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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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Keywords

nucleotide-binding and oligomerization domain 2; 2'-5'-oligoadenylate synthetase type 2; innate immunity; RNase-L; proteomics

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- κ B 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 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 I κ B α), 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 HEK 293 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). HEK 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), at 37°C in 5% CO₂. THP-1 cells were cultured in RPMI medium 1640 supplemented with 24 mM NaHCO₃, 10% FBS and 1% P/S, at 37°C in 5% CO₂.

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 Na₃VO₄ 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 to 10 ml of cell lysate was mixed with 200 µl of streptavidin-Sepharose beads, and incubated at 4°C, with shaking, for 60 minutes. 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 CaCl₂ 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 minutes. Beads were again pelleted by centrifugation and washed extensively with 150LYB supplemented with 1 mM CaCl₂. 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.

2.5. Mass-spectroscopy identification of purified proteins

Proteins extracted after tandem purification were isolated and identified by the Proteomics Shared Resource laboratory at Oregon Health & Science University. TCA precipitated and acetone washed protein pellets were resolubilized in 10 µl of 8 M urea, 1.0 M Tris-HCl, pH 8.5, 8 mM CaCl₂ and 0.2 M methylamine. One µl of 0.2 M dithioerythritol was added, and the samples incubated at 50°C for 15 min. One µl of 0.5 M iodoacetamide was then added, the samples incubated at room temperature for 15 min, an additional 2 µl 0.2 M dithioerythritol solution added, and the sample incubated for an additional 15 min. Sixteen µl of water was next added, followed by 10 µl of 0.1 mg/ml modified sequencing grade trypsin dissolved in 1 mM HCl (Trypsin Gold, ProMega). Following an overnight incubation at 37°C, 2 µl of formic acid was added to the digests and peptides were analyzed by LC-MS/MS using an Agilent 1100 series capillary LC system (Agilent Technologies Inc, Santa Clara, CA) and an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Electrospray ionization was performed with an ion max source fitted with a 34-gauge metal needle (Thermo Fisher Scientific, cat. no. 97144-20040) and 2.7 kV source voltage without sheath gas. The entire digest of each sample was applied at 20 µL/min to a trap cartridge (Michrom BioResources, Inc, Auburn, CA), and after 5 min the flow diverted to a 0.5 × 250 mm Zorbax SB-C18 column (Agilent Technologies) using a mobile phase containing 0.1% formic acid, 7–30% acetonitrile gradient over 95 min, and 10 µL/min flow rate. Data-dependent collection of MS/MS spectra used the dynamic exclusion feature of the instrument's control software (repeat count equal to 1, exclusion list size of 50, exclusion duration of 30 sec, and exclusion mass width of -1 to +4) to obtain MS/MS spectra of the three most abundant parent ions following each survey scan from m/z 400–2000. The tune file was configured with no averaging of microscans, a maximum inject time of 200 msec, and AGC targets of 3 × 10⁴ in MS mode and 1 × 10⁴ in Msn mode. Peptides were identified by comparing the observed MS/MS spectra to theoretical MS/MS spectra of peptides generated from a protein database using the program Sequest (Version 27, rev. 12, Thermo Fisher Scientific). DTA files were created with BioWorks 3.2 (Thermo Fisher Scientific) with a molecular weight range of 550 to 4000, an absolute threshold of 500, group scan setting of 1, and a minimum of 25 ions. In the Sequest searches, parent ion

and fragment ion mass tolerances were 3.8 and 1.0 Da, respectively, monoisotopic masses were selected, y- and b-ion series were used in the scoring, and a static modification of 57.02 was used due to alkylation of cysteine residues. A human only version of the Swiss-Prot database (Swiss Institute of Bioinformatics, Geneva, Switzerland) was used containing 13,748 entries. Lists of identified proteins were assembled using the program Scaffold (version 01_07_00, Proteome Software, Portland, OR). Thresholds for peptide and protein probabilities were set in Scaffold at 90 and 95%, respectively, as specified using the Peptide Prophet algorithm (Keller et al., 2002) and the Protein Prophet algorithm (Nesvizhskii et al., 2003) respectively, and requiring a minimum of 2 peptides matched to each protein entry. Differences in numbers of assigned spectra to each protein were used to assess the relative abundance differences of individual proteins in each experimental group.

2.6. Immunoprecipitation

Cells were lysed in 150LYB, supplemented with protease and phosphatase inhibitors, to a final protein concentration of 2–5 µg/µl. Up to 1 ml of lysate was mixed with 500 ng of antibody, and incubated overnight at 4°C. Then, the antibody-immunoprecipitated protein complexes were collected using 20 µl of protein G-Sepharose beads. After 30 minutes of incubation with protein G-Sepharose at 4°C with shaking, antibody-protein complexes were precipitated by centrifugation. After extensive washing with 150LYB, protein G-sepharose beads were resuspended in 30 µl 150LYB, and 10 µl 4X SDS-PAGE sample buffer (240 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 10% β-mercaptoethanol and 0.01% bromophenol blue). Samples were heated at 90°C for 4 minutes; then immunoprecipitated proteins were separated by SDS-PAGE, transferred to PVDF membranes, and identified by immunoblotting with specific antibodies

2.7. 2'-5'-oligoadenylate synthetase type 2 enzymatic assay

Cell lysates were mixed with human OAS2-specific antibody 56/3, or with human IkBα-specific monoclonal antibody H4, as a control, and protein-antibody complexes were collected with protein G-Sepharose. The immunoprecipitates were washed and resuspended in reaction buffer containing 20 mM HEPES, pH 6.9, 25 mM magnesium acetate, 50 mM KCl and 7 mM β-mercaptoethanol. The resuspended immunoprecipitates were split in two, and either poly (I:C) (50 µg/ml) or solvent control was added to each half. Reactions were started by addition of ATP (500 µM) and 10 µCi of [³²P]-α-ATP, followed by incubation at 30 °C overnight. Reaction products were separated by electrophoresis through 8M urea/15% polyacrylamide gels and visualized by autoradiography. For quantification, reaction products were loaded onto Bio-Gel P6 columns (Bio-Rad Laboratories, Hercules, CA), and radioactivity present in the flow-through quantified by scintillation counting.

2.8 Northern blot analysis

Northern blots for detecting cleaved products of 18S rRNA were performed using a digoxigenin-labeled(Roche) 18S specific probe (5'-GGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGT- 3'). After incubation with the probe, blots were developed with anti-digoxigenin antibody conjugated to alkaline phosphatase and a chemiluminescent protocol (Roche). Statistical analysis of densitometry (Scion Image, Frederick, MD) was performed by Student's *t* test using the statistics function in Excel.

3. Results

3.1 Identification of NOD2-OAS2 association by proteomics

THP-1 cells were stably transduced with a YFP-expressing retrovirus containing calmodulin binding peptide (CBP)-streptavidin binding peptide (SBP)-NOD2, CBP-SBP-NOD2-R334Q

and CBP-SBP without an insert (an empty vector control). Single cells expressing YFP were isolated by fluorescence-activated cell sorting and cultured. A clone representative of each construct, selected on the basis of high YFP expression, became the starting material for the proteomics analysis. Western blotting confirmed the presence of NOD2 in the NOD2 and NOD2-R334Q transfectants, which is in contrast to the vector control, that contained no detectable NOD2 (Fig. 1).

To identify proteins interacting with NOD2, we used tandem affinity purification and mass spectroscopic identification of purified proteins. CBP and SBP tagged-NOD2 allowed for sequential purification of NOD2 under conditions that allowed interactions occurring *in vivo* to remain intact yet eliminate non-specific interactions. The streptavidin binding peptide allowed for a first purification step using streptavidin-agarose and biotin elution. The calmodulin binding peptide allowed for a second purification step on calmodulin-Sepharose, followed by elution with the calcium chelator EGTA. The resulting mixture of NOD2 and associated proteins was digested with trypsin, fractionated by HPLC and subjected to analysis by mass spectrometry.

Five independent experiments were performed, starting with cultured cells and concluding with analysis of peptide spectra. As would be expected, the dominant spectra in all experiments was NOD2 itself. In all 5 independent experiments, we detected the presence of erbin (also referred to as LAP2) as the most dominant binding partner. Erbin is a known NOD2-binding partner previously detected using proteomics on HEK cells (McDonald et al., 2005) and by a yeast two-hybrid approach using a cDNA library from activated human leukocytes (Kufer et al., 2006). In addition to erbin, we detected several previously unreported binding partners; the characterization of one of these, OAS2, is described here. OAS2 bound in comparable amounts to both the wild type and R334Q form of NOD2. We did not find any examples of proteins uniquely binding to R334Q-NOD2, nor cases where proteins bound to wild type NOD2 failed to bind to R334Q-NOD2.

3.2 Confirmation of the NOD2-OAS2 interaction by NOD2 pull down

HEK293 cells were stably transduced to express CBP-SBP-NOD2 or the CBP-SBP construct without an insert (vector control). These cells were transiently transfected with an expression plasmid containing myc-tagged OAS2 (p69 isoform). Analysis of cell lysates by immunoblotting confirmed the appropriate expression of CBP-SBP-NOD2 and OAS2 (Fig. 2A). CBP-SBP was then precipitated from the lysates with streptavidin-Sepharose beads and immunoblotted with anti-NOD2 and anti-myc. As shown in Fig. 2B, myc-OAS2 co-fractionated NOD2 as expected for an OAS2-NOD2 complex. These same experiments have been repeated with NOD2 constructs lacking CBP and SBP using antibodies to NOD2 for immunoprecipitation with similar results (not shown) confirming that the CBP-SBP was not responsible for the interaction.

3.3 Confirmation of the NOD2-OAS2 interaction by OAS2 pull down

To further confirm the specificity of NOD2 and OAS2 interaction, we studied whether NOD2 could be detected in immunoprecipitates of endogenous OAS2. The anti-OAS2 monoclonal antibody 56/3 is reported to be effective for immunoprecipitation (IP). We verified the reactivity of 56/3 in OAS2-transfected cells by preparing immunoprecipitates and measuring OAS2 activity in the immunoprecipitates. Activated OAS2 catalyzes the formation of oligomers of ATP that can be monitored using [α - 32 P]ATP, polyacrylamide gel electrophoresis and autoradiography (Sanchez and Mohr, 2007). HEK293 cells were transiently transfected with pOAS2, lysates were immunoprecipitated with anti-OAS2 (56/3) or an isotype control monoclonal antibody (anti-IkB α), and precipitates were compared for the ability to incorporate ATP into high molecular weight oligomers (Fig. 3A). As shown in Fig. 3A, 2'-5'-

oligoadenylate synthetase activity was immunoprecipitated specifically with the 56/3 antibody. Note in Fig. 3A that poly(I:C), a critical co-factor for OAS2, must be added to the immunoprecipitates to activate OAS2. Confirming that we had a reagent that immunoprecipitated OAS2, we asked if NOD2 could be co-precipitated with endogenous OAS2 in THP-1 cells. Using THP-1 cells stably transduced with NOD2 and THP-1 cells transduced with an empty retroviral control, we found that NOD2 was readily detectable in complexes immunoprecipitated with anti-OAS2 (56/3) (Fig. 3B, **lane 5**) whereas only a faint band was seen in control (anti-I κ B α) immunoprecipitates (Fig. 3B, **lane 6**). As another specificity control, the same samples were immunoblotted with an antibody specific for β -actin. Trace levels of β -actin-immunoreactivity could be detected in the immunoprecipitates; these had an intensity similar to that of the NOD2-immunoreactivity in the anti-I κ B α control (lane 6) but well below the intensity seen of NOD2-immunoreactivity in anti-OAS2 immunoprecipitates (lane 5).

3.4 Over-expression of NOD2 increases RNase-L activity

In order to determine if the observed interaction between NOD2 and OAS2 had functional significance, we compared the activity of OAS in THP-1 cells stably transduced with NOD2 with that in cells transduced with vector control. Cells stimulated with poly(I:C), which is a mimic of double-stranded RNA virus infection. Total RNA was then isolated and analyzed for rRNA cleavage products indicative of RNase-L activation by northern blot analysis using a probe specific for 18S rRNA. As shown in Fig. 4, poly(I:C) triggered the expected cleavage of rRNA in the vector control (VC) cells treated with poly(I:C). Note that the amount of cleavage products increased significantly in cells over-expressing NOD2 (right hand lane, Fig. 4). In 4 independent experiments densitometry measurements for the larger RNA cleavage product showed an average increase of 2.2-fold (standard deviation ± 0.45) ($p = 0.008$) and the lower cleavage product showed a 3.52-fold increase (± 0.71) ($p = 0.03$) in NOD2-expressing cells compared to vector control.

4. Discussion

The data presented here add to our understanding of the numerous proteins that have been identified that bind to NOD2. Ipaf/CLAN, CENTB1, TAK1 and erbin have been shown to physically interact with NOD2 and can downregulate NF- κ B activation (Chen et al., 2004; Damiano et al., 2004; Kufer et al., 2006; McDonald et al., 2005; Yamamoto-Furusho et al., 2006). GRIM19 interacts with NOD2 and is required for activation of NF- κ B (Barnich et al., 2005b). NF- κ B-inducing kinase (NIK) can also physically associate with NOD2, and in a setting where NOD2 and TLR4 are both activated, promote induction of a B lymphocyte chemoattractant (Pan et al., 2006). HSP90 and SGT1 can bind to NOD2 and stabilize protein levels (Mayor et al., 2007). Several recent reports provide evidence for a direct interaction between NOD2 and caspase-1, and between NOD2 and NALP1, leading to IL-1 β secretion in response to MDP (Hsu et al., 2008; Maeda et al., 2005; Pan et al., 2007); however, there are also studies claiming no direct role for NOD2 in MDP-induced caspase-1 activation (Marina-Garcia et al., 2008). Most recently, an association between NOD2 and angio-associated migratory cell protein was identified (Bielig et al., 2009). The varied effects of NOD2 activation in different cells is most likely related to the numerous levels of regulation of NOD2 function by these various binding partners.

Our work also adds to the understanding of the many positions within the cell where different pathways connected to innate immune receptors intersect, leading to enhanced or suppressed function. It is well established that TLRs and NLRs synergize in their responses to invading pathogens (Fritz and Girardin, 2005). TLRs and NLRs also share many intracellular signaling molecules. Protein ubiquitination enzymes and deubiquitinating enzymes are critical in both

activating and restricting signaling through NOD2 (Abbott et al., 2007; Bertrand et al., 2009; Hitotsumatsu et al., 2008; LeBlanc et al., 2008). MDP-activated NOD2 interacts with the scaffolding protein kinase receptor-interacting protein 2 (RIP2, also known as RICK), through homotypic CARD interactions (Ogura et al., 2001b). RIP2 then recruits cIAP1 and cIAP2 (E3 ubiquitin ligases) which triggers RIP2 ubiquitination, followed by ubiquitination of IKK γ (NEMO), a process aided by either scaffolding or ubiquitinating properties of TRAF6 (Abbott et al., 2007; Bertrand et al., 2009). Ubiquitinated IKK γ recruits the TAK1/TAB complex (which leads to activation of TAK1/TAB kinase activity by further ubiquitin-dependent events) allowing TAK1 to phosphorylate IKK α and β (Abbott et al., 2007). Active IKK phosphorylates I κ Bs leading to their polyubiquitination and degradation and subsequent translocation of NF- κ B to the nucleus. MDP-induced NF- κ B signaling can be down-regulated by A20, a ubiquitin-modifying enzyme that functions by deubiquitinating RIP2 (Hitotsumatsu et al., 2008) and by caspase-12, which binds to RIP2 and displaces TRAF6 from the signaling complex (LeBlanc et al., 2008). These signaling interactions occur on the cytoplasmic side of the plasma membrane (Barnich et al., 2005a), and erbin, β -PIX and Rac1 all play a role in membrane recruitment and regulation of NOD2 (Eitel et al., 2008). NOD2-RIP2 can also interact with another CARD-containing protein, CARD9, which activates the MAP kinases (p38, ERK and JNK) via phosphorylation (Hsu et al., 2007). Activation of NF- κ B and MAPK pathways triggers cytokine release and activation of antimicrobial peptides (Kanneganti et al., 2007).

While OAS2 is known to bind viral double-stranded RNA, which in turn activates its enzymatic activity, to date proteins capable of interacting with OAS2 have not been reported. OAS1 associates with the prolactin receptor resulting in the inhibition of downstream signaling pathways (McAveney et al., 2000). OASL has been shown to interact with methyl CpG-binding protein 1 and both genes were upregulated in interferon-stimulated cells (Andersen et al., 2004). Non-protein interactions have been described for OAS1 as mRNA molecules for Raf kinase inhibitor protein and p15(rC)-binding protein 2 from prostate cancer cell lines can bind to and activate OAS1 (Molinaro et al., 2006).

Numerous studies have shown the role of NLR proteins in activation of innate immune response to bacterial infections and an extensive literature exists on the recognition of viral PAMPs by TLR (3, 7 and 8) and RLR (RIG-I and MDA-5). In contrast, much less is known about innate responses to viral infection that involve NLR proteins, as shown for the role of NLRP3 in the recognition of influenza A (Allen et al., 2009). The results presented here are the first evidence for cross talk between NOD2 and components of the RLR pathway. In addition to detecting a physical association between NOD2 and OAS2, we show that over-expression of NOD2 leads to enhanced rRNA degradation, a readout for activation of the OAS-[2-5A]-RNase-L pathway. Whereas the biologic significance of this observation is under further investigation, both with regards to how OAS2 could alter known functions of NOD2 and how NOD2 could influence OAS2 function in the setting of viral and bacterial infections, it has recently been recognized that the RNase-L pathway plays a role in host responses to bacteria. Mice deficient in RNase-L show increased mortality compared to wild type mice in response to challenge with bacteria and showed reduced expression of proteins involved in clearance of intracellular bacterial infection (Li et al., 2008). One possible role for the NOD2-OAS2 association reported here is to further activate RNase-L in the setting of bacterial infection, leading to upregulation of genes such as cathespin E, which is important in clearing phagocytosed bacteria.

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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

Figure 1.

Transduced THP-1 cells over-express NOD2. Lysates from THP-1 cells stably transduced with CBP-SBP vector control (lane 1), CBP-SBP-NOD2 (lane 2) and CBP-SBP-NOD2-R334Q (lane 3) analyzed by immunoblotting with anti-NOD2 antisera.

Figure 2.

OAS2 is detected in NOD2 precipitates. HEK-293 cells were stably transduced with retroviral constructs for CBP-SBP-NOD2 or the empty CBP-SBP construct serving as a vector control. HEK cells were then transiently transfected with a plasmid expressing myc-tagged OAS2. NOD2 and OAS2 status is indicated above each lane. **A)** The expression of NOD2 and OAS2 in lysates of transfected cells was determined by immunoblotting with anti-NOD2 (polyclonal from Cayman) and anti-myc (9E10, Sigma-Aldrich). As a loading control, the membrane was immunoblotted with a β -actin antibody. Lysates from untransduced and untransfected HEK cells were included as a reference. **B)** Precipitates were prepared by addition of streptavidin-Sepharose beads, and bound proteins were then identified by immunoblotting with NOD2- and myc-specific antibodies (shown adjacent to each panel).

Figure 3.

NOD2 co-immunoprecipitates with endogenous OAS2. A) Lysates from HEK293 cells transfected with myc-tagged human OAS2 were immunoprecipitated with 56/3 (left two lanes) or with control monoclonal antibody (anti-IκBα) (right two lanes) and assayed for OAS2 activity in the absence or presence of poly I:C (50 μg/ml) (indicated at bottom of each lane) and [α - 32 P]ATP. Reaction products were separated by electrophoresis and detected by autoradiography. The location of 2'-5'-oligoadenylates containing 2, 3, 4,...etc adenylate units are indicated by arrows. B) THP1 cells were stably transduced with empty retroviral vector (V) or vector containing wild type NOD2 (N) (indicated above each lane). Lysates immunoblotted with anti-NOD2 or anti-β-actin confirmed appropriate expression of transduced NOD2 (lanes 1 and 2). Lysates were then immunoprecipitated with anti-OAS2 (56/3) (lanes 3 and 5) or control antibody anti-IκBα (lanes 4 and 6), electrophoresed and immunoblotted with anti-NOD2 or anti-β-actin.

Figure 4.

Overexpression of NOD2 enhances RNase-L function in response to poly(I:C). THP-1 cells stably transduced with empty vector control (VC) or NOD2 were then treated for 3 hours with poly(I:C) (25 μ g/ml) in Mirus TKO transfection reagent. Total RNA was isolated and northern blot analysis was performed with an oligonucleotide probe specific for 18S rRNA.