with the susceptibility for Crohn's disease (CD), a common inflammatory bowel disease [80-82]. 25 to 50 % of CD patients have at least one allele carrying one of three major mutations L1007fsinsC, G908R, or R702W [83-86]. Homozygotic or complex heterozygotic individuals with these alleles have 20-to 50-fold higher risks for CD than those with standard alleles, whereas simple heterozygotic individuals have 1.5-to 2-fold higher risks [82-84]. Further detailed analysis showed that the Nod2 mutations are associated strongly with ileal disease and weakly with right colonic disease [83-85]. These Nod2 mutations are associated with early onset of CD [86] and with a greater need for surgical intervention [85]. L1007fsinsC also increases the risk of colorectal cancer in CD patients [87]. These three disease-related Nod2 mutants are defective in ligand-dependent NF- κ B activation, as demonstrated by ectopic expression of Nod2 in culture cells treated with bacterial LPS and peptidoglycan fractions and synthetic MDP [42,46,81,88]. Using this methodology, more than 10 minor Nod2 polymorphisms present in CD patients were identified as mutations defective in ligand response [89]. Mononuclear cells isolated from the peripheral blood of individuals homozygous for L1007fsinsC are defective in MDP response, resulting in loss of NF- κ B activation and induction of NF- κ B target genes, IL-1 and A1 by MDP but not TLR4 ligand, LPS [42]. Therefore defective host recognition of bacterial components by Nod2 mutations seems to be associated with the susceptibility of ileal CD.

Although all reports except reference 90 noted that Nod2 mutations have no association with ulcerative colitis (UC), another type of common inflammatory bowel disease [80-82,83-86,91], loss-of-function type Nod2 mutations might affect other inflammatory diseases. For example, following the studies of Nod2 mutations in CD and UC, several groups reported the association of the three major Nod2 mutations with psoriatic arthritis and allergic diseases [92,93], although other studies show no significant association of the Nod2 mutations with psoriasis, ankylosing spondylitis, and rheumatoid arthritis were found [94-99]. Very low weight infants with L1007fsinsC have a significantly higher rate of blood culture-proven sepsis [93]. Therefore it is interesting

to further investigate the relationship between other inflammatory diseases and Nod2 function as well as CD.

Whereas Nod2 mutations are associated with the susceptibility for CD, loss-of-function mutation of CIITA, another Nod2 family member, results in type II bare lymphocyte syndrome (BLS-II) [4]. Both deletional mutations and point mutations which result in exon organization, are present in BLS-II patients [4, 100-102] (Fig. 1). These mutations of CIITA result in loss of MHC-II expression, which cause severe immunodeficiency [4,100-102]. Both BLS and the susceptibility for CD are autosomal recessive, consistent with the fact that these diseases are associated with loss-of-function type mutations in the Nod proteins. Mutations of NAIP in A/J mice also seem to be loss-of-function type and the phenotype is also autosomal recessive [77], although critical point mutations in the NAIP5 have not been identified yet.

Mutational analysis of Nod1, Nod2 and CIITA has revealed the molecular basis for loss-of-function effects of these mutations [41,42,46,81,88,103]. The LRR-containing domains are critical for their functions and the smallest deletions close to their C-termini resulted in loss-of-function [42,46,81,88,103]. Even introduction of small tag sequences at C-termini of Nod2 and CIITA result in loss of their function (Inohara, Núñez, Chang CH, unpublished data). Detailed analysis of the point mutations in the LRR domain of Nod2 that lost MDP response shows that they can be classified into two groups [103]. In the first groups, the residues are located on the sheet surface of the predicted LRR structure, which are shown to be critical for ligand recognition in other LRR-containing proteins. The second groups of residues important for ligand response are located on the opposite outer surface of the predicted LRR structure [103]. These residues seem to be involved in interaction with the C-terminal part of NOD as suggested by functional complementation between mutations in the regions. One point mutant in the surface of the LRR domain resulted in loss of MDP response, but additional mutations in NOD recovered the MDP responsiveness. Similarly, point mutations in the same region of the NOD resulted in loss of MDP responsiveness, but additional mutations in LRR domain recovered it. R702W, one of major CD-associated mutations, are also located in the same “switching” region (Fig. 1). This suggests that the mutant R702W cannot undergo the switching to turn on completely.

HUMAN DISEASES ASSOCIATED WITH GAIN-OF-FUNCTION MUTATION OF NOD PROTEINS

On the other hand, another type of mutations in the same switching regions of Nod1 and Nod2, which resulted in constitutive NF- κ B activation, were found by the systemic mutational analysis [103]. The switching regions, which function by interacting with LRRs, are conserved in almost all LRR-containing Nod proteins including cryopyrin, a Nod family member. Mutations of cryopyrin are associated with three related but distinguishable genetic systemic inflammatory diseases, Familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and chronic infantile neurological cutaneous and articular syndrome (CINCA) [5, 104, 105]. Notably, several of the

cryopyrin mutations found in CINCA and MWS are located in the switching regions [105] (Fig. 1). The positions of these mutations are identical or very close to those of the Nod1 and Nod2 mutations, which result in constitutive NF- κ B activation [103]. This finding has led to the hypothesis that these disease-associated cryopyrin mutants might be deficient in switching and this results in constitutive activation of cryopyrin-mediated signaling. Indeed, expression of disease-associated cryopyrin mutants R260W, D303N, and E637G were found to induce elevated NF- κ B activation in HEK293T cells and secretion of IL-1 in monocytic THP-1 cells [106]. Although an upstream factor of cryopyrin has not been identified, these studies suggest that constitutive activation of the cryopyrin-mediated signaling pathway is associated with human inflammatory diseases.

Notably, the position of R260W cryopyrin mutation is identical to R334W Nod2 mutations found in patients of Blau syndrome, another systemic inflammatory disease [91,107] (Fig. 1). Whereas Nod2 mutations found in Crohn's disease patients result in defects in MDP responsiveness, mutations in Blau syndrome result in elevated levels of NF- κ B activation in the absence of MDP, and hyper-responsiveness to MDP [103]. Thus these Nod-associated human genetic inflammatory diseases seem to be based on a common molecular mechanism for constitutive activation of inflammatory signaling pathways by the mutations of these Nod family members. Elevated inflammation signaling by Nod mutations without ligand stimuli is also consistent with the fact that these inflammatory diseases are autosomal dominant. The R260 of cryopyrin and R334 of Nod2 might be involved in conformational change during functional ligand/LRR interaction rather than ATP(GTP) hydrolysis or oligomerization because Nod1, Nod2 and CIITA mutants at the residues, which are predicted to be critical for ATP/GTP hydrolysis, unlike R260W of cryopyrin and R233W of Nod2, resulted in loss-of-function and the R233W Nod2 mutant can still respond to MDP [104, 108].

Elevated inflammatory signaling mediated by Nod proteins is not only caused by gain-of-function type mutations of Nod proteins but also probably by loss of negative feedback of the signaling pathways. Pypin, is a key negative regulator of the signaling pathways mediated by cryopyrin and probably also Ipaf. Pypin inhibits NF- κ B activation and caspase-mediated apoptosis in cryopyrin and Ipaf signaling pathways [34,40]. This inhibitory effect of pypin is at least in part caused by competition with cryopyrin on binding to ASC and decreased recruitment of ASC to cryopyrin [34,40]. Mutations of pypin are commonly found in patients of familial Mediterranean fever, a systemic inflammatory disease [109,110] (Fig. 1). Because pypin is NF- κ B-inducible gene [111], pypin mutations seems to result in loss of negative feedback of signaling pathways mediated by cryopyrin and Ipaf.

PERSPECTIVE AND DIRECTION FOR FUTURE STUDY

Until recently, the causes of the Nod-associated immune diseases described above have been unclear and the

recognition of the association of Nod protein mutations with these diseases is a remarkable step to understand the pathogenesis of these diseases. Genetic test for mutations of Nod proteins is a powerful tool for diagnosis of the Nod-associated diseases, especially those associated with the gain-of-function mutations, which show dominant phenotypes. The patients with the cryopyrin mutations show elevated levels of serum IL-1 and administration of IL-1 receptor antagonist (IL-1Ra) have been shown to improve the diseases [112]. Although IL-1 is a general critical mediator of inflammation and seem to be secreted as downstream events of all kind of inflammatory responses, IL-1Ra therapy might also be effective for Blau syndrome.

On the other hand, the finding that of association between CD and Nod2 mutations suggests the mechanism for development of CD is different from that for diseases associated with their gain-of-function mutations of Nod proteins, because loss-of-function phenotype of Nod2 mutations cannot explain elevated inflammation in CD. The elevated levels of inflammatory cytokines in CD must be induced by other proinflammatory signaling pathways. One possibility is that loss of first defense, which is provided by the Nod2-mediated signaling pathway, may allow for more pathogen invasion and thereby creating more stimuli, which cause inflammation through signaling pathways that are dependent on other pathogen-recognizing molecules including IGs, TLRs and TCRs. Another possibility is that the loss of Nod2-mediated signaling pathway may somehow make the immune system hypersensitive against the stimuli. Induction of the signaling factors, which mediate negative feedback of inflammation and immuno-tolerance, are also known to be dependent on several same factors which induce inflammation [113-115].

Individuals who have Nod2 mutations and lost MDP responsiveness do not always develop CD [41,95,116]. Similar to human individuals with Nod2 functional deficiency, Nod2-deficient mice grown in a pathogen free condition develop normal morphology and do not develop inflammation in intestinal area [49]. These findings suggest that additional environmental and/or genetic factors are required for the development of CD in Nod2-deficient individuals. Polymorphisms in OCTN (IBD5), DLG5 and CD14 genes are suggested to affect the CD development of individuals carrying Nod2 mutations [117-120], although they alone cannot explain all genetic backgrounds for CD. Bacterial microflora, especially in intestines, is likely to be an environmental factor that affects development of CD, because Nod2 is involved in host recognition of bacterial components. Notably, in healthy intestine, Nod2 is expressed only in Paneth cells, which are important for mucosal innate immunity [121,122]. Thus impaired control of intestinal bacteria by Paneth cells might be associated with the development of CD. Further analysis will be required to understand the development of CD.

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