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Short communication

Nucleotide oligomerization domain-2 interacts with 2′-5′-oligoadenylate synthetase type 2 and enhances RNase-L function in THP-1 cells

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ABSTRACT

Nucleotide-binding and oligomerization domain-2 (NOD2) is an intracellular protein involved in innate immunity and linked to chronic inflammatory diseases in humans. Further characterization of the full spectrum of proteins capable of binding to NOD2 may provide new insights into its normal functioning as well as the mechanisms by which mutated forms cause disease. Using a proteomics approach to study human THP-1 cells, we have identified 2′-5′-oligoadenylate synthetase type 2 (OAS2), a dsRNA binding protein involved in the pathway that activates RNase-L, as a new binding partner for NOD2. The interaction was confirmed using over-expression of OAS2 and NOD2 in HEK cells. Further confirmation was obtained by detecting NOD2 in immunoprecipitates of endogenous OAS2 in THP-1 cells. Finally, over-expression of NOD2 in THP-1 cells led to enhanced RNase-L activity in cells treated with poly(I:C), a mimic of double-stranded RNA virus infection. These data indicate connectivity in pathways involved in innate immunity to bacteria and viruses and suggest a regulatory role whereby NOD2 enhances the function of RNase-L.

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1. Introduction

The innate immune system is activated when conserved structures on invading pathogens are recognized by pattern-recognition receptors such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), C-type lectin receptors and RigI-like receptors (RLRs) (Kanneganti et al., 2007). Nucleotide-binding and oligomerization domain-2 (NOD2), an NLR family member, is an intracellular protein that is activated by muramyl dipeptide (MDP), a breakdown product of bacterial peptidoglycan. This leads to activation of the NF-κB transcription factor and mitogen-activated protein kinases and release of cytokines associated with the initial steps of innate immunity and host defense. Mutations in NOD2 are associated with chronic inflammatory disorders including Crohn's disease and Blau syndrome (Hugot et al., 2001; Miceli-Richard et al., 2001; Ogura et al., 2001a). The mechanisms linking mutations in NOD2 with human diseases are not completely understood and observations made in animal models of Crohn's disease do not always match the comparable analysis in humans with the disease (Kim et al., 2008; Maeda et al., 2005; Noguchi et al., 2009; Watanabe et al., 2008).

A unique feature of NOD2 is its tripartite structure consisting of two N-terminal CARD (caspase activation and recruitment domain) motifs, a central nucleotide oligomerization domain and a C-terminal leucine-rich repeat domain. As CARD domains are known to promote protein–protein interactions, a full description of NOD2-binding partners will elucidate the intracellular pathways where NOD2 is playing a contributory role, as well as possibly

Abbreviations: CARD, caspase activation and recruitment domain; CBP, calmodulin binding peptide; MDP, muramyl dipeptide; NLRs, non-like receptors; NOD2, nucleotide-binding and oligomerization domain-2; OAS, oligoadenylate synthetases; OAS2, 2′-5′-oligoadenylate synthetase type 2; poly(I:C), polyinosinic acid:polycytidylic acid; RLRs, RigI-like receptors; SBP, streptavidin binding peptide; TLRs, Toll-like receptors; VC, vector control.

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leading to new insights of disease pathogenesis. Here we report the identification of a novel interaction between NOD2 and 2′-5′-oligoadenylate synthetase type 2 (OAS2). The observation was made by a proteomics analysis of the human monocyte line, THP-1.

Viral infections activate innate immunity by triggering type I interferons. One group of interferon-induced antiviral proteins is the 2′-5′-oligoadenylate synthetases (OAS) (Hovanessian, 2007). In humans, there are 4 OAS genes, with OAS2 having the highest level of induction by interferons (Sanda et al., 2006). OAS enzymes are inactive following their induction by interferons. Their enzymatic activity is turned on when they bind to non-self, viral double-stranded (ds) RNA. OAS–dsRNA complexes have the ability to convert ATP into PP_i and 2′-5′ linked oligomers (2-5A) ranging from dimers to 30-mers (Sarkar et al., 1999). 2-5A oligomers then bind to and activate RNase-L, an endoribonuclease, triggering the so-called OAS-[2-5A]-RNase-L pathway. While OAS deficient mice have not been reported, mice deficient in RNase-L produce less IFN-β in response to viral infection compared to wild-type mice, indicating the importance of the OAS-[2-5A]-RNase-L pathway in host defense (Malathi et al., 2007). It has recently been shown that in addition to cleaving viral RNA, the majority of cleavage products generated by active RNase-L come from self-RNA (Malathi et al., 2007). In fact, self-rRNA is partially degraded when the OAS-[2-5A]-RNase-L pathway is activated, leading to a discrete banding pattern detectable by northern blotting using rRNA-specific probes (Silverman et al., 1983). This serves as an assay for OAS activation. Herein we show that over-expression of NOD2 enhances rRNA degradation in response to polyinosinic acid:polycytidylic acid [poly(I:C)], used as a mimic of viral dsRNA.

2. Materials and methods

2.1. Materials

Streptavidin- and calmodulin-sepharose beads were purchased from GE HealthCare (Piscataway, NJ). Protein G-Sepharose beads and PVDF membranes (Invitrolon) were purchased from Invitrogen (Carlsbad, CA). Complete EDTA-free protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Polyinosinic acid:polycytidylic acid [poly(I:C)] was purchased from Sigma–Aldrich (St. Louis, MO). Monoclonal antibody H4 (specific for IκBα), monoclonal antibody C2 (specific for actin), monoclonal antibody 9E10 (specific for the myc epitope tag), monoclonal antibody 56/3 (specific for human OAS2), and monoclonal antibody 2D9 (specific for human NOD2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) (H4 and C2), Sigma–Aldrich, R&D Systems (Minneapolis, MN) and Novus Biologicals (Littleton, CO), respectively. A polyclonal antibody specific for human NOD2 was purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Plasmids

RT-PCR was used to generate cDNA for the NOD2 gene from human peripheral blood mononuclear cells. The cDNA was subcloned into pcDNA4HisMax (Invitrogen) and sequenced to confirm that it matched the human NOD2 sequence previously reported. Using a commercial kit purchased from Promega (Madison, WI), site-directed mutagenesis was performed to create a common mutation found in Blau syndrome, NOD2-R334Q. Wild type NOD2 and NOD2-R334Q were subcloned into pNTAP-B (Stratagene, LaJolla, CA), a plasmid allowing calmodulin binding peptide (CBP) and streptavidin binding peptide (SBP) to be placed in frame at the amino-terminus of NOD2. In order to generate stable human cell lines expressing CBP–SBP-NOD2, the construct was removed from the pNTAP-B vector and subcloned into pGCY, a retroviral expression vector containing an internal ribosome entry site and the gene for yellow fluorescent protein (YFP) (Costa et al., 2000). Recombinant pGCY was packaged into amphotropic (Phoenix-A) packaging cells using a standard calcium phosphate precipitation protocol that yielded high titer retroviral stocks. THP-1 cells were stably transduced with retrovirus containing CBP–SBP-NOD2, CBP–SBP-NOD2-R334Q and CBP–SBP without an insert (an empty vector control). Using the YFP reporter, cells were cloned by cell sorting and clones expressing high levels of YFP were established from single cells for use in all studies.

To generate vector pmycOAS2p69, the human OAS2 coding sequence was amplified from plasmid pSPORT6hOAS2 (MGC51939, Invitrogen), using primers 5′-AATCTCGAATTCGGATGGGAAATGGGGAGTCCCA-3′ and 5′-TCTGGCGAATTCGCTTAGATGACTTTTACCGGCA-3′. The approximately 2000 bp PCR product was digested with EcoRI and inserted in frame with the myc tag sequence of pCMV-Myc (BD Biosciences, San Jose, CA), using the EcoRI site of the vector multiple cloning site. Insert orientation was determined by restriction analysis. Vector pUNO-NOD2, for mammalian expression of the untagged, native human NOD2 gene, was purchased from Invivogen (San Diego, CA).

2.3. Cell culture

The human embryonic kidney cell line HEK293 and the human acute monocytic leukemia cells THP-1, a human cultured monocytic cell line displaying properties of isolated human peripheral monocytes were purchased from the American Tissue Culture Collection (ATCC) (Manassas, VA). HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), at 37 °C in 5% CO_2. THP-1 cells were cultured in RPMI medium 1640 supplemented with 24 mM $NaHCO_3$, 10% FBS and 1% P/S, at 37 °C in 5% CO_2.

2.4. Tandem affinity purification

THP-1 cells were stably transduced with retroviral plasmid pTAP-NOD2, or with the empty pTAP vector as control. Exponentially growing cells were lysed in immunoprecipitation lysis buffer (150LYB: 50 mM Tris/HCl pH 7.5, 150 mM NaCl, 20% glycerol, 0.1% NP-40, 5 mM NaF, 1 mM Na_3VO_4 and a complete, EDTA-free protease inhibitor cocktail tablet diluted as per the manufacturer's (Roche) instructions). Final protein concentrations in the crude lysates ranged from 2 to 5 μg/μl. For tandem purification of TAP-NOD2 and associated proteins, 5–10 ml of cell lysate was mixed with 200 μl of streptavidin-sepharose beads, and incubated at 4 °C, with shaking, for 60 min. Then, beads were pelleted by centrifugation and washed extensively with 150LYB. TAP-NOD2 and associated proteins were eluted with 150LYB containing 1 mM biotin. The eluates were supplemented with $CaCl_2$ to a final concentration of 1 mM, mixed with 200 μl of calmodulin-sepharose beads, and then incubated at 4 °C, with shaking, for 60 min. Beads were again pelleted by centrifugation and washed extensively with 150LYB supplemented with 1 mM $CaCl_2$. TAP-NOD2 and associated proteins were eluted with 1 ml 150LYB supplemented with 2 mM EGTA, precipitated with 10% TCA and washed with acetone. The washed TCA pellets were subjected to proteomic analysis.

For copurification of myc-tagged human OAS2 protein with TAP-NOD2, 1 ml of 150LYB crude lysate of cells expressing TAP-NOD2 and myc-tagged OAS2 plasmids was bound to 25 μl of streptavidin-sepharose as before, and TAP-NOD2 and associated proteins were eluted with 30 μl 150LYB containing 1 mM biotin. Protein complexes were then analyzed by western immunoblotting using antibodies specific for NOD2, the myc epitope tag and actin.