Biochemical and Biophysical Research Communications 456 (2015) 298–304

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

RIPK3 regulates p62–LC3 complex formation via the caspase-8-dependent cleavage of p62

Yu Matsuzawa [a], Shigeru Oshima [a,*], Yoichi Nibe [a], Masanori Kobayashi [a], Chiaki Maeyashiki [a], Yasuhiro Nemoto [a], Takashi Nagaishi [a], Ryuichi Okamoto [a,b], Kiichiro Tsuchiya [a,c], Tetsuya Nakamura [a,c], Mamoru Watanabe [a,*]

[a] Department of Gastroenterology and Hepatology, Graduate School, Tokyo Medical and Dental University (TMDU), Tokyo, Japan
[b] Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University (TMDU), Tokyo, Japan
[c] Department of Advanced Therapeutics for GI Diseases, Tokyo Medical and Dental University (TMDU), Tokyo, Japan

ARTICLE INFO

Article history:
Received 17 November 2014
Available online 24 November 2014

Keywords:
RIPK3
Necroptosis
Autophagy
p62
LC3
Caspase-8

ABSTRACT

RIPK3 is a key molecule for necroptosis, initially characterized by necrotic cell death morphology and the activation of autophagy. Cell death and autophagic signaling are believed to tightly regulate each other. However, the associated recruitment of signaling proteins remains poorly understood. p62/sequestosome-1 is a selective autophagy substrate and a selective receptor for ubiquitinated proteins. In this study, we illustrated that both mouse and human RIPK3 mediate p62 cleavage and that RIPK3 interacts with p62, resulting in complex formation. In addition, RIPK3-dependent p62 cleavage is restricted by the inhibition of caspases, especially caspase-8. Moreover, overexpression of A20, a ubiquitin-editing enzyme and an inhibitor of caspase-8 activity, inhibits RIPK3-dependent p62 cleavage. To further investigate the potential role of RIPK3 in selective autophagy, we analyzed p62–LC3 complex formation, revealing that RIPK3 prevents the localization of LC3 and ubiquitinated proteins to the p62 complex. In addition, RIPK3-dependent p62–LC3 complex disruption is regulated by caspase inhibition. Taken together, these results demonstrated that RIPK3 interacts with p62 and regulates p62–LC3 complex formation. These findings suggested that RIPK3 serves as a negative regulator of selective autophagy and provides new insights into the mechanism by which RIPK3 regulates autophagic signaling.

1. Introduction

Receptor-interacting protein kinase 3 (RIP3/RIPK3) is a critical regulator of necroptosis. Necroptosis is a programmed necrotic cell death process driven by a defined molecular pathway. Necroptosis was originally observed under caspase inhibition in vitro [1,2] and confirmed in caspase-8-deficient mice [3,4]. Importantly, necroptosis can also occur in the absence of caspase inhibition in various situations [5,6], and it has an important pathophysiological role in pancreatitis [7,8], inflammatory bowel disease [9,10], and many other disorders. RIPK3 has an active kinase domain in its N-terminus that is conserved in other RIPKs and is required for necroptosis [7,11]. RIPK3 forms an intracellular complex with RIPK1 to assemble the necrosome [12]. RIPK3 also associates with the RIPK1/FADD/caspase-8 complex [7]. Recent studies have identified the mixed lineage kinase domain-like protein as an interacting partner of RIPK3 [13].

Autophagy is the common name for lysosome-based degradation of cytosolic cargos [14]. Autophagy is considered to be a nonselective, bulk process. A small portion of the cytoplasm is engulfed by an isolation membrane, which results in the formation of an autophagosome. Microtubule-associated protein 1 light chain 3 (LC3) is a marker of the autophagosome. LC3-I is subsequently conjugated with phosphatidylethanolamine (PE) to become LC3-II (LC3-PE). In contrast to the cytoplasmic localization of LC3-I, LC3-II associates with both the outer and inner membranes of the autophagosome [15]. Selective autophagy ensures the recognition and removal of various cytosolic cargos [16]. Selective autophagy is mediated by selective autophagy receptors, such as p62 (also known as sequestosome-1) and NBR1. p62 is a stress-inducible intracellular protein

Abbreviations: UBA domain, ubiquitin-associated domain; LIR, LC3-interacting region; LRS, LC3 recognition sequence; PLA, proximity ligation assay; TNT, in vitro translation.

* Corresponding authors at: Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University (TMDU), 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81 3 5803 0268.

E-mail addresses: soshima.gast@tmd.ac.jp (S. Oshima), mamoru.gast@tmd.ac.jp (M. Watanabe).

http://dx.doi.org/10.1016/j.bbrc.2014.11.075
0006-291X/

known to regulate various signal transduction pathways involved in cell survival and death [17]. By directly binding to LC3 via its LC3-interacting region (LIR) motif, p62 becomes a selective autophagy receptor, transporting ubiquitinated protein aggregates to the autophagosome. As p62 was identified as one of the specific substrates degraded through the autophagy–lysosomal pathway [18,19], the total cellular expression of p62 can be used to monitor autophagic flux [15]. However, recent studies indicated that p62 expression can be also regulated by transcription and protein cleavage [20,21]. In addition, p62 can suppress autophagy [22]. Although autophagy modulates the levels of p62 protein, there are potential limitations of evaluating autophagic flux by p62.

Dying cells often display an accumulation of autophagosomes and hence adopt a morphology called autophagic cell death. However, in many cases, autophagic cell death is called cell death with autophagy rather than cell death by autophagy [23]. Necroptosis is originally characterized by necrotic cell death morphology and the activation of autophagy [5], and interplay between autophagy and RIPK1-dependent necroptotic cell death has been reported [24]. However, how necroptotic and autophagic signaling proteins are recruited remains poorly understood. Given the potential importance of RIPK3 in necroptosis and the potential importance of p62 in autophagy, we investigated whether RIPK3 can regulate p62.

2. Materials and methods

2.1. Cell culture and reagents

HEK293T cells were cultured in DMEM (Sigma) with fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C. Mouse p62 cDNA was kindly provided by Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science). Mouse p62 cDNA was cloned into the p3XFLAG-CMV-10 expression vector (Sigma). Mouse Myc-RIPK3 and mouse Flag-A20 expression vectors were kindly provided by Dr. Averil Ma (UCSF). Human RIPK3 cDNA was amplified by RT-PCR from the total RNA of Jurkat cells and then cloned into pCMV-3tag-2C plasmids (Agilent Technologies). The pan-caspase inhibitor Z-VAD-FMK was purchased from Peptide Institute. The caspase-8 inhibitor Z-IETD-FMK was purchased from R&D SYSTEMS.

2.2. DNA and siRNA transfection

HEK293T cells were transiently transfected empty plasmid and/or expression plasmids using TransIT-LT1 (Mirus) as indicated by the supplier. OnTarget Plus SMARTpool siRNA oligonucleotides specific for human caspase-8 and nontargeting siRNA (control siRNA) were purchased from ThermoFisher Scientific. HEK293T cells seeded on 6-well plates were firstly transfected with 150 pmol of siRNA using Lipofectamine RNAiMAX (Invitrogen); after 8 h, p62 and RIPK3 expression plasmids were transfected using TransIT-LT1, according to the manufacturer's protocol.

2.3. Immunoblotting and immunoprecipitation

Cells were incubated in lysis buffer [either 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% CHAPS, 10% glycerol, 2 mM NEM, Halt protease, and phosphatase inhibitor cocktail (Pierce) or 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 2 mM NEM, and protease inhibitors] on ice for 20 min and centrifuged at 14,000×*g* for 20 min. For Flag immunoprecipitation, cell lysates were incubated with anti-Flag M2 beads (Sigma) for 3 h at 4 °C. Samples were resolved on NuPage precast 4–12% Bis–Tris gels (Invitrogen) and transferred to a PVDF membrane. The following antibodies and reagents were used for immunoprecipitation and immunoblotting studies: anti-β-actin and anti-Flag (SIGMA); anti-LC3 (PD014, MBL), anti-A20 (5630, Cell Signaling), anti-Ub (P4D1), and anti-Myc (A-14) (Santa Cruz).

2.4. In situ proximity ligation assay (PLA)

To detect protein interactions in HEK293T cells, the Duolink PLA in situ kit (SIGMA–ALDRICH, 92101) was used, according to the manufacturer's instructions. To analyze the interaction between RIPK3 and p62 (Fig. 2), the primary antibodies were rabbit anti-c-Myc antibody (A-14, Santa Cruz) and mouse anti-Flag antibody (F3165, SIGMA). To analyze the interaction between LC3 and p62 (Fig. 4B), the primary antibodies were rabbit anti-Flag antibody (F7425, SIGMA) and mouse anti-LC3 antibody (M152-3, MBL). As a control, the primary antibody was mouse control IgG (Vector Laboratories, I-2000). Images were acquired with a confocal laser microscope (FV10i, Olympus) using a ×60 oil-immersion objective lens.

3. Results

3.1. RIPK3 mediates p62 cleavage

To investigate the potential roles of RIPK3 in regulating autophagy, we introduced Myc-mRIPK3 and Flag-p62 into HEK293T cells. HEK293T cells do not express any detectable endogenous human RIPK3 [7]. Immunoblotting analyses were performed with extracts from HEK293T cells with or without Flag-p62. A band corresponding to ~62 kDa was detected with anti-Flag antibody. This result indicated that the ~62-kDa band was the full-length p62 protein. Surprisingly, ~45-kDa and ~35-kDa bands were also observed using the same anti-Flag antibody in the presence of mouse RIPK3 (Fig. 1A). This result suggested that the ~45-kDa and ~35-kDa fragments were the products of p62 cleavage (Fig. 1A). As we performed this analysis in human HEK293T cells, we then investigated whether this p62 cleavage is specific for mouse RIPK3. We cloned human RIPK3 into an expression vector, and then human RIPK3 was transfected with Flag-p62 into HEK293T cells. Similar observations were also made using human RIPK3. Again, the ~45-kDa and ~35-kDa bands were detected in the presence of human RIPK3 (Fig. 1B). Taken together, these results suggested that RIPK3 mediates p62 cleavage in HEK293T cells.

3.2. RIPK3 forms a complex with p62

p62 regulates various signal transduction pathways and interacts with many molecules [17]. To understand how RIPK3 mediates p62 cleavage, we investigated whether RIPK3 binds to the p62 complex. We used PLA to detect whether Myc-RIPK3 interacts with Flag-p62. In this assay, a pair of oligonucleotide-labeled secondary antibodies (PLA probes) generates an individual fluorescent signal when bound to two primary antibodies in close proximity [25]. Although no PLA foci were detected in the presence of Flag-p62 or Myc-RIPK3 alone, many PLA foci were observed in the presence of both Flag-p62 and Myc-RIPK3 (Fig. 2). When we used control mouse IgG instead of mouse anti-Flag antibody, PLA foci were not observed even in the presence of both Flag-p62 and Myc-RIPK3. These data illustrated that Flag-p62 and Myc-RIPK3 interact in situ.

3.3. p62 is cleaved by caspase-8

Self-oligomerization of p62 is essential for its localization to the autophagosome formation site [26]. p62 also interacts with