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ATP binding by NLRP7 is required for inflammasome activation in response to bacterial lipopeptides

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ABSTRACT

Nucleotide-binding oligimerization domain (NOD)-like receptors (NLRs) are pattern recognition receptors (PRRs) involved in innate immune responses. NLRs encode a central nucleotide-binding domain (NBD) consisting of the NAIP, CIITA, HET-E and TP1 (NACHT) domain and the NACHT associated domain (NAD), which facilitates receptor oligomerization and downstream inflammasome signaling. The NBD contains highly conserved regions, known as Walker motifs, that are required for nucleotide binding and hydrolysis. The NLR containing a PYRIN domain (PYD) 7 (NLRP7) has been recently shown to assemble an ASC and caspase-1-containing high molecular weight inflammasome complex in response to microbial acylated lipopeptides and *Staphylococcus aureus* infection. However, the molecular mechanism responsible for NLRP7 inflammasome activation is still elusive. Here we demonstrate that the NBD of NLRP7 is an ATP binding domain and has ATPase activity. We further show that an intact nucleotide-binding Walker A motif is required for NBD-mediated nucleotide binding and hydrolysis, oligomerization, and NLRP7 inflammasome formation and activity. Accordingly, THP-1 cells expressing a mutated Walker A motif display defective NLRP7 inflammasome activation, interleukin (IL)-1β release and pyroptosis in response to acylated lipopeptides and *S. aureus* infection. Taken together, our results provide novel insights into the mechanism of NLRP7 inflammasome assembly.

1. Introduction

The intracellular nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) gene superfamily represents evolutionary-conserved innate immune pattern recognition receptors (PRRs). They detect pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs, respectively), and have been determined to trigger diverse pro-inflammatory host defense signaling pathways that contribute to pathogen clearance and wound healing, including inflammasome activation, activation of NF-κB and MAPK, and transcriptional control of MHC and related genes. NLRs are composed of a characteristic tripartite domain architecture featuring C-terminal ligand sensing and auto-regulatory leucine-rich repeats (LRRs), a central nucleotide-binding domain (NBD) comprised of the NACHT (NAIP, CIITA, HET-E and TP1) domain and NACHT-associated domain (NAD), and a variable N-terminal effector domain responsible for homo-typic protein–protein interactions, which enable downstream signaling. The 22 human members are sub-classified according to their effector domain as NLRA (containing an activation domain, AD), NLRB (containing a baculovirus inhibitor of apoptosis domain, BIR), NLRCs (containing a caspase activation and recruitment domain, CARD), NLRX (containing an X domain) and NLRPs (containing a PYRIN domain, PYD).

Abbreviations: acLP, acetylated lipopeptides; ASC, apoptosis-associated speck-like protein containing a CARD; ATPase, adenosine triphosphatases; CARD, caspase recruitment domain; DAMP, damage associated molecular pattern; FLICA, fluorescent labeled inhibitor of caspase-1 assay; HEK293, human embryonic kidney 293 cells; HKAL, heat killed *Acholeplasma laidlawii*; HM, hydatidiform moles; IL-1β, interleukin-1β; LRR, leucine rich repeat; NACHT, NAIP, CIITAHET-E and TP1; NAD, NACHT associated domain; NBD, nucleotide binding domain; NLR, nucleotide binding oligomerization domain (NOD)-like receptor; NLRP7, NLR containing a PYD domain 7; PAMP, pathogen associated molecular pattern; PI, Propidium iodide; PRR, pattern recognition receptor; PYD, PYRIN domain; *S. aureus*, *Staphylococcus aureus*; WA, Walker A.

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Several NLRs assemble inflammasomes, which are large, multi-protein signaling complexes consisting of NLRs, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1 (Martinon et al., 2002; Ratsimandresey et al., 2013; Schroder and Tschopp, 2010; Khare et al., 2010). Infection and tissue damage, as well as pathogen-, environmental- or host-derived danger signals trigger inflammasome formation, which subsequently leads to caspase-1 activation. Active caspase-1 is required for the proteolytic maturation and release of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (Kostura et al., 1989; Thornberry et al., 1992; Fantuzzi et al., 1999; Ghayur et al., 1997; Kuida et al., 1995; Li et al., 1995), induction of pyroptosis (Fink and Cookson, 2006) and the release of alarmins and danger signals, including IL-1α, HMGB1 and inflammasome particles (Baroja-Mazo et al., 2014; Franklin et al., 2014; Groß et al., 2012; Lamkanfi et al., 2010; Willingham et al., 2009). The mechanism for inflammasome assembly involves ASC nucleation and the subsequent self-perpetuating polymerization of ASC (Cai et al., 2014; Lu et al., 2014; Man et al., 2014; Chu et al., 2015). Consequently polymerized ASC nucleates caspase-1 polymerization, which results in its activation (Stehlik et al., 2003a,b; Srinivasula et al., 2002). ASC nucleation is facilitated by NLRs and their ASC nucleation efficiency is enhanced upon NBD-dependent NLR oligomerization (Lu et al., 2014). NLRs belong to the signal transduction adenosine triphosphatases (ATPases) with numerous domains (STAND) subfamily within the ATPases associated with various cellular activities (AAA+) superfamily (Leipe et al., 2004; MacDonald et al., 2013). Since the 3D structure of a prototypical NACHT-NAD domain has not been resolved yet, it has only been characterized *in silico* by multiple sequence homology alignments, secondary structure prediction analyses as well as homology modeling (MacDonald et al., 2013; Proell et al., 2008). The overall secondary structure is conserved between the NACHT-NAD domain and the NB-ARC domain of the apoptotic ATPase apoptotic peptidase activating factor 1 (APAF-1), another STAND family member (Reubold et al., 2011; Riedl et al., 2005). The NACHT domain consists of two distinct conserved regions. (1) The Walker A motif [GxxxxGK(S/T)], which forms the integral phosphate-binding loop (P-loop) and coordinates the β and γ phosphates of the nucleotide during hydrolysis (Eibl et al., 2012; MacDonald et al., 2013). Particularly, the highly conserved lysine residue is responsible for the coordination of the γ-phosphate (Traut, 1994). (2) The Walker B motif [(R/K)xxxxGxxxxLhhhhD], which is situated downstream of the Walker A motif in most P-loop proteins, is involved in the coordination of Mg^{2+} ions and contributes to ATP binding and ATPase catalytic activity (Iyer et al., 2004). The NAD is a C-terminal extension of the NACHT domain and shares significant homology with domains of APAF1 and contains a particular proline residue that is proposed to interact with the adenine ring of bound ATP (MacDonald et al., 2013). STAND proteins function as molecular switches, with the "OFF" position corresponding to a monomeric, resting ADP-bound form and the "ON" position corresponding to an ATP-bound, oligomeric form, competent of downstream signaling through the effector domains. Experimentally this model has been confirmed for NLRC4, where the LRR sterically blocks the NBD and sequesters NLRC4 in a monomeric state (Danot et al., 2009; Hu et al., 2013). Binding of an inducer to the sensor domain promotes ADP/ATP exchange and induces a conformational change resulting in protein activation by removing intramolecular inhibitory interactions, and hence facilitating oligomerization (Danot et al., 2009; Hu et al., 2013). Evidence for this nucleotide-dependent oligomerization model has been observed in several NLRPs. NLRP1 binds ATP, GTP, CTP, TTP and UTP to form a caspase-1-activating complex *in vitro* (Faustin et al., 2007), NLRP12 requires ATP binding activity in order to elicit its anti-inflammatory role (Ye et al., 2008) and NLRP3 inflammasome activation also depends on ATP binding and ATPase activity (Duncan et al., 2007). If the Walker A motif is experimentally mutated, these NLRs lose their function and even hereditary gain-of-function mutations in NLRP3, which cause Cryopyrinopathies, depend on nucleotide binding and lose their activity as a consequence of mutation of the Walker A motif (Duncan et al., 2007). However, Walker A mutations in NLRP1B result in an over-active protein, suggesting that mechanistic differences exist within the NLR family NBDs (Faustin et al., 2007).

NLRP7 belongs to the NLRP subfamily and forms an ASC and caspase-1-containing inflammasome in human macrophages in response to microbial acylated lipopeptides (acLP) and bacterial infection with *Staphylococcus aureus* and *Listeria monocytogenes* (Khare et al., 2012; Radian et al., 2013). Accordingly, NLRP7 is present in high molecular weight complexes following *S. aureus* infection (Khare et al., 2012). It has also been reported to co-localize with the Golgi and microtubules and inhibit IL-1β (Messaed et al., 2011a,b; Kinoshita et al., 2005). NLRP7 contains a predicted NBD domain and has recently been shown to self-associate through its NAD domain (Singer et al., 2014). However, it is currently unknown, if the NLRP7 NBD has ATPase activity and if this is involved in inflammasome activation.

In this study, we demonstrate for the first time that the NBD of NLRP7 binds ATP and exhibits ATPase activity and that this activity is required for NLRP7 oligomerization. We further show that an intact Walker A motif is necessary for NLRP7 inflammasome activation by acLPs and *S. aureus*. Thus, our results provide novel insights into the activation mechanism of NLRP7 and contribute to a better understanding of NLR activation.

2. Materials and methods

2.1. Materials and cell culture

The human embryonic kidney (HEK) 293 cell line was obtained from ATCC and maintained in DMEM containing 10% FBS and 100 IU/ml penicillin, 1 mg/ml streptomycin. THP-1 cells were obtained from ATCC and maintained in RPMI 1640 media, supplemented with 10% FBS, 1 mM sodium pyruvate, 1 mM HEPES buffer, 100 IU/ml penicillin, 1 mg/ml streptomycin, 2 mM glutamine, and 0.05 mM 2-mercaptoethanol. Cells were routinely tested for Mycoplasma contamination by PCR and TLR2 activation assay (Invivogen). Recombinant lentivirus was produced in HEK293-lenti cells (Clontech) by Xfect-based transfection (Clontech) with pLEX expression plasmids encoding myc-NLRP7 or myc-$NLRP7^{WA}$ and the packaging plasmids pMD.2G and psPAX2 (Addgene plasmids 12259 and 12260), followed by filtration (0.45 μm). THP-1 cells were stably transduced with lentiviral particles in the presence of polybrene (0.5 μg/ml) using magnetic beads (ExpressMag, Sigma) or spinoculation and selected with Puromycin (0.5 μg/ml) for 2 weeks. Expression of NLRP7 and $NLRP7^{WA}$ was verified by immunoblot. Cells were lysed in Laemmli buffer and cleared samples were separated by SDS-PAGE, transferred to PVDF membranes and analyzed by immunoblotting with anti-myc antibodies (Santa Cruz Biotechnology) and HRP-conjugated secondary antibodies (GE Healthcare), ECL detection (Pierce), and image acquisition (Ultralum). Cells were treated with FSL-1 (0.1 mg/ml), Pam3CSK4 (2 mg/ml), or HKAL (2×10^5 cfu/ml).

2.2. Plasmids

pcDNA3-based expression constructs for ASC, NLRP3, pro-caspase-1, pro-IL-1β and NLRP7 were described earlier (Bryan et al., 2010; Dorfleutner et al., 2007a,b; Khare et al., 2012; Stehlik et al., 2003a,b). $NLRP7^{GKT183,184,185AAA}$ ($NLRP7^{WA}$) was generated by site directed mutagenesis and $NLRP7^{NBD}$