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Lys63/Met1-hybrid ubiquitin chains are commonly formed during the activation of innate immune signalling

Christoph H. Emmerich [1], Siddharth Bakshi [1], Ian R. Kelsall, Juanma Ortiz-Guerrero, Natalia Shpiro, Philip Cohen*

MRC Protein Phosphorylation and Ubiquitylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, UK

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ABSTRACT

We have reported previously that activation of the MyD88-signaling network rapidly induces the formation of hybrid ubiquitin chains containing both Lys63-linked and Met1-linked ubiquitin (Ub) oligomers, some of which are attached covalently to Interleukin Receptor Associated kinase 1. Here we show that Lys63/Met1-Ub hybrids are also formed rapidly when the TNFR1/TRADD, TLR3/TRIF- and NOD1/RIP2-signaling networks are activated, some of which are attached covalently to Receptor-Interacting Protein 1 (TNFR1 pathway) or Receptor-Interacting Protein 2 (NOD1 pathway). These observations suggest that the formation of Lys63/Met1-Ub hybrids are of general significance for the regulation of innate immune signaling systems, and their potential roles in vivo are discussed. We also report that TNFα induces the attachment of Met1-linked Ub chains directly to TNF receptor 1, which do not seem to be attached covalently to Lys63-linked or other types of ubiquitin chain.

1. Introduction

The innate immune system is vital for defense against infection by microbial pathogens, especially in young children [1]. In this system, components of these microbes activate Pathogen Recognition Receptors, such as TLRs and NOD proteins, triggering the production of pro-inflammatory cytokines, such as IL-1 and TNFα, which mount responses to combat the invading microbes.

Most TLRs, as well as the IL-1R, initiate signal transduction by recruiting the adaptor protein MyD88, which is followed by the binding of IRAK4 to MyD88, and then the association of other IRAK family members with IRAK4 to form an oligomeric complex, termed the Myddosome [2,3]. This leads within minutes to the interaction of IRAKs 1 and 2 with the E3 ubiquitin ligase TRAF6 [4,5] and to the formation of Lys63-linked ubiquitin and Met1-linked ubiquitin chains (K63-Ub, M1-Ub chains). The M1-Ub chains (also called linear Ub chains) are formed by the E3 ligase LUBAC [6], while K63-Ub chains can be formed by the action of TRAF6 in combination with the E2-conjugating complex Ubc13-Uev1a (also called UBE2N-UBE2V1) [7,8].

We found that the M1-Ub chains formed upon activation of the MyD88 signaling network are attached covalently to preformed K63-Ub chains, forming ubiquitin chains containing both types of linkage, hereafter called K63/M1-Ub "hybrids" [9]. Some of the K63/M1-Ub hybrids present in extracts prepared from IL-1 receptor-expressing HEK293 cells (IL-1R cells) or human THP1 monocytes were attached to IRAK1, but some were not anchored to any other protein [9]. HOIP, the catalytic subunit of LUBAC, interacts with K63-Ub oligomers specifically, but not with M1-Ub oligomers [9], which may help to explain, at least in part, why K63-Ub oligomers (and not ubiquitin monomers) are the preferred substrate for LUBAC in the MyD88-dependent signaling network.

The formation of K63/M1-Ub hybrids provides a platform for the

Abbreviations: AMSH-LP, AMSH-like protein; λ-PPase, bacteriophage λ protein phosphatase; BMDM, bone marrow-derived macrophages; JNK, c-Jun N-terminal kinase; cIAP, cellular Inhibitor of Apoptosis; DUB, deubiquitylase; dsRNA, double-stranded RNA; GST, glutathione-S-transferase; HRP, horseradish peroxidase; IKK, IκB kinase; IL-1, interleukin-1; IL-1R, IL-1 receptor; IRAK, IL-1R-Associated Kinase; LUBAC, Linear UBiquitin Assembly Complex; K63-Ub, Lys63-linked ubiquitin; M-CSF, Macrophage Colony Stimulating Factor; M1-Ub, Met1-linked ubiquitin; MyD88, Myeloid Differentiation primary response gene 88; NEMO, NF-κB Essential Modifier; NOD, Nucleotide Oligomerisation Domain; PRRs, Pathogen Recognition Receptors; PNGase F, Protein Asparaginyl Glycosidase F; RIP, Receptor-Interacting Protein; TAK1, TGFβ-activated kinase 1; TAB, TAK1-binding protein; TRAF, TNF-Receptor-Associated Factor; TLR, Toll-Like Receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β; TNFα, Tumour Necrosis Factor α; TNFR1, TNFα Receptor 1; TRADD, TNF-receptor associated death domain protein; Ub, Ubiquitin; USP, Ub-specific protease.

* Corresponding author.
E-mail address: p.cohen@dundee.ac.uk (P. Cohen).
[1] Joint First Authors.

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co-recruitment of two or more proteins that bind specifically to either K63-Ub or M1-Ub oligomers, which include the two "master" protein kinase complexes of the MyD88 signaling network, the TAK1 and canonical IKK complexes. The TAB2 and TAB3 components of TAK1 complexes [10] interact specifically with K63-Ub oligomers [11,12] while NEMO, a regulatory component of the canonical IKK complex, binds to M1-Ub dimers with far higher affinity than it binds to K63-Ub dimers [13,14]. We have suggested that the co-recruitment of these kinases to K63/M1-Ub hybrids may increase the efficiency with which TAK1 initiates activation of the IKK complex [15].

Met1-linked ubiquitin chains are also formed when other innate immune signaling networks are activated, such as the TNFα [16] and NOD2 [17] signaling pathways and, similar to the MyD88 pathway, activation of TNFR1 or NOD2 induces the activation of TAK1 and the canonical IKK complex. However, whether the M1-Ub chains formed when other innate immune signaling pathways are activated become attached covalently to K63-Ub and/or other types of ubiquitin linkage, has not yet been investigated. Here we demonstrate the rapid formation of K63/M1-Ub hybrids when TNFR1, TLR3 and NOD1 signaling is activated, indicating that the production of these molecules is a general feature of innate immune signaling pathways.

2. Methods

2.1. Proteins

Proteins were of human origin and full length, unless stated otherwise and were expressed in *Escherichia coli* and purified by the Protein Production Teams of the MRC Protein Phosphorylation and Ubiquitylation Unit (MRC-PPU) coordinated by James Hastie and Axel Knebel. The proteins were:- λPPase (DU4170), GST-Otulin (DU43487), AMSH-LP[264–436] (DU15780), vOTU (DU45351), GST-OTUD3 (DU21323), His_6-TRABID[245–697] (DU22468), GST-Cezanne (DU20899), OTUB1 (DU19741) and Rat USP2[271–618] (DU35832). The expression vectors and proteins generated with their assigned [DU] numbers can be ordered from the reagent's section of the MRC-PPU website (https://mrcppureagents.dundee.ac.uk/). Murine TNFα was obtained from Peprotech (#315-01A) and poly(I:C) from InvivoGen (tlrl-pic). Lys63-linked ($K63_{2-7}$) and Met1-linked ($M1_{2-7}$) ubiquitin oligomeric standards were purchased from Boston Biochem. The Halo-NEMO and Halo-TAB2 beads were prepared [9] and the NOD1 agonist KF1B synthesized as described [18,19]. PNGase F was from New England Biolabs (#P0704S).

2.2. Antibodies

An antibody recognizing IKKα phosphorylated at both Ser176 and Ser180 and IKKβ phosphorylated at Ser177 and Ser181 (#2697) and antibodies recognizing IRAK1 (#4504), K63-Ub linkages (#5621) and GAPDH (#2118) were from Cell Signaling Technology. A phospho-specific antibody recognizing JNK1 and JNK2 phosphorylated at Thr183 and Tyr185 (#44682) was obtained from Invitrogen and an anti-RIP2 antibody from Abcam (ab8428). Anti-ubiquitin was from Dako (#Z0458) and anti-RIP1 (#610459) from BD Biosciences. An antibody recognizing M1-Ub chains specifically [20] was generously provided by Vishva Dixit, Genentech, USA. Anti-TNFR1 (sc-8436) was from Santa Cruz. Secondary antibodies coupled to HRP were from Thermo Scientific.

2.3. Mice, cell culture, cell stimulation and cell lysis

Heterozygous knock-in mice expressing an E3-ligase inactive mutant of HOIP (HOIP[C879S]) [9] were crossed to TNFR1 knockout mice. Macrophages were obtained by differentiation of foetal livers from E13.5 embryos or bone marrow obtained from the femur and tibia of mice, as described [4]. Adherent BMDM were re-plated into 12-well tissue culture plates (5×10^5 cells) or 10 cm tissue culture grade plates (5×10^6 cells) using fresh culture medium. After re-plating, liver macrophages or BMDM were stimulated with 10 μg/ml poly(I:C) or RAW macrophages with 25 μM KF1B. Monocytes were purified from human peripheral blood mononuclear cells and differentiated into macrophages as described [21]. The human monocyte cell line THP1 was maintained in RPMI medium supplemented with 5% foetal bovine serum (FBS), 2 mM L-glutamine and antibiotics (100 Units/ml penicillin, 0.1 mg/ml streptomycin) and cultured at 37 °C in an 8% CO_2 humidified atmosphere. HeLa cells and MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM). THP-1 and HeLa cells were stimulated with 10 ng/ml human TNFα or MEFs with 5 ng/ml mouse IL-1α or 10 ng/ml mTNFα.

The cells were rinsed in ice-cold PBS and, unless stated otherwise, were extracted in ice cold lysis buffer (50 mM Tris/HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 10 mM sodium 2-glycerophosphate, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, plus 100 mM iodoacetamide to inactivate deubiquitylase activities. Cell lysates were clarified by centrifugation at 14,000 × g for 30 min at 4 °C and the supernatants (cell extracts) were collected and their protein concentrations determined by the Bradford procedure.

2.4. Capture of ubiquitin chains or ubiquitylated proteins

To capture ubiquitin chains, ubiquitylated proteins and other proteins with which they interact, cell extracts (2–3 mg protein) were incubated overnight at 4 °C with either Halo-NEMO or Halo-TAB2 beads (20 μl packed volume) as described [9,22]. The beads were washed three times with 1 ml lysis buffer containing 500 mM NaCl and once with 1 ml 10 mM Tris/HCl pH 8.0 and the captured proteins were released by denaturation in SDS. To analyze ubiquitylation events triggered by the NOD1 agonist KF1B, Halo-NEMO beads were first washed twice, each time for 3 min, with 50 mM Tris/HCl pH 7.5 containing 0.1% (w/v) SDS and then three times with 50 mM Tris/HCl pH 7.5, 500 mM NaCl, 1% (v/v) Triton X100, and once with 50 mM Tris/HCl pH 7.5. The brief wash with 0.1% (v/v) SDS did not interfere with the capture of ubiquitin chains and ubiquitylated proteins from the cell extracts but removed an impurity in the bacterially expressed Halo-NEMO preparation that was recognized by anti-RIP2 and was of similar molecular mass to RIP2.

2.5. Treatment with deubiquitylases, phosphatase and PNGase F

Proteins captured by Halo-NEMO beads were washed three times with 1 ml of lysis buffer containing 0.5 M NaCl and once with 1 ml of 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 2 mM DTT. The beads were resuspended in 30 μl of 50 mM HEPES pH 7.5, 100 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM $MnCl_2$, 0.01% (w/v) Brij-35) with λPPase (100 units/reaction) and incubated for 1 h at 37 °C with or without the deubiquitylases (DUBs) USP2 (1.0 μM), AMSH-LP (0.2 μM), vOTU (0.1 μM), Otulin (1.0 μM), OTUB1 (2.0 μM), Cezanne (5.0 μM), OTUD3 (2.0 μM) or TRABID (1.0 μM). To deglycosylate TNFR1, SDS and DTT were added to final concentrations of 0.5% (w/v) and 40 mM, respectively, after DUB treatment and heated for 10 min at 100 °C. After cooling to 21 °C, NP-40 and sodium phosphate buffer pH 7.5 were added to final concentrations of 1% (v/v) and 50 mM, respectively, followed by incubation for 1 h at