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Expression, Purification, and Characterization of Recombinant NOD1 (NLRC1), a NLR Family Member

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Abstract

NOD1 (NLRC1) is a member of the NLR family of innate immunity proteins, which are important cellular sensors of various pathogens. Deregulated NOD1 signaling is involved in various autoimmune, inflammatory, and allergic diseases, making it a potential target for drug discovery. However, to date, the successful high-yield purification NOD1 protein has not been reported. Here we describe the large-scale expression of recombinant NOD1 protein in non-adherent mammalian cells. One-step immunoaffinity purification was carried out, yielding highly pure protein with excellent yields. Gel-sieve chromatography studies showed that the purified NOD1 protein eluted almost exclusively as a monomer. Addition of the NOD1 ligand, (γ -Tri-DAP), stimulated NOD1 protein oligomerization. Using purified NOD1 protein for nucleotide binding studies by the Fluorescence Polarization Assay (FPA) method, we determined that NOD1 binds preferentially to ATP over ADP and AMP or dATP. We also documented that purified NOD1 protein binds directly to purified pro-apoptotic protein Bid, thus extending recent data that have identified Bid as an enhancer of NOD1 signaling. This expression and purification strategy will enable a wide variety of biochemical studies of mechanisms of NOD1 regulation, as well as laying a foundation for future attempts at drug discovery.

Keywords

NOD1; Protein Expression; FPA; NLR; Nucleotide binding; Bid

1. Introduction

Innate immunity represents the first line of defense against pathogens. The human genome contains at least 22 genes encoding NLR (NACHT and Leucine Rich Repeat [LRR]) proteins, having a conserved architecture of a nucleotide-binding NACHT domain and variable numbers of LRRs (Martinon et al., 2002; Stehlik and Reed, 2004). NLRs are intracellular proteins, typically found in the cytosol, representing intracellular analogs of the TLR (Toll-like receptors), important mediators of innate immunity. NLRs recognize exogenously derived Pathogen Associated Molecular Patterns (PAMPs) and endogenously generated Damage Associated Molecular Patterns (DAMPs), which convert NLRs from inactive monomers to activated oligomers. Intracellular bacteria, various microbe-derived

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molecules, and the presence of viral DNA in the cytosol have all been shown to trigger activation of certain NLR family members (Ting et al., 2010). Oligomerized NLRs serve as platforms for activating signaling proteins, including inflammatory caspase-family proteases, involved in cytokine processing, such as activation of Interleukin-1 β (IL-1 β) and IL-18. The caspase-activating NLR protein complexes have been dubbed “inflammasomes.”

The NLR family member NOD1 (NLRC1) is a 108-kD protein which consists of three major domains: a Caspase Recruitment Domain (CARD), a Nucleotide Binding Domain (NBD, also known as NACHT), and 10 leucine-rich repeats (LRRs) (Chamaillard et al., 2003). NOD1 is prototypically activated by γ -tri-DAP, a component of peptidoglycans (PGNs) found in bacterial cell walls (Shaw et al.; Strober et al., 2006; Tattoli et al., 2007). When activated, NOD1 transduces signals leading to induction of protein kinases that drive activation of NF- κ B, AP-1/c-Jun, and IRF family transcription factors. Insertion/deletion polymorphisms in the *NOD1* gene have been associated with various disorders, including sarcoidosis, Crohn's disease, asthma and autoimmune uveitis (Carneiro et al., 2008; Eckmann and Karin, 2005; Franchi et al., 2008; Strober et al., 2006; Tattoli et al., 2007), making this protein a potential target for drug discovery.

To enable detailed biochemical characterization of the mechanisms controlling NOD1 activity, as well as development of drug screening assays, we sought to develop a process for production of recombinant NOD1 protein. To date, production and purification only of the CARD domain of NOD1 has been reported (Srimathi et al., 2007). This short 106 amino acid-containing domain (13 kDa) was expressed in *E. coli*. However, the full-length NOD1 protein, similar to other NLR proteins, is a large complex protein that does not express well in bacteria, requiring eukaryotic expression systems (Duncan et al., 2007; Faustin et al., 2007). Herein, we report the expression of NOD1 using mammalian cells and large-scale purification of the NOD1 protein using a one-step immunoaffinity chromatography method. The resulting recombinant NOD1 protein was employed for studies of interactions with nucleotides, bacterial ligands, and NOD1 partner proteins.

2. Materials and Methods

2.1. Cell Culture

For virus production, HEK293T cells were grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% antibiotics (penicillin and streptomycin), and L-glutamine (All from CellGro). These cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. 293 Freestyle cells (Life Technologies, Carlsbad, CA) were grown in 293 Freestyle medium without serum (Gibco) in Erlenmeyer flasks. Flasks were under constant shaking (127 rpm) and were maintained at 37°C in an 8% CO₂ humidified atmosphere.

2.2. Viral NOD1 expression vector construction and cell infections

Full-length cDNA encoding NOD1 (Supplementary Fig. 1a) (6 $\times$ His- and flag-epitope tagged at the N-terminus) was subcloned into *Xba*I and *Xho*I sites of a CMV-driven lentiviral construct (pCSC-SP-PW, gift from Dr. Robert Marr, The Salk Institute) (Supplementary Fig. 1a–b). The lentiviral plasmids contain the cytomegalovirus (CMV) promoter upstream of introduced cDNAs, as well as a central polypurine tract (cPPT) of HIV-1 and a posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE). Self-inactivating mutations were inserted in the promoter/enhancer sequences of the 3' LTR, resulting in transcriptional inactivation of the integrated proviruses in host cells (Supplementary Fig. 1b).

Vesicular stomatitis virus G envelope protein-pseudotyped lentiviruses were prepared and purified as described (Naldini et al., 1996; Pfeifer et al., 2002; Pfeifer and Verma, 2001).

Vector concentrations were estimated according to biological titer provided by GFP-expressing lentiviruses (control).

To infect 293 Freestyle cells, we used a lentivirus containing the NOD1 Open Reading Frame (ORF) N-terminally fused with 6×His-flag and flag-tag under the CMV promoter). 2×10^6 cells were infected with 35×10^8 viruses in 20 ml culture volume. Virus-containing medium was replaced after 16 hours of incubation.

2.3. Protein expression and purification

293 Freestyle cells stably expressing NOD1 were grown to a concentration of $3\text{--}4 \times 10^6$ cells/ml, typically in 600 ml cultures in 2 liter screw-cap Erlenmeyer flasks. Cells were sedimented by centrifugation for 5 minutes $500 \times g$ at 4°C . Cell pellets were resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA) supplemented with Complete protease inhibitors tablets (Roche). The ratio of lysis buffer to cell number was 125 μl lysis solution per 5×10^6 cells. Lysis was performed under gentle agitation in 4°C for 1 hour. Lysates were sonicated followed by centrifugation at $20,000 \times g$ for 30 minutes in 4°C . Cleared lysates (i.e. soluble fraction) were then incubated with anti-flag antibody-conjugated beads (Sigma) for 3–4 hours under gentle shaking in 4°C . Then, the lysate-beads mixture was loaded onto a Poly-Prep Chromatography Column (Bio-Rad) and unbound material was allowed to flow through the column. Beads were then washed with 500 column volumes (CV) of wash buffer A (50 mM Tris-HCl pH 7.4, 150 mM NaCl), followed by another wash step using wash buffer B, which has double the salt concentration (50 mM Tris-HCl pH 7.4, 300 mM NaCl). Then, a final wash step was performed with $20 \times \text{CV}$ wash buffer A. Elution was carried out using $14 \times \text{CV}$ elution buffer (0.1M Glycine pH 3.5), and eluates were collected in tubes containing $0.1 \times \text{CV}$ neutralizing buffer (0.5M Tris-HCl pH 7.4, 1.5M NaCl). Aliquots of fractions were subjected to SDS-PAGE and stained with GelCode Blue Safe Protein Stain (Thermo Scientific), a Coomassie-based dye, to assess the purification yield and purity. Additionally, immunoblot analysis was performed using anti-flag antibodies. Fractions containing NOD1 protein were pooled and concentrated 7–14-fold using Vivaspin concentration columns (GE Healthcare) with a cutoff of 50-kDa.

2.4. Protein Gel Electrophoresis and Immunoblot analysis

The specified volumes (Typically 1–2% of each fraction) of purified NOD1 were size-fractionated by gel-electrophoresis using 10% (w/v) polyacrylamide gels and analyzed by either staining with GelCode Blue Safe Protein Stain (Thermo Scientific) or by immunoblotting using mouse anti-flag antibody (Sigma-Aldrich) followed by fluorescently-labeled goat anti-mouse IgG (Li-Cor Biosciences) and detection with a fluorimeter scanner (Li-Cor Biosciences). For protein band quantification, Odyssey V3.0 software was used (Li-Cor Biosciences).

2.5. Gel filtration

Gel filtration analysis was performed by injection of 500 μl purified Flag-6His-tagged NOD1 (900 $\mu\text{g}/\text{ml}$) onto a Superdex 200 HR 10–30 column (24 ml, GE Healthcare) at a flow rate of 0.5 ml/min in TBS. The column was connected to an AKTAPurifier FPLC machine (GE Healthcare). Fractions of 0.4 mL volume were collected with a fraction collector. Eluted proteins were detected by UV absorbance at 280 nm. Prior to injecting the sample, 125 μl of Bio-Rad Gel Filtration Molecular Weight Standards (Bio-Rad) was applied to the column under the same conditions.

2.6. Protein quantification

The micro bicinchoninic acid (BCA) protein assay kit was used (Thermo Scientific) per the manufacturer's instructions for measuring total protein concentration.

2.7. Fluorescence Polarization Assay (FPA)

Various concentrations of immunoaffinity-purified NOD1 or 6His-purified Bcl-XL (negative control) proteins were incubated with 10 nM fluorescein isothiocyanate (FITC)-conjugated ATP in TBS for 5 minutes at room temperature. Fluorescence polarization was measured using an Analyst AD Assay Detection System (LJL Biosystems).

3. Results

3.1. NOD1 expression in a mammalian cell system

Expression of NOD1 in 293 Freestyle cells was achieved using infections with a recombinant lentivirus. The lentiviral vector contained a NOD1 cDNA comprising the open reading frame (ORF) with 6×His- and flag-epitope tag appended at the N-terminus under control of a CMV immediate-early region promoter (Sup. Fig. 1). During the viral infection, the cell concentration was never lower than 1×10^5 cells/ml, which improved cell survival. To avoid dilution of the cells beyond 10^5 cells/ml, we used highly concentrated virus, employing highly purified viral stocks of 10^9 to 10^{10} infectious units/ml (as determined by virus titer assessment). Cells were infected overnight, followed by medium replacement. Cells were allowed to recover (typically 3–4 days) and were diluted regularly when the cell concentrations reached $1.5\text{--}2.5 \times 10^6$ cells/ml.

To increase expression levels, multiple sequential infections were carried out. Each infection procedure was performed after the cells fully recovered from the previous infection cycle (i.e. normal proliferation was observed and the cells looked morphologically healthy). A MOI of 20 was used for each round of infection, performing a total of 4 infections with the goal to obtain multiple virus integrations per cell. The increase in NOD1 protein expression achieved with increasing number of viral infections is shown in Figure 1.

3.2. Purification of NOD1 protein

For the NOD1 purification, cells that were infected 4 times were used. The stably infected 293 Freestyle cells were propagated in Erlenmeyer flask cultures. To ensure aeration, culture volumes did not exceed approximately one-third of the total volume of the Erlenmeyer flask (e.g. 1 liter culture in a 3 liter Erlenmeyer). Cells were expanded every 2–3 days by dilution at a 1:2 – 1:3 ratio, such that the cell concentration was 8×10^5 - 3×10^6 cells/ml. When cultures reached a volume of 2 liters (representing two 3 liter flasks, each containing 1 liter cell culture), the cells were grown to a concentration of $3.5\text{--}4 \times 10^6$ cells/ml and thereafter collected by mild centrifugation.

Cleared lysates were incubated with the anti-flag antibody-coated beads in a batch mode for 4 hours in 4°C. A wash step of $500 \times \text{CV}$ was performed under a controlled flow rate of 600 $\mu\text{l}/\text{min}$ with TBS buffer. Subsequently, another step of wash was performed, with a buffer containing 300 mM NaCl, in order to reduce interactions of recombinant Flag-6×His-NOD1 with other cellular proteins present in lysates. Next, a $50 \times \text{CV}$ wash was performed with TBS to reduce the excess salt. Elution of protein from antibody-coated beads was performed using low pH glycine solution (0.3 M pH 3.5). Following collection of fractions, eluted Flag-6×His-NOD1 protein was detected by Coomassie-stained SDS-PAGE (Fig. 2a) and by immunoblotting using anti-flag antibody (Fig. 2b). As shown in Fig. 2, the majority of NOD1 eluted in fractions 1–6, with later fractions containing much less protein. Although NOD1 could not be detected by Coomassie in fractions 10–13 (Fig. 2a), it could still be

detected either by using anti-flag antibody immunoblotting or by using a highly sensitive BCA protein quantification method in those fractions (Fig. 2b and Fig. 2c, respectively). As estimated from imaging of protein-stained gels, purified recombinant 6×His-flag-NOD1 was >90% pure and was obtained with a yield of approximately 1 mg per 1 liter of culture (Table 1 and Fig. 3).

A sample of the flag antibody beads from the column, which was boiled in SDS-sample solution and analyzed by gel-electrophoresis, still contained NOD1 in significant amounts (Fig. 2b). As low pH may denature some proteins, inducing their aggregation, we attempted to elute NOD1 by a milder elution scheme, using flag peptide as the eluent. Elution with the flag peptide was performed in the same manner as the glycine elution, with the same number of fractions collected and in the same volumes. However, similar NOD1 amounts remained with the beads following elution with flag peptide (data not shown). This result suggests that NOD1's retention in the column is not due to the elution conditions. Because NLR proteins are known to easily become aggregated (Faustin et al 2007), we presume that the retained protein is insoluble (aggregated) NOD1, incapable of being eluted.

3.2. Studies of recombinant NOD1 protein oligomeric status

It is known that NLR family proteins, including NOD1, form large oligomers upon activation by various PAMPs and DAMPs. To determine the oligomerization status of the purified Flag-6×His-NOD1 protein, the purified material was loaded onto a Superdex 200 gel filtration column and its elution was followed by UV absorption at 280 nm (Figure 4a). One distinct peak with retention time compatible with the monomeric form of the protein was consistently observed (Figs. 4a and 4b). No higher molecular weight forms of NOD1 were detected. Analysis of elution fractions by SDS-PAGE and Coomassie blue staining confirmed that UV absorption measurements corresponded to NOD1 protein (Figure 4a).

3.3. The peptidoglycan-derived ligand γ -Tri-DAP induces NOD1 oligomerization

NOD1 is activated by γ -Tri-DAP, a bacterial dipeptide found in peptidoglycan (Strober et al., 2006; Tattoli et al., 2007). Recently, it was reported that γ -Tri-DAP interacts with the LRR domain of NOD1, resulting in the increase of NOD1 binding to RICK and a subsequent elevation of RICK's phosphorylation (Laroui et al.). To test the ability of γ -Tri-DAP to induce NOD1 oligomerization, we incubated purified NOD1 protein with either Mg/ATP or γ -Tri-DAP (200 μ g/ml) and Mg/ATP for 30 minutes at room temperature, and subjected the sample to gel sieve chromatography (Fig. 5). Whereas non-treated NOD1 eluted mainly as a monomer, γ -Tri-DAP treatment shifted NOD1's elution towards the void volume fractions, indicating that γ -Tri-DAP induces oligomerization.

3.5. ATP binding studies

Because NOD1 contains a nucleotide-binding domain (see Supplementary Fig. 1a), we sought to measure the binding of various adenosine phosphonucleotides to the purified protein. To this end, increasing concentrations of purified Flag-6×His-NOD1 protein were incubated with Fluorophore-conjugated ATP (FL-ATP), measuring ATP binding by Fluorescence Polarization Assay (FPA) (Faustin et al., 2007). NOD1 protein induced a concentration-dependent increase in FL-ATP fluorescence polarization (Fig. 6a), thus demonstrating ATP binding to the recombinant purified Flag-6×His-NOD1 protein. In contrast, purified 6×His-Bcl-XL, which does not contain a nucleotide-binding domain, did not bind FL-ATP as determined by the FPA method. Binding of NOD1 to FL-ATP was concentration-dependent and saturable, with an apparent K_D of 25 nM.

To verify FL-ATP binding specificity, we performed competitive displacement experiments where increasing concentrations of non-labeled ATP were incubated with purified NOD1

plus FL-ATP (Fig. 6b). Unlabeled ATP displaced FL-ATP with an IC_{50} of ~15 nM (Fig. 6b). These results show that the binding of FL-ATP to Flag-6×His-NOD1 is highly specific, as evidenced by low IC_{50} value for competitive displacement by unlabeled ATP and by the ability of unlabeled ATP to drive the fluorescence polarization signal essentially to baseline values (for FL-ATP measured alone, without NOD1 protein). Additional studies where FL-ATP was bound to NOD1 prior to addition of unlabeled ATP also showed that the binding is reversible.

We also measured the ability of other adenosine phosphates such as ADP, AMP and dATP to compete with FL-ATP for NOD1 binding (Fig. 6b). Interestingly, although ADP effectively displaced FL-ATP, its efficiency was much lower, with an IC_{50} of 190 nM (Fig. 6b), suggesting a markedly lower affinity of ADP compared to ATP for binding to flag-His6-NOD1 protein. In contrast, AMP and deoxyATP (dATP) did not displace FL-ATP from NOD1, as measured by FPA.

3.6. Purified NOD1 binds to Bid

Recently, it was shown that the pro-apoptotic Bcl-2 family protein Bid interacts with NOD1 and is required for optimal NOD1 signaling in cells (Yeretssian et al.). However, interaction of NOD1 with Bid has not been demonstrated using purified proteins in an in vitro reconstituted system. To investigate the ability of the purified NOD1 to bind to purified Bid, we incubated purified recombinant Flag-6×His-NOD1 protein with bacterially-expressed purified recombinant 6×His-Bid (Zhai et al., 2005) or with 6×His-Bcl-XL (another Bcl-2 family member used here as a negative control) (Fig. 7). Flag-6×His-NOD1 was immunoprecipitated with anti-flag antibody and binding of 6×His-Bid or 6×His-Bcl-XL was analyzed by immunoblotting. Flag-6×His-NOD1 protein formed stable complexes with 6×His-Bid but not with 6×His-Bcl-XL, suggesting that the purified NOD1 protein. In addition to documenting a direct interaction of Bid with NOD1 without requirement for additional proteins, these results also provide evidence that recombinant Flag-6×His-NOD1 is correctly folded such that it can specifically interact with a known binding partner.

4. Discussion

Purifying NLR-family proteins poses a considerable challenge, as these proteins have high molecular weights and are multidomain proteins, typically containing ≥ 3 discrete types of protein domains. As most NLRs are activated by bacterial components (e.g. PGN, flagellin, etc.), expressing them in bacterial systems is problematic, since they might be activated spontaneously when expressed in prokaryotic systems. In fact, our attempts to express several NLRs in *E. coli* resulted in poor yields, with most the protein present in insoluble fractions (data not shown). The mammalian expression system used in this study overcomes this problem. Although other expression systems were previously reported to be useful for the expression and purification of some NLRs, particularly insect cells (Faustin et al., 2007), this is the first time a mammalian expression system has been successfully used for this purpose.

To express the NOD1 protein, we employed 293 Freestyle mammalian cells. A mammalian expression system was chosen for several reasons, including: (1) Full-length NOD1 protein was not expressed well in *E. coli*, when attempted (data not shown); (2) Insect cell-based expression systems previously used to express NLRs (Baculovirus), although successful, require viral amplification for each purification attempt, thus increasing the procedure time as well as making reproducibility variable; and (3) As a mammalian protein, a mammalian expression system would ensure proper functional folding as well as any posttranslational modifications.

Unlike conventional HEK293 cells, 293 Freestyle cells are grown in suspension to relatively high cellular concentrations (up to 4×10^6 cells/ml). To avoid multiple transfections of NOD1-encoding plasmid and to reduce batch to batch variability, cells were stably infected with a recombinant lentivirus expressing NOD1 protein with a N-terminal flag-6×His double tag (supplemental Fig. 1). Using a stable transduction approach also simplified subsequent scale-up relative to transient transfection approaches, while also avoiding costly transfection reagents.

Our approach offers several advantages over other expression systems. First, since the cells were infected with recombinant lentiviruses, they stably express NOD1, obviating the need for infecting or transfecting cells before each round of scale up cell culture, unlike the insect cell-based baculovirus system where virus must be produced each time a protein preparation is desired. The cells are simply grown to the desired volume and are then harvested. This reduces the labor and logistics of the protein expression process significantly. Avoiding the need to introduce the DNA construct in cells before each purification by means of transfection also reduces costs, as well as labor.

Previous attempts to express NOD1 protein were only partially successful. Srimathi *et al* expressed a short, 106 amino acid stretch of NOD1 in *E. coli* (Srimathi et al., 2007). Although crystallized, this small portion of NOD1 contains only the CARD domain, and the activity of this purified domain was not determined. A recent study by Laroui *et al* used commercially available full-length NOD1 protein, purchased from Novus Biologicals, Littleton, CO (Laroui et al., 2011). The manufacturer, however, states that their NOD1 preparation is not active and should not be used for experiments requiring activity (http://www.novusbio.com/NOD1-Recombinant-Protein_H00010392-P01.html). Since this commercially available NOD1 protein was expressed in bacteria (which contain γ -Tri-DAP in the cell wall), it is plausible that it is highly aggregated.

To the best of our knowledge, this is the first time a protein is reported to be expressed intracellularly in 293 Freestyle cells. These cells are typically used for expression of secreted proteins, by adding a secretion signal to the protein of interest. However, when secreted expression was attempted with NOD1, no protein elaboration into culture supernatants was observed and the protein was also not detected intracellularly (data not shown). Since 293 Freestyle cells grow robustly in suspension, scale-up production in a bioreactor may be feasible for preparing even larger amounts of NOD1 protein.

Although flag tag-based immunoaffinity purification proved successful for NOD1, other tags were not as useful. Specifically, our attempts to employ the 6×His tag for Nickel chelation affinity purification by applying the lysate to a Ni-NTA column resulted in a high amount of impurities. This is probably due to the relatively high abundance of His-tag-containing proteins present in mammalian cells, compared to bacterial or insect cells. Though Ni-chelation chromatography is generally less expensive than antibody-based immunoaffinity chromatography, the anti-flag column can be regenerated and reused. Also, because we used low pH rather than flag peptide for elution, our approach still maintains low cost. It should be noted however that a considerable amount of recombinant NOD1 protein remained associated with the anti-flag beads after glycine elution, suggesting that future improvements in yield might be obtained by using alternative elution strategies.

The NOD1 recombinant protein prepared as described here is capable of binding to ATP with high affinity, as measured by FPA using fluorochrome-conjugated ATP. Competitive displacement experiments indicate that NOD1 binds ADP with approximately 10-fold lower affinity compared to ATP, while displaying essentially no affinity for AMP or dATP. We surmise from these observations that the ribosyl and phosphate moieties of ATP contribute

to the binding affinity, since dATP (deoxyribosyl form of ATP) and AMP do not compete with ATP for binding. These results are in agreement with data obtained using partially purified NLRP3 (Duncan et al., 2007), which showed much higher affinity (~1000-fold) for ATP compared to ADP. However, as opposed to NLRP3, NOD1 showed no detectable binding to dATP (Duncan et al., 2007). Given these differences in the specificity of NLR family members for binding to nucleotides, it is tempting to speculate the selective or semi-selective small molecule inhibitors could be generated that compete for ATP binding at the NACHT domains of these proteins.

The ability of monomeric NOD1 to bind ATP stands in contrast to purified recombinant NLRP1, which requires prior activation with a bacterial ligand (muramyl dipeptide) to become competent to bind ATP (Faustin et al., 2007). For NLRP1, a two-step activation mechanism was proposed, wherein monomeric NLRP1 binds bacterial ligand (presumably via LRRs), then ATP binds to induce oligomerization. For NOD1, a two-step mechanism may still apply, however, the sequence of events may be different, where ATP binding to monomeric NOD1 occurs, followed by bacterial ligand binding to result in oligomerization. In this regard, we showed here that the reported NOD1 ligand γ -Tri-DAP added to purified recombinant monomeric NOD1 stimulates its oligomerization, as measured by gel-sieve chromatography.

The purified NOD1 protein described here is able to bind recombinant purified Bid, a Bcl-2 family member that enhances NOD1 activity through unclear mechanisms. We presume that Bid bound to monomeric NOD1, since nearly all NOD1 is monomeric when produced by the protocol described. With availability of purified recombinant NOD1 protein, it may now be possible to elucidate the mechanism by which Bid enhances NOD1's activity. In this regard, Bid conceivably could (a) increase the affinity of NOD1 for ATP or for γ -Tri-DAP, (b) promote NOD1 oligomerization directly or by accelerating ATP hydrolysis to drive NOD1 oligomerization; or (c) operate as a nucleotide binding exchange factor for NOD1.

In summary, we have devised reagents and methods for high-yield expression and purification of recombinant full-length NOD1 protein. The method employed entails stable expression of NOD1 from a lentivirus vector in mammalian cells adapted for non-adherent suspension growth in large volume cultures. Recombinant NOD1 protein produced by this method is monomeric, competent to bind ATP, capable of oligomerizing in response to γ -Tri-DAP, and binds cofactor Bid. The protein is suitable for more detailed biochemical analysis of the mechanisms regulating NOD1 and for devising high throughput assays for drug screening (Correa et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Carneiro LA, Magalhaes JG, Tattoli I, Philpott DJ, Travassos LH. Nod-like proteins in inflammation and disease. *J Pathol.* 2008; 214:136–148. [PubMed: 18161746]
- Chamaillard M, Girardin SE, Viala J, Philpott DJ. Nods, Nalps and Naip: intracellular regulators of bacterial-induced inflammation. *Cell Microbiol.* 2003; 5:581–592. [PubMed: 12925128]

- Correa RG, Khan PM, Askari N, Zhai D, Gerlic M, Brown B, Magnuson G, Spreafico R, Albani S, Sergienko E, Diaz PW, Roth GP, Reed JC. Discovery and Characterization of 2-Aminobenzimidazole Derivatives as Selective NOD1 Inhibitors. *Chem Biol*. 2011; 18:825–832. [PubMed: 21802003]
- Duncan JA, Bergstralh DT, Wang Y, Willingham SB, Ye Z, Zimmermann AG, Ting JP. Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proc Natl Acad Sci U S A*. 2007; 104:8041–8046. [PubMed: 17483456]
- Eckmann L, Karin M. NOD2 and Crohn's disease: loss or gain of function? *Immunity*. 2005; 22:661–667. [PubMed: 15963781]
- Faustin B, Lartigue L, Bruey JM, Luciano F, Sergienko E, Bailly-Maitre B, Volkmann N, Hanein D, Rouiller I, Reed JC. Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol Cell*. 2007; 25:713–724. [PubMed: 17349957]
- Franchi L, Park JH, Shaw MH, Marina-Garcia N, Chen G, Kim YG, Nunez G. Intracellular NOD-like receptors in innate immunity, infection and disease. *Cell Microbiol*. 2008; 10:1–8. [PubMed: 17944960]
- Laroui H, Yan Y, Narui Y, Ingersoll SA, Ayyadurai S, Charania MA, Zhou F, Wang B, Salaita K, Sitaraman SV, Merlin D. L-Ala-g-D-Glu-meso-DAP interacts directly with the leucine-rich region domain of nucleotide-binding oligomerization domain 1, increasing the phosphorylation activity of receptor-interacting serine/threonine-protein kinase 2 and its interaction with nucleotide-binding oligomerization domain 1. *J Biol Chem*.
- Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell*. 2002; 10:417–426. [PubMed: 12191486]
- Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272:263–267. [PubMed: 8602510]
- Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci U S A*. 2002; 99:2140–2145. [PubMed: 11854510]
- Pfeifer A, Verma IM. Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet*. 2001; 2:177–211. [PubMed: 11701648]
- Shaw PJ, Barr MJ, Lukens JR, McGargill MA, Chi H, Mak TW, Kanneganti TD. Signaling via the RIP2 adaptor protein in central nervous system-infiltrating dendritic cells promotes inflammation and autoimmunity. *Immunity*. 34:75–84. [PubMed: 21236705]
- Srimathi T, Robbins SL, Dubas RL, Seo JH, Park YC. Purification, crystallization and preliminary crystallographic characterization of the caspase-recruitment domain of human Nod1. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2007; 63:21–23.
- Stehlik C, Reed JC. The PYRIN connection: novel players in innate immunity and inflammation. *J Exp Med*. 2004; 200:551–558. [PubMed: 15353551]
- Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol*. 2006; 6:9–20. [PubMed: 16493424]
- Tattoli I, Travassos LH, Carneiro LA, Magalhaes JG, Girardin SE. The Nodosome: Nod1 and Nod2 control bacterial infections and inflammation. *Semin Immunopathol*. 2007; 29:289–301. [PubMed: 17690884]
- Ting JP, Duncan JA, Lei Y. How the noninflammasome NLRs function in the innate immune system. *Science*. 2010; 327:286–90. [PubMed: 20075243]
- Yeretssian G, Correa RG, Doiron K, Fitzgerald P, Dillon CP, Green DR, Reed JC, Saleh M. Non-apoptotic role of BID in inflammation and innate immunity. *Nature*. 474:96–99. [PubMed: 21552281]
- Zhai D, Luciano F, Zhu X, Guo B, Satterthwait AC, Reed JC. Humanin binds and nullifies Bid activity by blocking its activation of Bax and Bak. *J Biol Chem*. 2005; 280:15815–15824. [PubMed: 15661737]

Highlights

- We describe methods for high yield expression of NOD1 in mammalian cells
- NOD1 was purified using a one-step immunoaffinity purification procedure
- The purified NOD1 protein is mainly monomeric
- Recombinant purified NOD1 binds ATP and with lower affinity ADP but not deoxyATP or AMP
- Treatment with a NOD1 ligand derived from bacterial peptidoglycan (γ -tri-DAP) stimulates the protein's oligomerization

Figure 1. Effect of the number of infections on NOD1 expression

Non-infected 293Freestyle cells (N/I), or cells infected once, twice, 3 or 4 times with $3\text{--}3.5 \times 10^7$ virus particles per infection were lysed with sample buffer (1×10^6 cells/200 μl SB) and boiled for 10 minutes. Sample of 30 μl of each were size-fractionated by 10% SDS-PAGE and transferred to nylon membrane. After transfer, membranes were incubated with anti-His antibodies (upper panel) or anti-actin antibodies (lower panel). Band intensities were measured using the Odyssey software (LiCor Biosystems). Data are representative of 2 experiments.

Figure 2. NOD1 immunoaffinity purification

(A) Sample representing 9 μ l aliquots of 550 μ l elution fractions (i.e. 1.6% from each fraction) were size fractionated by 10% SDS-PAGE. Numbers correspond to elution fraction number. M, molecular weight marker (Benchmark, Invitrogen). B, A sample of the anti-flag beads ($\sim$ 15 μ l) was boiled, SB-resuspended. Gel was stained with GelCode Blue (a Coomassie based dye). The asterisk shows the anti-flag antibody's heavy chain. (B) Immunoblotting of the same fractions as in (A). Anti-flag was used to detect 6 $\times$ His-Flag-NOD1. (C) Protein content quantification of fractions 1–13, using the sensitive BCA method.

Figure 3. Tracking NOD1 purity and yield through the purification

Lane 1: Total Lysate. Lane 2: Flow through. Lane 3: Washing buffer. Lane 4: Flag-purified protein 6xHis-flag-NOD1. M, molecular weight standards.

Figure 4. Oligomerization studies of NOD1

(A) Chromatogram of Superdex 200 HR10–30 (24 ml) gel filtration (UV_{280nm}) performed on 6His-Flag-NOD1 protein eluted from anti-flag column. Injection: 0.5 ml. Flow rate: 0.5 ml/min. (B) Samples from every other fraction of the peak shown in (A) were loaded on a 10% SDS-PAGE. Gel was stained with GelCode Blue. (B) The same column as in (A) was calibrated with bovine thyroglobulin (670 kDa), bovine γ -globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa) molecular mass standards. Arrow indicates the elution volume corresponding to the peak shown in (A).

Figure 5. γ -tri-DAP induces NOD1 oligomerization

NOD1 was incubated with γ -tri-DAP (200 μ g/ml) and Mg/ATP for 30 minutes at room temperature (black line) or left untreated (gray line). Thereafter, samples were injected to a S-200 gel sieve column. Oligomerization status is indicated by arrowheads.

Figure 6. NOD1 ATP binding studies

(A) The indicated concentrations of purified NOD1 or Bcl-XL (negative control) were incubated with 10 nM of FITC-conjugated ATP for 10 minutes in RT. (B) Various concentrations of nucleotides were incubated with recombinant NOD1 (50 nM) for 2 min, then 10 nM FITC-conjugated ATP was added in HEPES buffer containing 0.005% Tween 20. ATP binding was analyzed by FPA. mP, millipolars.

Figure 7. Purified NOD1 binds Bid

Purified NOD1 was mixed with purified His-Bid or His-Bcl-XL. Anti-flag beads were added and mixtures were incubated overnight in 4°C with gentle agitation. Upon thorough washing, immunoprecipitates were analyzed by SDS-PAGE/immunoblotting. Membranes were incubated with the specified antibodies. IP, immunoprecipitation.

Table 1

Purification of 6×His-flag-NOD1 from 293Freestyle mammalian cells

The estimated purity and yields of recombinant NOD1 protein are provided for the cell lysates before and after one-step immunoaffinity purification.

	Total protein (mg)	Target protein (mg)	Purity (%)	Overall yield (%)
Soluble lysate	1276.8	98.5	7.71	100
Flag-purified	1.976	1.8	91.1	1.83