FEBS Open Bio 5 (2015) 348–356

journal homepage: www.elsevier.com/locate/febsopenbio

Oligomerized CARD16 promotes caspase-1 assembly and IL-1β processing

Tadayoshi Karasawa [a,*], Akira Kawashima [a], Fumitake Usui [a], Hiroaki Kimura [a], Koumei Shirasuna [a], Yoshiyuki Inoue [a], Takanori Komada [a], Motoi Kobayashi [a], Yoshiko Mizushina [a], Junji Sagara [b], Masafumi Takahashi [a,*]

[a] Division of Inflammation Research, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan
[b] Department of Molecular Oncology, Shinshu University Graduate School of Medicine, Nagano, Japan

ARTICLE INFO

Article history:
Received 6 March 2015
Revised 17 April 2015
Accepted 17 April 2015

Keywords:
Caspase
Cytokine
Inflammation
Interleukin

ABSTRACT

Increasing evidence indicates that caspase recruitment domain (CARD)-mediated caspase-1 (CASP1) assembly is an essential process for its activation and subsequent interleukin (IL)-1β release, leading to the initiation of inflammation. Both CARD16 and CARD17 were previously reported as inhibitory homologs of CASP1; however, their molecular function remains unclear. Here, we identified that oligomerization activity allows CARD16 to function as a CASP1 activator. We investigated the molecular characteristics of CARD16 and CARD17 in transiently transfected HeLa cells. Although both CARD16 and CARD17 interacted with CASP1CARD, only CARD16 formed a homo-oligomer. Oligomerized CARD16 formed a filament-like structure with CASP1CARD and a speck with apoptosis-associated speck-like protein containing a CARD. A filament-like structure formed by CARD16 promoted CASP1 filament assembly and IL-1β release. In contrast, CARD17 did not form a homo-oligomer or filaments and inhibited CASP1-dependent IL-1β release. Mutated $CARD16_{D27G}$, mimicking the CARD17 amino acid sequence, formed a homo-oligomer but failed to form a filament-like structure. Consequently, $CARD16_{D27G}$ weakly promoted CASP1 filament assembly and subsequent IL-1β release. These results suggest that oligomerized CARD16 promotes CARD-mediated molecular assembly and CASP1 activation.

1. Introduction

Activation of caspase-1 (CASP1) and subsequent processing of interleukin (IL)-1β are essential for initiation of the inflammatory response. In this process, large molecular complexes, known as the inflammasomes, serve as platforms for CASP1 activation [1,2]. Several types of inflammasomes have been reported and typically contain one of the NLR family proteins, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and cysteine protease CASP1. Among the inflammasomes, the NLRP3 inflammasome is the most extensively studied; it recognizes danger signals and induces the sterile inflammatory response in various diseases. NLRP3 is composed of C-terminal leucine-rich repeats (LRRs), a central nucleotide domain termed the NACHT domain, and an N-terminal effector domain [pyrin domain (PYD)] [2]. ASC contains an N-terminal PYD and a C-terminal caspase recruitment domain (CARD) [3]. CASP1 consists of a CARD and catalytic domains (p10 and p20) [4,5]. Notably, each inflammasome component possesses oligomerization activity. NLRP3 homo-oligomerizes via its NACHT domain when stimulated by danger signals and the NLRP3 PYD homotypically interacts with that of ASC after which CARD of ASC recruits and binds to CASP1. These interactions finally assemble the NLRP3 inflammasome, leading to the formation of the active CASP1 tetramer, which processes pro-IL-1β into its biologically active mature form. Interestingly, both PYD and CARD of ASC were originally identified as the

Abbreviations: ANOVA, analysis of variance; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; Bcl10, B-cell lymphoma/leukemia 10; BS^3, bis(sulfosccinimidyl)suberate; CARD, caspase recruitment domain; CARMA1, CARD-membrane-associated guanylate kinase 1; COPs, CARD-only proteins; CASP1, caspase-1; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; LPS, lipopolysaccharide; LRRs, leucine-rich repeats; NLRs, nucleotide-binding domain leucine-rich repeat containing receptors; NF-κB, nuclear factor kappa beta; PYD, pyrin domain

* Corresponding authors at: Division of Inflammation Research, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. Tel.: +81 285 58 7446; fax: +81 285 44 5365.

E-mail addresses: tdys.karasawa@jichi.ac.jp (T. Karasawa), masafumi2@jichi.ac.jp (M. Takahashi).

http://dx.doi.org/10.1016/j.fob.2015.04.011
2211-5463/

domains that oligomerize to form filament-like aggregates [3]. Although their physiological relevance remains unclear, a recent investigation revealed that both PYD and CARD of ASC and CASP1 form filaments and that the endogenous NLRP3 inflammasome forms a large filamentous complex [6].

NLRP3 inflammasomes are activated by various endogenous danger signals such as extracellular adenosine triphosphate (ATP), monosodium urate, and cholesterol crystals and induce IL-1β release and subsequent inflammatory responses that contribute to disease development [7–9]. We previously demonstrated the importance of the NLRP3 inflammasome in the pathophysiology of myocardial ischemia–reperfusion, vascular injury, abdominal aneurysm, and chronic kidney disease [10–13]. Furthermore, recent clinical studies have demonstrated the therapeutic effects of blocking IL-1 in several types of sterile inflammatory diseases [14,15].

Several CARD-only proteins (COPs) are CASP1 CARD homologs. Of these, previous investigations suggested that CARD16 (also known as pseudo-ICE), CARD17 (also known as INCA), and CARD18 (also known as Iceberg) can function as negative regulators of CASP1 activity by binding the CARD of CASP1 [16–19]. All of these COPs are located on chromosome 11q22.3 and share highly conserved amino acid sequences with CASP1 [20]. CARD16 consists of 97 amino acids and shows 92% amino acid identity with the CARD of CASP1. CARD17 also shares 81% sequence identity with the CARD of CASP1. In contrast, CARD18 is less similar to CASP1 than CARD16 and has 53% identity with the CARD of CASP1. These COPs are not present in the rodent genome but are present in the human genome, suggestive of the complexity of CASP1 regulation in humans. Although the CARD interaction is essential for the inflammasome assembly, the functional difference among these COPs remains unclear. In the present study, we investigated the molecular characteristics of CARD16 and CARD17 with respect to the recruitment and activation of CASP1.

2. Materials and methods

2.1. Plasmids

The polymerase chain reaction (PCR)-generated cDNAs encoding human CARD16, CARD17, CASP1, CASP1CARD (1–102), ASC, ASCPYD (1–100), and ASCCARD (101–195) were subcloned into the N-terminal 3 × Flag-tagged pCDNA3.1 vector or N-terminal Myc-tagged pMG2 vector. Enzymatically inactive $CASP1_{C285A}$, mutated $CARD16_{D27G}$, and mutated $CARD16_{R45C}$ were generated using the PrimeSTAR Mutagenesis Basal kit (Takara Bio, Shiga, Japan).

2.2. In silico analysis of promoter region

The promoter region of human CASP1 (2000 base pairs upstream of the initiation codon) was compared with that of CARD16, CARD17, and CARD18 using a BLAST algorithm (http://blast.ncbi.nlm.nih.gov).

2.3. Isolation of human PBMCs and cell culture

HEK-293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal calf serum (FCS) and antibiotics. THP-1 cells, HL60 cells, and human PBMCs were cultured in RPMI1640 (Sigma, St Louis, MO, USA) supplemented with 10% FCS and antibiotics. PBMCs were isolated from five healthy male volunteers (25–40 years old) with Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK). This study protocol was approved by the ethical committee of Jichi Medical University and all subjects provided written informed consent. THP-1 macrophages were differentiated with 200 nM PMA for 24 h. Transfection was performed using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) according to manufacturer's protocol or PEI MAX (Polysciences, Warrington, PA, USA) as described previously [21].

2.4. Reverse transcription and real-time PCR

Total RNA was prepared using the ISOGEN system (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's instructions. Total RNA was reverse transcribed using a Super Script VILO cDNA Synthesis kit (Life Technologies). Real-time PCR were performed using SYBR Premix Ex Taq II (Takara Bio). The primers used in the assay were as follows: CASP1 (forward, 5′-GAAGCTCAAAGGATATGGAAACAAA-3′; reverse, 5′-AAGACGTGTGCGGCTTGACT-3′), CARD16 (forward, 5′-TGCTCCCCTTGCATAAAGGA-3′; reverse, 5′-CCAGTTTGCAACTCTTTACCTAAACC-3′), CARD17 (forward, 5′ CTTCCTTCCTAGGTTCAACTTTCATT-3′, Reverse; 5′-GTGCTGGGCATCTGTGCTT-3′), CARD18 (forward, 5′–AAGATGGGTTTGCACTAAGAGAGAA-3′; reverse, 5′- TGGAAGAAGCTCTGGGAAGTCT-3′), and ACTB (forward, 5′- GGCACTCTTCC AGCCTTCCTTC-3′; reverse, 5′-GCGGATGTCCACGTCACACTTCA-3′). A dilution series of the pCR2.1 plasmid encoding the target sequence was used as the standard for absolute quantification.

2.5. IL-1β measurement

The IL-1β level was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems, Minneapolis, MN, USA). The supernatants were precipitated with ice-cold acetone and resolved in 1 × Laemmli buffer for western blot analysis.

2.6. In vitro protein interaction assays

HeLa cells in 12-well culture plates were transfected with 1.6 μg of the indicated plasmids for the co-immunoprecipitation assay. After 24 h, they were lysed in Nonidet P (NP)-40 buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1% NP-40) supplemented with a protease inhibitor cocktail (Sigma). After centrifugation, the supernatants were subjected to immunoprecipitation, and the precipitated proteins were analyzed as the insoluble fraction. The supernatants were immunoprecipitated using specific antibodies in combination with protein G or protein A-Sepharose (GE Healthcare). HeLa cells were lysed in cross-linking buffer (20 mM phosphate buffer, pH 8.0, 150 mM NaCl, and 1% NP-40) for the cross-linking assay. After centrifugation, the supernatants were placed on ice with 2 mM bis(sulfosccinimidyl)suberate (BS^3) for 2 h, and the crosslinking reaction was terminated by adding an excess of glycine.

2.7. Western blot analysis

Samples were separated by SDS–PAGE and transferred to PVDF membranes. After blocking with Tris-buffered saline containing 2% casein, the membranes were incubated with the following primary antibodies: anti-β actin monoclonal antibody (Ab) (clone AC-15; Sigma), anti-Flag monoclonal Ab (clone M2; Sigma), anti-IL-1β polyclonal Ab (H153; Santa Cruz Biotechnology, Dallas, TX, USA), anti-Myc polyclonal Ab (MBL, Nagoya, Japan).

2.8. Immunocyotochemistry

Cells were cultured in 8-well slide glass chambers and fixed with 10% neutral buffered formalin for 10 min at room temperature, and then permeabilized with PBS containing 0.1% Triton