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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.

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 toll-like receptors (TLR), 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 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-1β (IL-1β) and IL-18. The caspase-activating NLR protein complexes have been dubbed "inflammasomes."

The NLR family member NOD1 (NLRC1) is a 108-kDa 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., 2011; Strober et al., 2006; Tattoli et al., 2007). When activated, NOD1 transduces signals leading to induction of protein kinases that drive activation of NF-κB, 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

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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 (FBA), 1% antibiotics (penicillin and streptomycin), and L-glutamine (all from CellGro). These cells were maintained at 37 °C in a 5% CO_2 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% CO_2 humidified atmosphere.

2.2. Viral NOD1 expression vector construction and cell infections

Full-length cDNA encoding NOD1 (Supplementary Fig. 1A) (6× His- and Flag-epitope tagged at the N-terminus) was subcloned into *XbaI* and *XhoI* sites of a CMV-driven lentiviral construct (pCSC-SP-PW, gift from Dr. Robert Marr, The Salk Institute) (Supplementary Fig. 1A and 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- and Flag tags 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 h of incubation.

2.3. Protein expression and purification

293 Freestyle cells stably expressing NOD1 were grown to a concentration of 3–4 $\times$ 10^6 cells/ml, typically in 600 ml cultures in 2 l screw-cap Erlenmeyer flasks. Cells were sedimented by centrifugation for 5 min 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 $\times$ 10^6 cells. Lysis was performed under gentle agitation in 4 °C for 1 h. Lysates were sonicated followed by centrifugation at 20,000 $\times$ *g* for 30 min in 4 °C. Cleared lysates (i.e. soluble fraction) were then incubated with anti-flag antibody-conjugated beads (Sigma) for 3–4 h 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× CV wash buffer A. Elution was carried out using 14× CV elution buffer (0.1 M glycine pH 3.5), and eluates were collected in tubes containing 0.1× CV neutralizing buffer (0.5 M Tris–HCl pH 7.4, 1.5 M 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, immmunoblot 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 μg/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 AKTA Purifier 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) as 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 fluoroscein isothiocyanate (FITC)-conjugated ATP in TBS for 5 min 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 (Supplementary Fig. 1). During the viral infection, the cell concentration was never lower than 1 $\times$ 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 10^9–10^{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)