The Nod-Like Receptor (NLR) Family: A Tale of Similarities and Differences

Martina Proell¹, Stefan J. Riedl², Jörg H. Fritz³, Ana M. Rojas⁴, Robert Schwarzenbacher¹ *

¹ Department of Molecular Biology, University of Salzburg, Salzburg, Austria, ² The Burnham Institute for Medical Research, La Jolla, California, United States of America, ³ Department of Immunology, University of Toronto, Toronto, Ontario, Canada, ⁴ Structural Computational Biology Group, Spanish National Cancer Research Center, Madrid, Spain

Abstract

Innate immunity represents an important system with a variety of vital processes at the core of many diseases. In recent years, the central role of the Nod-like receptor (NLR) protein family became increasingly appreciated in innate immune responses. NLRs are classified as part of the signal transduction ATPases with numerous domains (STAND) clade within the AAA+ ATPase family. They typically feature an N-terminal effector domain, a central nucleotide-binding domain (NACHT) and a C-terminal ligand-binding region that is composed of several leucine-rich repeats (LRRs). NLRs are believed to initiate or regulate host defense pathways through formation of signaling platforms that subsequently trigger the activation of inflammatory caspases and NF-kB. Despite their fundamental role in orchestrating key pathways in innate immunity, their mode of action in molecular terms remains largely unknown. Here we present the first comprehensive sequence and structure modeling analysis of NLR proteins, revealing that NLRs possess a domain architecture similar to the apoptotic initiator protein Apaf-1. Apaf-1 performs its cellular function by the formation of a heptameric platform, dubbed apoptosome, ultimately triggering the controlled demise of the affected cell. The mechanism of apoptosome formation by Apaf-1 potentially offers insight into the activation mechanisms of NLR proteins. Multiple sequence alignment analysis and homology modeling revealed Apaf-1-like structural features in most members of the NLR family, suggesting a similar biochemical behaviour in catalytic activity and oligomerization. Evolutionary tree comparisons substantiate the conservation of characteristic functional regions within the NLR family and are in good agreement with domain distributions found in distinct NLRs. Importantly, the analysis of LRR domains reveals surprisingly low conservation levels among putative ligand-binding motifs. The same is true for the effector domains exhibiting distinct interfaces ensuring specific interactions with downstream target proteins. All together these factors suggest specific biological functions for individual NLRs.

Introduction

Eukaryotes have evolved complex systems to detect microbial infection and other potential threats to the host. Recognition of microbes relies on the sensing of microbe associated molecular patterns (MAMPs) by germline-encoded host pattern recognition molecules (PRMs), which include various families of leucine-rich repeat (LRR) bearing proteins in plants and animals. While Toll-like receptors (TLRs) constitute the main sensors for detection of extracellular microbes, recent findings suggest that two distinct protein families, the RIG-like helicases (RLHs) and the Nod-like receptors (NLRs), act as intracellular surveillance molecules [1–3]. Several proteins of the highly conserved NLR family have been shown to function as intracellular PRMs for the initiation of innate and adaptive immune responses upon pattern-specific sensing of microbes [4].

Like TLRs, NLRs are thought to recognize microbial products, as well as other intracellular danger signals, thereby initiating host defense pathways through the activation of the NF-kB response and inflammatory caspases [5]. Moreover, the NLR family has gained increased attention, since polymorphisms in certain NLR genes are linked to inflammatory disorders such as Blau syndrome, Crohn’s disease or early-onset sarcoidosis [6].

Structurally, NLRs are large multi-domain proteins with a tripartite architecture. NLR proteins typically contain a central nucleotide-binding domain termed NACHT domain (often also referred to as NOD domain), N-terminal effector domains (PYRIN, caspase recruitment domain CARD, or baculovirus inhibitor of apoptosis protein repeat BIR domain) for binding downstream signaling molecules, while the C-terminal part consists of a receptor domain, which is characterized by a series of leucine-rich repeats (LRRs). It is hypothesized that the crucial step in NLR activation lies in the oligomerization of the NACHT-domain, thereby forming an active signaling platform (e.g. the inflammasome or nodosome [7,8], respectively), which allows binding of adaptor molecules and effector proteins, ultimately leading to an inflammatory response.

To date, 22 members of the human NLR protein family have been reported, which can be distinguished depending on the...
presence of a PYRIN, CARD, BIR, and a yet unclassified effector domain (Table 1).

According to the current general paradigm, NLR signaling is believed to be initiated by the C-terminal LRR region through the recognition of molecules triggering NLR activation. However, the actual molecular switch, namely the oligomerization of the NLR, then is thought to be mediated by the NACHT domain in a nucleotide-dependent manner. Recent studies show that Ipaf [9] and NALP3 [10] selectively bind ATP/dATP and that nucleotide binding is essential for their function in downstream signaling. Once the switch has occurred, the signal is transferred to the effector proteins such as inflammatory caspases or adaptor molecules, via their effector domains. Thus, CARD-containing NLRs such as NOD1 and NOD2 are thought to interact with the CARD-containing kinase RICK [RIP2] leading to the activation of CARD9 and NF-kB pathways [11]. In contrast, several PYRIN domain containing Nalp proteins were found to form a signaling platform, dubbed inflammasome, and drive caspase-activation by binding to the adaptor protein ASC [1,7,12,13].

Despite the growing amount of research data, little is known about the precise molecular mechanism of NLR activation and the initiation of subsequent signaling cascades. Moreover, the structural and mechanistic data on NLR proteins is scarce and mainly limited to single effector domains. Recent studies by Albrecht et al discussed models of the NACHT and LRR domains of NOD2 and NALP3 in relation to disease associated SNPs and protein function [14]. Here, we provide further insights into structural and functional relationships of NLRs based on detailed sequence and modeling analyses of the whole NLR family. We show that although Apaf-1 shares less than 15% sequence identity to most NLRs and contains a different receptor domain (WD40 repeats), its structure, mode of action, and mechanistic principles can serve as a valuable working model for NLR signaling. In addition, we investigated the N-terminal effector domains (CARD and PYRIN) of the NLR protein family to construct a prediction for potential interfaces and interacting partners. Furthermore, we analyzed sequences of the LRR domains for conserved regions that may play a putative role in ligand binding and/or interaction with the NACHT and effector domains. Finally, we created a homology model of NOD2 based on Apaf-1 and the ribonuclease inhibitor (pdb id: 1dfj), which we used to depict disease related polymorphisms and mutations [6].

| Nomenclature | Synonyms | Domain structure | Chrom. location | Genebank |
|--------------|----------|------------------|-----------------|----------|
| **CARD domain** | | | | |
| NOD1 | NLRC1,CARD4 | CARD-NACHT-WH-SH-LRR | 7p14.3 | AF126484 |
| NOD2 | NLRC2, CARD15 | CARD-CARD-NACHT-WH-SH-LRR | 16q12.1 | AF178930 |
| CIITA type I | NLRA | CARD-(X-NACHT-WH-SH-LRR) | 16p13.13 | AF000002 |
| **PYRIN domain** | | | | |
| NALP1 | NLRP1, CARD7 | PYD-NACHT-WH-SH-LRR-FIIND-CARD | 17p13.2 | AB023143 |
| NALP2 | NLRP2, Pypaf2 | PYD-NACHT-WH-SH-LRR | 19q13.42 | AK000517 |
| NALP3 | NLRP3, Pypaf1 | PYD-NACHT-WH-SH-LRR | 1p44 | AF54176 |
| NALP4 | NLRP4, Pypaf4 | PYD-NACHT-WH-SH-LRR | 19q13.43 | AF479747 |
| NALP5 | NLRP5, NOD14, Pypaf8 | PYD-NACHT-WH-SH-LRR | 19q13.43 | AY154460 |
| NALP6 | NLRP6, Pypaf5 | PYD-NACHT-WH-SH-LRR | 11p15.5 | AF467665 |
| NALP7 | NLRP7, NOD12, Pypaf3 | PYD-NACHT-WH-SH-LRR | 19q13.42 | AY154463 |
| NALP8 | NLRP8, NOD16 | PYD-NACHT-WH-SH-LRR | 19q13.43 | AY154463 |
| NALP9 | NLRP9, NOD6 | PYD-NACHT-WH-SH-LRR | 19q13.43 | AY154464 |
| NALP10 | NLRP10, NOD8, Pynod | PYD-NACHT-WH-SH | 11p15.4 | AY154465 |
| NALP11 | NLRP11, NOD17, Pypaf6 | PYD-NACHT-WH-SH-LRR | 19q13.42 | AY095145 |
| NALP12 | NLRP12, Pypaf7, RNO | PYD-NACHT-WH-SH-LRR | 19q13.42 | AY095146 |
| NALP13 | NLRP13, NOD14 | PYD-NACHT-WH-SH-LRR | 19q13.42 | AY154468 |
| NALP14 | NLRP14 | PYD-NACHT-WH-SH-LRR | 11p15.4 | BK001107 |
| **BIR domain** | | | | |
| NAIP | NLRB1, BIRC1 | BIR-NACHT-WH-SH-LRR | 5q13.1 | U19251 |
| **untypical CARD** | | | | |
| Ipaf | NLRC4, Card12, Clan | Card?-NACHT-WH-SH-LRR | 2p22-p21 | AF376061 |
| NOD4 | NLRC5, NOD27 | Card7-NACHT-WH-SH-LRR | 16q13 | AF389420 |
| NOD3 | NLRC3 | Card7-NACHT-WH-SH-LRR | 16p13.3 | BK001112 |
| **undefined** | | | | |
| NOD5 | NLRX1, NOD26, NOD9 | X-NACHT-WH-SH-LRR | 11q23.3 | AB094095 |
| CIITA | NLRA | X-NACHT-WH-SH-LRR | 16p13.13 | U16259 |

Protein name and synonyms (new synonyms in bold), accession number, and chromosomal location according to http://www.genenames.org/genefamily/nlr.php followed by domains as defined by FFAS. CARD, caspase activation and recruitment domain; PYD, pyrin; NACHT, domain present in NAIP, CIITA, HET-E, TP-1; NALP, NACHT-LRR-PYD-containing protein; WH, winged helix domain; SH, superhelical domain, LRR, leucine-rich repeats.

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Results and Discussion

1.1 NLR domain structure

To elucidate relations of NLR proteins, we used NLR sequences (see Table 1) as separate queries for FFAS searches [15] and secondary structure prediction with the PredictProtein Server [16]. Furthermore, a domain profile search using Interpro and SMART [17] was performed to verify the domain structure of individual NLRs. Comparative sequence analyses (Figure 1, Table 2) revealed that all NLR proteins belong to the AAA+ ATPase superfamily [18] where they are further classified as signal transduction ATPases with numerous domains (STAND) [19]. The STAND proteins are distinguished from other P-loop NTPases by the presence of unique sequence motifs associated

Figure 1. Multiple sequence alignment of NLR NACHT-WH-SH domains and the Apaf-1 NACHT-WH-SH domain. Degree of conservation is shown as blue shading. The secondary structure of Apaf-1 is shown above the Apaf-1 sequence. Arrows underneath the alignment indicate domain boundaries. Conserved sequence features important for catalytic activity are shown in black boxes. Orange and magenta boxes depict interfaces residues while the orange ones contribute to interactions with the left partner and the magenta residues are thought to interact with the right partner in the oligomer. Green boxes indicate additional motifs as described in the text.

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with the N-terminal helix and the core β-strand-4, as well as a C-terminal helical bundle that is fused to the NTPase domain [19].

A sequence profile based search using FFAS identifies Apaf-1 as a distantly related homologue of NLRs within the human genome. The main difference between Apaf-1 and NLRs is the lack of a LRR domain. Instead, Apaf-1 utilizes two sets of WD40 repeats as additional domains and within the length of the linker from the effector domain to the NACHT domain. Good examples for this are NOD2, which contains two N-terminal CARD domains, NOD5 and CIITA, which show an undefined N-terminal region. Other examples are the untypical secondary structure prediction for the CARD domain of Ipaf, or the partial sequence of the type 1 isoform of CIITA (accession number: AF000002) [21], which contains an alternative 5′ region that encodes a CARD domain.

In respect to the C-terminal LRR region NALP10 is the only NLR member that has no or only a very short occurrence of LRR repeats. NALP1 on the other hand shows a typical LRR region, but contains an alternative 5′ isoform of CIITA (accession number: AF000002) [21], which contains an undefined N-terminal region. Other examples are the untypical secondary structure prediction for the CARD domain of Ipaf, or the partial sequence of the type 1 isoform of CIITA (accession number: AF000002) [21], which contains an alternative 5′ region that encodes a CARD domain.

### Table 2. Key elements of Apaf-1 and NLR NACHT-WH-SH domains extracted from the multiple sequence alignment in Figure 1.

| Seq-ID | Walker A | Walker B | Sensor 1 | WH His | WH cons |
|--------|----------|----------|----------|--------|---------|
| Apaf-1 | -        | GKS      | LLDDLWDSW | TTR    | H438    | METEEV  |
| NOD1   | 9%       | GKS      | LFTFDGLDEEH | TAR    | H517    | FTQEEV  |
| NOD2   | 8%       | GKS      | LLTFDGDEFK | TSR    | H560    | FSAQQL  |
| Ipaf   | 9%       | GKS      | LFLFDGYNEFK | TTT    | H443    | FELODV  |
| NALP1  | 11%      | GKS      | LFLFDVDEPG | TAR    | H623    | FSPDDL  |
| NOD5   | 12%      | GKS      | LFVLHLHELHN | TTR    | -       | FSEEDV  |
| CIITA  | 11%      | GKS      | LLUDAFEELE | TAR    | -       | LQED--  |
| NALP5  | 10%      | GKS      | LFLFDGFDLGG | TVR    | H584    | FDGDDL  |
| NALP12 | 11%      | GKS      | LFLFDGFDELK | TTR    | H514    | FEEQDL  |
| NALP6  | 12%      | GKT      | LFLFDGADELP | TTR    | -       | FAKEL   |
| NALP7  | 10%      | GKT      | LFVDGLDELKL | TTR    | H468    | FHREDL  |
| NALP8  | 9%       | GKT      | LLLLLGFEEET | MIR    | -       | LGKEDL  |
| NALP9  | 8%       | GKT      | LFIIDFGEQQLK | ALG    | H445    | FSHGDL  |
| NALP10 | 13%      | GKT      | LFLFDGFDLQ  | TTR    | H466    | FEIEAL  |
| NALP13 | 9%       | GKT      | LFIIDFGEEl  | TIK    | H536    | FKEDTT  |
| NALP14 | 10%      | GKT      | LFIIDFDELN  | TTR    | H480    | FYREN   |
| NALP11 | 9%       | GKT      | LFILEDLDINR | SSR    | H450    | FSQEDL  |
| NA1P   | 14%      | GKT      | LFLFDYKIEC  | AVR    | -       | FNDDDL  |
| NOD3   | 12%      | GKT      | LLUDGLDECR  | TSR    | H502    | FYEQDM  |
| NOD4   | 8%       | GKT      | LLUDGLDEAL  | TSR    | H491    | FYAKDI  |
| NALP2  | 10%      | GKT      | LFVFDGFDEL  | TTR    | H503    | LHREDL  |
| NALP3  | 10%      | GKT      | LFLFDGFDLQQ | TTR    | H520    | FEEQDL  |
| NALP4  | 10%      | GKT      | LFVFDGFEEQ  | AIK    | H452    | FCECDL  |

Sequence identities shown are derived from pairwise alignments using FFAS between Apaf-1 and individual NLRs. WH His and WH cons show the conserved histidine and the conserved METEEV sequence patch, respectively which are located in the winged helix (WH) domain.

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1.2 NLR evolutionary profiles

Detailed sequence comparisons of 22 human NLR members reveal an overall sequence identity in the range of 10–30% amongst pairs. Since domain shuffling is a eukaryotic hallmark and has created a large complexity of functions in proteins, it hampers evolutionary analysis of full length proteins. In particular effector domains are subject to domain accretion, and/or domain shuffling or duplication for the acquisition of new domain architecture. Taking this into account, we have chosen the
The NLR Family

1.3 The NLR NACHT-WH-SH region shows distinct adaptations in NLR function

Members of the AAA+ superfamily feature a so-called ATPase, P-loop or Rossmann-fold which adopts a three-layered α-β sandwich configuration. This fold contains recurring regulatory units with the β-strands forming a central, parallel β-sheet, which is embedded between α-helices on both sides (scop id C.37.1.20). The parallel β-sheet forming the core of the ATPase domain assumes a 51432 topology [22]. This fold contains several characteristic motifs, namely the Walker A/P-loop and Walker B motif, and the Sensor 1 and Sensor 2 motif. These motifs are involved in ATP-binding and hydrolysis of the β-γ phosphate diester bond [19] leading to specific conformational changes.

To decipher the structure and mechanism of the NLR protein family we performed a multiple sequence alignment of NLR proteins and Apaf-1 (Figure 1) focusing on the NACHT-WH-SH domains using the program muscle [23]. The outcome was then compared with FFAS [15] search results. Subsequently, we utilized secondary structure prediction and homology modelling (Figure 3) to decipher the presence of critical ATPase motifs like Walker A/P-loop, Walker B, Sensor 1 and Sensor 2 to deduce putative functional features unique to NLRs. As shown in the multiple sequence alignment (Figure 1), the overall secondary structure features of the NACHT domain are conserved among NLR proteins and Apaf-1 (Table 2). We observed that the only main difference is constituted by a deletion before β-strand 3 and a 20 residue insertion after β-strand 3 in the NACHT domain.

The Walker A motif is composed of the characteristic consensus pattern GxxxxGKT/S (x represents any amino acid), where the lysine residue directly interacts with a phosphate moiety.
The Mg²⁺ proximal aspartate residue is crucial for coordinating binding of hhhDD/E (h represents a hydrophobic amino acid). The binding site is characterized by the conserved sequence pattern found in active ATPases, the detailed catalytic consequences of cons in humans. Although, both residues, T and S, have a signature. Then, NALP1, NALP5 and NALP12 represent the tree (Figure 2B, where orange fonts indicate a presence of S groups (Table 2). This separation is reflected in the evolutionary members of the NLR protein family can be subdivided into two cases. In comparison to other AAA+ ATPases, Apaf-1 displays the involvement of the WH domain in the coordination of ADP, instead of the missing Sensor 2 motif, with H438 and S422 contributing two hydrogen bonds to the coordination of the phosphate groups. Of particular interest in this case is H438, which can be regarded as replacement of the Sensor 2 motif, when compared to the structures of other AAA+ superfamily members [29]. Our structural alignments of NLR proteins with Apaf-1 reveal that Sensor 2 is also replaced by a conserved histidine in the WH domain of NLR proteins (Table 2, Figure 3). Importantly, we observed that the conserved histidine is part of a highly conserved sequence patch among NLR family members (Figure 1). This patch is characterized by the consensus sequence FxHxxQEhxA, which has been described as a unique feature of the NAIP-like subfamily among the STAND clade [19] and now points to a common involvement of this patch in NLR acting in a Sensor 2-like manner. We observed that almost all NLRs harbour this conserved sequence with slight variations concerning the glutamate residue. Exceptions are NALP6, NALP8, NOD5, CIITA, and NAIP, where the conserved histidine is not present (Figure 1). It is not clear whether these NLRs replace the histidine by another feature or are incapable of ATP hydrolysis. As mentioned above, the Sensor 2 motif in AAA+ ATPases is composed of a conserved arginine residue that completes the active site of the neighbor molecule in the oligomer, where it is supposed to be involved in nucleotide-binding [24]. In fact, some NLR proteins such as NAIP, NALP2, NALP4, and NOD1 display an arginine residue downstream of the Sensor 1 motif that could function as a Sensor 2 motif. However, the conserved histidine residue present in the WH domain of NALP2, NALP4, and NOD1 may still be capable to substitute the function of Sensor 2.
1.4 Additional domains and motifs

The GxP signature is a conserved motif located in the small helical subdomain (C-domain) [18] at the C-terminal region of the NACHT domain (Figure 1) [19]. Interestingly, the conserved proline interacts with the adenine moiety of the bound ATP molecule (Figure 3). Our alignment analyses revealed that most NLRs display this highly conserved proline residue (with the exception of NAIP (T) and NALP 11 (A)), but lack the conserved glycine residue (Figure 1), suggesting a key feature assigned to the proline among NLRs. As described, additional domains following the NACHT domain are the WH domain, also referred to as HETHS domain [19] containing the conserved histidine motif, and the SH domain, which consists of eight alpha helices in a superhelical arrangement of yet unknown function.

Additional NLR sequence motifs are the cysteine rich region in the NACHT domain, containing a VCWxVCT motif located adjacent to the nucleotide-binding site (Figure 1), which plays a role in nucleotide recognition. Another feature is a highly conserved patch located in the WH domain of Apaf-1. This feature displays the sequence METEEV (Figure 1, Table 2) where the second glutamate is part of the interface to the adjoining CARD domain and forms hydrogen bonds to backbone atoms in the loop connecting helices 3 and 4 of the CARD domain. This interaction, which may lead to the stabilization of the dormant form seems to be conserved in the whole NLR family. In NLR proteins there is in place of the methionine a highly conserved phenylalanine residue (Figure 1, Table 2). Only NALP2, NALP8, and CIITA contain a leucine instead of the phenylalanine residue. Also the glutamate residues are conserved to a certain degree or substituted by an aspartate residue within NLR proteins. These conserved motifs are most likely involved in intra- and intermolecular interactions required for stabilization of the closed form and formation of the active signaling platform.

1.5 Features important for intermolecular interactions and oligomerization

Since our detailed sequence analyses revealed that most NLRs and Apaf-1 share the same domain architecture and many secondary structure features, the availability of structural and mechanistic data for Apaf-1 provides the opportunity to link conserved sequence features of NLRs to functional aspects of NLR signaling. Cytochrome c activated Apaf-1 has been shown to undergo an ATP-hydrolysis-dependent conformational rearrangement in order to form heptamers through an interaction of its NACHT domains. Interestingly, the heptamers were proposed to arrange in a ring-like structure, which is usually found in AAA+ ATPases such as RuvB or NtrC1 [30–32]. We consequently generated homology models for the NACHT-WH-SH regions and analyzed the distribution of conserved motifs and residues in order to deduce a putative mechanism for NLR oligomerization (Figure 4A). Although an alternative ring formation has also been proposed [33], we used the typical AAA+ like arrangement in which the interface is formed of surface residues in the NACHT domain (see orange and magenta boxes in Figures 2).

Based on the fact that all structural features required for oligomerization are present (Figure 4B), we hypothesize that NLRs...
are in principle capable of building signaling platforms like Apaf-1. This suggests that NLRs also use the ring-like arrangement of effector domains to recognize and activate signaling partners.

2. NLR effector domains and their corresponding binding partners

**CARD-CARD interactions.** Structural and mutational studies of the CARD domains of Apaf-1 and pro-caspase-9 have identified the essential motifs for pro-caspase-9 activation by Apaf-1 [34]. The interface of these two proteins has been shown to be mainly constituted by electrostatic interactions between an acidic and convex surface patch (helices 1 and 4) within the CARD domain of Apaf-1 and by a basic and concave surface patch (helices 2 and 3) within the CARD of pro-caspase-9. Among this homophilic CARD-CARD interaction, it has been shown that the crucial residues D27, E39, E40, and E41 are localized within the acidic region of Apaf-1 [35]. Furthermore, on the NLR protein NOD1, residues D42, D48, E53, D54, and E56 of the NOD1 CARD were suggested to mediate its interaction with its effector protein RICK. Complementary residues R444, K480, R483, R488 on the CARD domain of RICK were found in the putative interaction surface [35].

Based on these findings, we examined whether these residues, which are necessary for homophilic CARD-CARD interactions, are conserved among NLR CARDs and the CARDs of their effector proteins, respectively, by means of multiple sequence alignment (Figure 5A and 5B). Although the primary sequence conservation between CARD domains is generally low, we observed that the domains display a high degree of structural homology. Importantly, the known interface residues of the homophilic CARD-CARD interactions of NOD1/RICK and Apaf-1/C9 are to a high degree conserved among NLR effector domains, caspases, and adaptor proteins (Table 3). Notably, the first and last residues of the acidic as well as the basic patch are highly conserved among the analyzed CARD domains, suggesting a pattern of interaction similar to the one described for Apaf-1/C9 or NOD1/RICK. These observations imply that the main principle of CARD-CARD interactions is based on the engagement of an acidic patch built of helices 1 and 4, with a basic patch composed of helices 2 and 3. However, the surrounding residues within this interface most likely define the specificity for interactions between CARD domains thereby ensuring the selectivity for the right interaction partner.

**Table 3. Conservation of interface residues in the acidic patch and the basic patch based on the multiple sequence analysis in Figure 5A and 5B.**

| Conserved residues Acidic patch | Basic patch |
|--------------------------------|-------------|
| **Apaf-1** | D27 | S38 | E39 | E40 | E41 | R12 | E46 | Q49 | A54 |
| **ASC** | D134 | E144 | Q145 | Y146 | Q147 | R15 | E152 | N155 | R56 |
| **Cardif** | E26 | R37 | D38 | Q39 | D40 | T8 | C46 | N51 | W56 |
| **Cardinal** | D367 | N378 | E379 | K380 | E381 | H352 | E386 | R389 | E394 |
| **Caspase 1** | D27 | E38 | E39 | M40 | E41 | R10 | N47 | V50 | R55 |
| **Caspase 5** | N69 | K80 | E81 | E82 | E83 | H48 | Y88 | K91 | A96 |
| **Caspase 9** | D27 | H38 | M39 | I40 | E41 | R13 | G47 | R51 | R56 |
| **Ipaf** | D25 | E36 | E37 | V38 | N39 | S8 | E44 | E47 | R52 |
| **NOD1** | D42 | E53 | D54 | A55 | E56 | R27 | C61 | Q64 | R69 |
| **NOD2** | D58 | E69 | D70 | Y71 | E72 | R38 | G78 | L81 | R86 |
| **RICK** | D461 | E472 | D473 | Y474 | E475 | R444 | K480 | R483 | R488 |

Bold residues contribute to the interface in NOD1/RICK and Apaf-1/Caspase9, respectively.

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PYRIN-PYRIN interactions. To date, neither information from crystal structures nor mutational analysis of PYRIN domains or PYRIN-PYRIN interactions have been reported [36]. However, utilizing NMR, a recent report observed a highly bipolar organization of the human ASC and ASC2 PYRIN domains [36,37], revealing that they resemble the molecular surface properties of CARD domains. These tertiary structure similarities between PYRIN and CARD domains indicate that like for CARD-CARD interactions, an electrostatic interface may play an important role for the biochemical properties and the interaction behavior of PYD-containing molecules [36,37]. Based on this hypothesis, we propose that the already described interaction between the CARDs of Apaf-1 and caspase-9 can be utilized as a working model for PYRIN-PYRIN interactions as well. Following on this suggestion, one would expect that the residues in helices 2 and 3 of one PYD build an interface with the residues in helices 1 and 4 of a complementary PYD [37].

By utilizing multiple sequence alignments (Figure 5A and 5B) of both, CARD and PYD domains, we observed that the residues involved in the homophilic domain interfaces are conserved among the NLR family. However, mutational studies showed that these residues, which are important in a certain CARD-CARD interaction, are dispensable in the homotypic interaction of other proteins (e.g. the D42 mutant in Nod1 does not impair binding to Rick, but its corresponding residue in Apaf-1 is essential for its interaction with procaspase-9).

The proposed model of CARD-CARD and PYD-PYD interactions is that the acidic patch of one domain interacts with the basic patch of the other protein. Hydrophobic residues of adjacent regions are also suggested to be important in this interaction. Nevertheless, it is not clear so far, if there is a limited repertoire of structurally conserved motifs that may mediate interactions among death domain superfamily members. Therefore, more structural studies and mutational analysis of complexes built of those domains are necessary to define the motifs and interacting residues involved.

3. The LRR receptor domain

Similarly to the WD-40 repeats in Apaf-1, leucine-rich repeats are the ligand sensing motif of NLR proteins, a property they share with members of the TLR and RLR (RIG-I-like receptors) families. LRRs in general consist of 2–45 motifs of 20–30 amino acids in length and exhibit a typical curved horseshoe-like structure with a parallel beta sheet on the concave side and helical elements on the convex side [38].

In NLRs the C-terminal LRR domain is thought to act as a sensor of bacterial products. Yet, little is known about how the PAMP is interacting with the LRR or even how the LRR region interacts with the remainder of the NLR, since no structural data is available on these questions. Recently, some insight into the possible mechanism of ligand-receptor binding was provided by the two LRR-ligand complex structures of TLR1:TLR2 [39] and TLR4:MD2 [40]. Within the proposed LRR-ligand complex, the ligand-binding site is located at the concave surface of the LRR domain.

In order to augment our understanding of the molecular mechanism of ligand recognition we generated a homology model of the NOD2 LRR domains based on the structure of the ribonuclease inhibitor (aa1–413, PDB id: 1bnh, seqID 33%) as a template. Additionally, we utilized the Consurf Server for the identification of functional regions in NLRs by surface mapping of phylogenetic information. Figure 6A shows the modeled LRR domains of NOD2 with highly conserved residues in the human NLR family colored in green and non-conserved residues shown in white. The figure clearly shows an extensive patch of conserved residues spanning the surface hinting to a function of these residues in signal sensing or the activation mechanism.

Our findings are in accordance with recently published work by Tanabe and colleagues, which showed loss of function mutations found in CD patients are depicted in red. (Core forming residues that do not contribute to the ligand-binding patch are highlighted in lightblue). C NOD2 homology model based on templates Apaf-1 (aa1–581, PDB id: 1z6t, seqID 11%) and ribonuclease inhibitor (aa1–413, PDB entry: 1bnh, seqID 33%) (33). NOD2 model depicted as a cartoon color-coded according to domain structure: blue CARD2 aa95–182, green linker aa183–240, purple NACHT aa241–465, orange winged helix and superhelical domain aa466–734, green-yellow LRR aa735–1040). An ADP molecule bound in the ATPase active site is depicted in sticks. The position of the SNP mutations P268S, R702W, and G908R, are shown as red spheres. The truncation due to mutation L1007fsinsC is colored red.

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in the LRR domain to be located on the convex surface with additional residues on the concave region [41]. However, only those residues that are predicted to contribute to the convex surface are conserved in the corresponding regions of LRR proteins, whereas the residues on the concave surface are not (Figure 6A and 6B). On the other hand the loss-of-function mutations on the outer surface in the LRR domain do not form a continuous patch. They are scattered all over the molecule and are therefore not likely to form the ligand-binding site.

Our homology model suggests a putative ligand-binding pocket situated in the concave surface and supports earlier observations, where the predicted loss-of-function-mutations W907L, V935M, E959K, C961Y, K969E, S991F as well as the Crohn’s disease related mutation G908 have been mapped to the same area [41]. These amino acid residues do form a contiguous patch and therefore may point to the putative ligand-binding site (Figure 6B). Supporting this, is the fact that the location of this particular surface patch corresponds to ligand-binding sites in other LRR proteins [42–44]. Taken together, these results point to a common putative binding pocket located at the concave surface of the LRR, which, however, differs from protein to protein. Whether the patches on the convex surface do contribute to ligand-binding or eventually contribute to locking the NLR proteins in the dormant form remains to be further investigated.

**Disease derived mutations of NLRs: implication on NLR function.** Several diseases were found to arise from aberrant NLR function [45–49]. More accurately, they are caused by SNPs (single nucleotide polymorphisms) leading to point mutations in NLR genes. One particular intriguing SNP is SNPS in NOD2 leading to Crohn’s disease. To analyze the position of the SNPS mutation P268S within NOD2, a NOD2 homology model was created based on templates Apaf-1 (aa1–581, PDB id: 1z6t, seqID 11%) and ribonuclease inhibitor (aa1–413, PDB id: 1mnh, seqID 33%). The SNPS mutation P268S resides in the linker region before the first helix of the NACH domain (Figure 6C). P268 constitutes part of the nucleotide-binding interface where it interacts with the adenine moiety of ADP. P268S disturbs the backbone conformation of the linker thus interferes with nucleotide binding and may alter the affinity and hydrolysis rate of the nucleotide-binding domain. Hence, SNPS impairs the fine-tuned conformational states of the active-inactive balance of the NOD2 receptor and has therefore most likely a direct impact on its signaling properties.

In summary, our study clearly shows that the overall architecture and secondary structure features of most NLRs resemble those of Apaf-1. From the structural point of view, most of the NLR family members are therefore Apaf-1-like, with deviations including NOD2 (2 CARD domains), NALP1 (additional FIIND and CARD domain), NALP10 (missing LRR region), NOD5 and CIITA (undefined N-terminal region). Analyses of multiple sequence alignments revealed that all NLRs contain the crucial features for ATP-binding. In comparison to Apaf-1, most NLRs display a modified Walker B box. Since all NLRs, except for NAIP and NALP11, do not contain the crucial Walker B glutamate or aspartate required to activate the water molecule, they seem to have developed a new motif, the extended Walker B box to retain ATP hydrolysis activity. This is supported by the observation that NALP12 is able to bind and hydrolyze ATP [25]. Thus, our sequence analysis now provides the basis for further studies to elucidate whether the modified/extended Walker B box is functional.

Additionally, we identified one of the most intriguing features, which is the conserved histidine in the WH-domain, to be conserved among members of the NLR family. NLRs displaying this feature most likely resemble similar to Apaf-1 and activate their targets by oligomerization. Interestingly, NOD5, CIITA, NALP6, and NALP8 do not contain the conserved histidine in their WH domain. Whether their oligomerization mechanism and ATP hydrolysis capacity differ remains an open issue.

Our analyses of the effector domains of NLRs as well as those of their adaptors and target caspases, or kinases reveal a common interface, which is composed of charged surface patches. The presence of acidic and basic surface patches theoretically renders all CARD and PYD domains compatible for interaction with each other. Yet their distinct profile and that of surrounding residues found in the described interfaces ensure the specificity for each interaction. This selectivity allows a well-balanced fine-tuning of the elicited immune response.

Finally, sequence comparison of LRRs in human NLRs does not reveal one particular region that serves as the general ligand-binding site. This suggests that individual NLRs evolved highly specialized modes to recognize specific ligands. However, conserved residues found within this domain may contribute to the intramolecular interaction or backfolding of the LRR region in order to regulate NLR activation. Our results serve as a basis for further mutational and functional analyses required to more precisely define the role of LRRs in ligand recognition and NLR activation.

**Methods**

**Sequence alignments**

NLR protein sequences (see Table 1) were submitted to profile-sequence searches with the FFAS server (http://ffas.ljcrf.edu) [15]. Secondary structure prediction was done using the predictprotein server (http://www.predictprotein.org) [16]. We analyzed the human sequences for NACHT domain paralogues (about 410 residues). Multiple alignments were created using muscle [23] and m-coffee [50] with default options in the aforementioned sequences and the Apaf-1 sequence. The alignment was manually adjusted according to secondary structure prediction.

**Evolutionary analysis**

The alignments were used to run phylogenetetic probabilistic analyses using the parallel implementation of MrBayes [51]. The sequence of Apaf-1 was used to root the tree in all cases. A total number of 200000 generations were run in 4 independent chains. The model used to set the priors for amino acid data was an average of all the available models and a sample was obtained each 10 generations. Once convergence was reached, a total of a credible 6973 trees were sampled and clade credibility values (probabilities) calculated. In order to check how the paralogues arrange in a bigger tree, homologous sequences were retrieved from Uniprot databases from close organisms. The new sequences (31) were re-aligned to the original multiple alignment using T-coffee. To keep the clarity of the tree, we used a final number of 54. As in previous cases, 200000 generations were run. The frequency of sampling was each 10 generations. A total of credible 2777 trees were then sampled.

**Homology modeling**

The protein structure with the highest scoring alignment from the FFAS-search was used as a template for modeling the structures with the SCWRL-Server (http://www1.jesg.org/scripts/prod/scwrl) [52]. Models were evaluated using PROSA (https://prosa.services.came.sbg.ac.at/prosa.php) manually inspected, analyzed, and figures were prepared using Pymol (http://pymol.sourceforge.net). The degree of conservation was suspected, analyzed, and figures were prepared using Pymol (https://pros.services.came.sbg.ac.at).
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Author Contributions

Conceived and designed the experiments: RS MP. Performed the experiments: MP. Analyzed the data: RS AR. Contributed reagents/materials/analysis tools: SR JF AR. Wrote the paper: RS MP. Other: Revised manuscript: JF SR.

The NLR Family