

Functional and Physiological Characteristic of RIPK and MLKL in TNF Signaling

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Receptor-interacting protein kinase 1 (RIPK1) and RIPK3 are members of the serine or threonine protein kinase superfamily that phosphorylates the hydroxyl group of serine or threonine through the highly conserved kinase region. The RIPK family plays a crucial role not only in inflammation and innate immunity, but also in mediating programmed cell death, such as apoptosis and necroptosis. The interaction between RIPK1 and other TNFR1-related proteins has been shown to assemble a signaling complex I that controls activation of the pro-survival transcription factor NF- κ B upon binding of cytokines to TNF receptor 1 (TNFR1). Moreover, RIPK1 and RIPK3 interact through their RIP homotypic interaction motifs (RHIMs) to mediate programmed necrosis, which has long been considered an accidental and uncontrolled cell death form with morphological characteristics differing from those of apoptosis. Highly conserved sequences of RHIM in RIPK1 and RIPK3 were shown to regulate their binary interaction, leading to assembly of a cytosolic amyloid complex termed the "necrosome". The necrosome also contains mixed lineage kinase domain-like protein (MLKL), which has been found recently to be a substrate of RIPK3 to mediate downstream signaling. This review provides an overview of the functional and physiological characteristics of RIPKs and MLKL in TNF signaling.

Key words : MLKL, programmed cell death, RHIM, RIPK, TNF signaling

Introduction

Stimulation of tumor necrosis factor (TNF)-like cytokines can initiate cellular signaling pathways that control homeostasis and viability in an organism [7, 40]. This stimulation is regulated by many protein complexes, including ligand-receptor complex, inhibitor κ B kinase (IKK) complex, death-inducing signaling complex, apoptosome, necrosome, transforming growth factor beta-activated kinase-1 (TAK1)/TAK1-binding protein 2/3, signaling complexes of death domain superfamily and other cytosolic complexes (Fig. 1) [1, 7, 19, 26, 50]. Receptor interacting protein kinase (RIPK) 1 and RIPK3 are two of the most thoroughly investigated serine/threonine-protein kinases of the TNF- α signaling pathway. RIP kinases are central molecules for activation of the pro-survival transcription factor NF- κ B that also regulate apoptotic and necrotic cell death. The extrinsic pathway of

programmed cell death is mediated via diverse factors, such as the TNF receptor (TNFR), caspase and RIPK superfamily [9, 23, 38].

The RIPK family members play an important role in TNF induced inflammation and other immune responses [12, 17]. They also have been shown to play a role in death receptor-dependent programmed cell death. Programmed cell death plays a key role in the elimination of reactive immune cells after massive clonal expansion in response to infection. Moreover, defects in normal programmed cell death mechanisms are related to autoimmune diseases, immunodeficiencies and cancer pathogenesis [10, 17, 34]. Apoptosis by cellular suicide mechanism is characterized by obvious morphological characteristics and energy dependent biochemical mechanisms, including cell shrinkage, DNA fragmentation, membrane blebbing and nuclear condensation [5]. Programmed cell death by the caspase-dependent pathway is triggered by extrinsic TNF-mediated signaling, whereas programmed necrosis via caspase-independent pathways is triggered when apoptosis is inhibited through cells that express mixed lineage kinase domain like protein (MLKL) and its up-regulators, such as RIPK3 [6, 47].

Recently, a complex functional interplay between RIP1 and RIP3 has been identified as a form of programmed necrosis regulated through toll-like receptor or TNFR1. This

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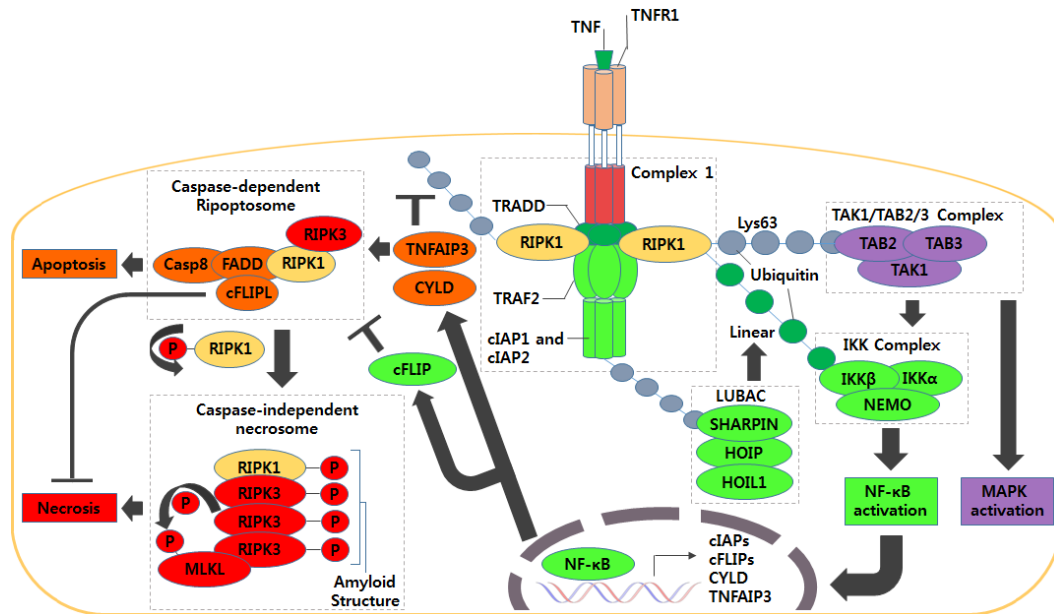


Fig. 1. TNFR1-induced signaling pathways and roles of RIPKs and signaling complexes in modulating balance between cell survival and death.

interplay is a crucial part of diverse physiological and pathological actions, including tissue damage response, development, and antiviral immunity. To elucidate the molecular mechanisms underlying the interplay between RIP1 and RIP3, we will discuss the current understanding of how these kinases regulate TNF/TNFR1-dependent programmed cell death.

TNFR1-induced signaling pathways through RIPK1: live or let die

TNF/TNFR1-mediated programmed necrosis shares initial signaling cascades with the NF-κB pathway and apoptotic signaling. RIPK1 and RIPK3 mediate several signal transduction cascades, controlling a broad spectrum to survival from death in cell signaling pathways. Upon binding of TNF-α to the extracellular cysteine-rich domains of TNFR1, TNF receptor-associated death domain protein (TRADD) recruits additional death domain (DD) protein such as the RIPK1 from the extreme C-terminal cytoplasmic region (also known as DD) [29, 47]. The interaction among these DD superfamily members has been shown to assemble a signaling complex I, which consists of the adaptor molecule TRAF-2 in lipid rafts on the plasma membrane [24, 35]. The cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1 and 2) is also enlisted into signaling complex I, which permits dimerization of two cIAP molecules. The ubiquitin ligase activity of the cIAPs is required to recruit the linear

ubiquitin chain assembly complex (LUBAC), which is composed of a SHANK-associated RH domain interacting protein (SHARPIN), heme-oxidized IRP2 ubiquitin ligase 1 (HOIL1), HOIL1-interacting protein (HOIP), and RING finger protein. This TNFR1 associated signaling complex I promotes the expression of pro-inflammatory proteins through the NF-κB and mitogen-activated protein kinase (MAPK) signaling cascade [31].

With the induced TNF/TNFR1 signal cascade, RIPK1 is rapidly polyubiquitinated at lysine 377 in an intermediate domain through cIAP 1 and 2. Interestingly, the programmed cell death can be inhibited by family members known as baculoviral IAP repeat-containing proteins (BIRCs) or cIAPs, which comprise a conserved domain region of roughly 70 amino acids known as baculoviral IAP repeat (BIR). The human genome encodes eight IAP family members that play key roles in regulation of cell division and the anti-apoptotic signaling pathway through direct binding to caspases. Both cIAP1 and 2 (also known as BIRC protein 2 and 3) share conserved sites, including N-terminal three baculoviral IAP repeats (BIRs), caspase activation and recruitment domain (CARD), and the C-terminal RING domain [3, 4, 15]. CARD is a DD superfamily member composed of six amphipathic α-helices folded in an antiparallel α-helical bundle. With the interaction between cIAPs and RIP1, polyubiquitin chains of RIP1 serve as a platform for recruitment of downstream components of cell survival

pathways such as IKK, LUBAC, NEMO and TAK1/TAB2/3 complex. The adaptor molecules, TAB2/3, bind to the ubiquitin chain to form complexes with TAK1 for IKK complex activation [20, 46, 50]. NF- κ B is activated through these complexes, which leads to the translocation of transcription factors to the nucleus and expression of cFLIP, cylindromatosis (CYLD) and tumor necrosis factor alpha-induced protein3 (TNFAIP3). These factors then negatively regulate NF- κ B signaling. CYLD and TNFAIP3 bind to RIPK1 and remove its polyubiquitin chain in the TNFR-induced signaling complex [13, 14, 39]. When RIPK1 is not ubiquitylated by removal of the E3 ligases cIAP 1 and 2 through genetic ablation, RNAi knockdown, or IAP antagonists, RIPK1 and RIPK3 are recruited to a cytoplasmic complex that is often referred to as the ripoptosome (Complex II) [18, 42, 48]. The ripoptosome consists of five subunits, caspase-8, FADD, RIPK1, RIPK3 and long isoform of cellular FLICE-like inhibitory proteins (cFLIPL) [2, 37]. The ripoptosome is involved in activation of the extrinsic apoptosis pathway when there is internalization of TNFR1. Non-ubiquitylated RIPK1 and its interaction molecules lead to inhibition of NF- κ B signaling and the formation of protein complexes that promote programmed apoptosis.

TNFR1-mediated programmed necrosis via RIPKs and MLKL

Necrotic cell death is considered an accidental and uncontrolled form of cell death with morphological characteristics differing from those of apoptosis. This unregulated process has been identified in numerous pathologies including ischemia-reperfusion injuries (brain, heart, kidney, and liver), eye diseases, brain trauma, acute inflammatory conditions, and viral infection [27, 32, 43]. Hallmarks of necrosis include swelling of the cytoplasm, endoplasmic reticulum, and mitochondria, with subsequent plasma membrane rupture [16]. Understanding of unique cellular events for necroptosis was initiated by the discovery that the RIPK1 is required for the necrosis of mouse embryonic fibroblasts (MEFs) through TNF signaling cascade [22]. Activation of RIPK1 and RIPK3 require auto/cross-phosphorylation and binary interaction through the RIP homotypic interaction motif (RHIM), which is the core domain that regulates necrotic cell death (Fig. 2A) [26]. Human RIPK1 encodes a protein composed of 671 amino acids with a kinase domain at the N-terminus and a C-terminal death domain. This domain is a conserved homology domain that acts as a protein interaction module

containing a hexahelical bundle of 80 amino acids [36]. In addition to its N- and C-terminal domain, RIPK1 contains an intermediate domain that also retains a RHIM. Both RIPK1 and RIPK3 share a conserved motif, including kinase domain and RHIM, whereas RIP3 lacks the C-terminal death domain. Both serine/threonine kinase proteins are highly conserved, with 31%/53% sequence identity for the kinase domains of human RIPK1 and RIPK3, 77%/90% sequence identity for the kinase domains of human and mouse RIPK1, and 71%/81% sequence identity for the kinase domains of human and mouse RIPK3 (Fig. 2B). Highly conserved isoleucine/valine-glutamine-isoleucine/leucine/valine-glycine sequences of RHIM in RIPK1 and RIPK3 were shown to regulate their binary interaction, which leads to assembly of a cytosolic amyloid signaling protein complex that has been called the 'necrosome' [26, 33]. The critical role of the RIPKs in programmed necrosis has been shown in cellular programs containing necrosis related factors [TNFR1; FAS receptor; nucleotide-binding and oligomerization, leucine-rich repeat and pyrin domains-containing protein 3 (NALP3); retinoic acid-inducible gene 1 (RIG1); toll-like receptor (TLR) 3; or TLR4] [25, 28, 30, 44, 45, 49]. RIPK1 was recently found to be dispensable for some programmed necrotic signaling, whereas RIPK3 is an essential upstream kinase in all extrinsic necrosis pathways. The necrosome also contains other molecules, such as mixed lineage kinase domain like protein (MLKL), which was recently discovered to be a substrate of RIPK3 that mediates downstream signaling [41, 52]. Recent cell studies suggest that activation results in plasma membrane translocation of trimerized MLKL protein, which promotes Ca^{2+} and Na^{+} influx, and eventually leads to membrane lysis [6, 8]. MLKL encodes a 471 amino acids protein with an N-terminal coiled-coil domain and a C-terminal kinase-like domain that is responsible for association with RIPK3. The interaction between MLKL and RIPK3 is dependent on RIPK3 kinase activity, and RIP3 needs to be phosphorylated at serine 227 of MLKL [41]. Conversely, RIPK3 phosphorylates MLKL at the threonine 357 and serine 358 sites in humans and serine 345, 347 and threonine 349 sites in mouse. Point mutation of MLKL phosphorylation sites or deletion of the MLKL inhibits programmed necrosis, but does not affect complex formation [9, 11, 21, 26, 47, 51]. These results provide molecular and functional characterization of homotypic interaction motifs of RIPKs and its substrates, which are associated with programmed necrosis.

Although many previous studies have focused on the

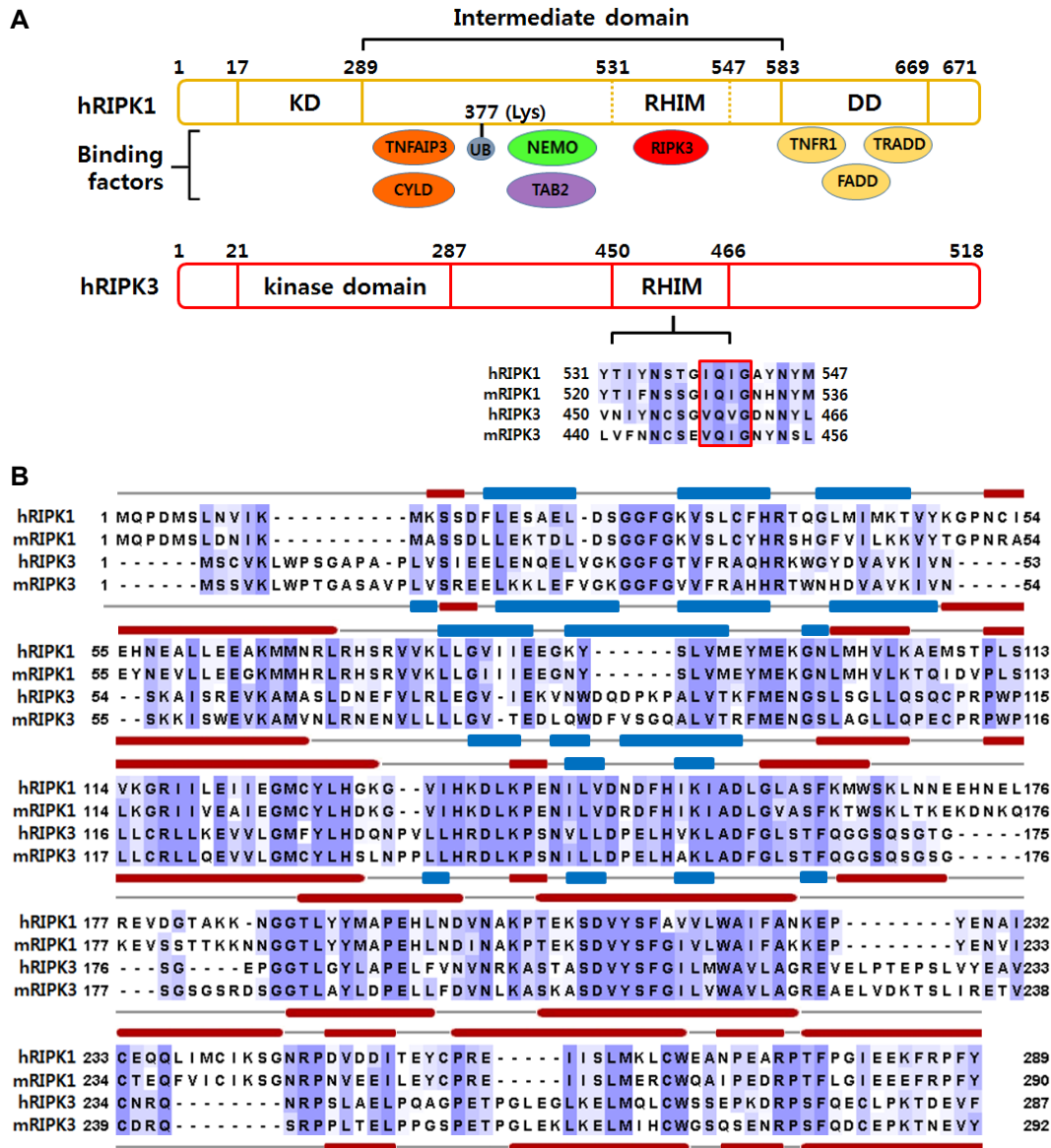


Fig. 2. Conserved domains and secondary structures of RIPK1 and RIPK3. (A) Domain structure of hRIPK1 and hRIPK3. The known binding factors of each domain are shown. Amino acid sequence alignment of RIPKs RHIM is also shown. (B) Amino acid sequence alignments of human RIPK1 (17-289), mouse RIPK1 (17-290), human RIPK3 (21-287) and mouse RIPK3 (22-292) kinase domain are shown. The secondary structural elements of hRIPK1 and mRIPK3 are indicated above and under the sequences. Alpha helices are presented as red ellipses and β -sheets as blue. Loop regions are presented as gray lines (KD, kinase domain; DD, death domain).

mechanism of RIPK family members in inflammation and innate immunity, it is clear that these proteins have many functional and physiological characteristics that are derived from their crucial mechanism in programmed cell death signaling. These studies confirm that RIPKs and its related molecules play a pivotal role in the assembly of death complexes, which are identified by nuclear magnetic resonance and X-ray diffraction studies. Furthermore, many of the regulatory mechanisms of RIPK1 in apoptosis are mediated by

ubiquitination of the kinase domain or combination through DD interaction. RIPK3 function is controlled by interaction proteins such as RIPK1 or MLKL through the RHIM or kinase domain, which are triggered to execute different death signaling outcomes such as programmed necrosis. Having been recognized for more than two decades, programmed cell death is now a main research topic in cell biology. This review serves as an information source to improve our mechanistic understanding of RIPK and its substrate in TNF

signaling.

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초록 : TNF 신호전달에서 RIPK와 MLKL의 기능적 생리적 특성

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수용체 상호작용 단백질 인산화 효소 RIPK1 (Receptor-interacting protein kinases 1)과 RIPK3은 고도로 보존된 인산화 효소 부위를 통하여 세린이나 트레오닌의 하이드록실기를 인산화하는 세린 또는 트레오닌-단백질 인산화 효소 군에 속한다. RIPK군은 염증이나 선천성 면역뿐 만 아니라 세포사멸이나 괴사와 같은 프로그래밍된 세포사멸을 중재하는데 중요한 역할을 담당한다. RIPK1과 다른 TNFR1 관련 단백질들의 상호작용은 TNF 수용체 1 (TNFR1)에 사이토카인이 결합할 때 생존 촉진 전사인자 NF- κ B의 활성을 조절하는 신호전달복합체 I을 조립하는 것으로 알려져 왔다. 뿐만 아니라, RIPK1과 RIPK3은 프로그래밍된 세포괴사를 중재하는 RIP 동형 상호작용 모티프(RHIM)를 통하여 상호작용하고, 이러한 괴사는 세포사멸의 유형과는 다른 형태학적 특징을 가진 돌발적이고 제어되지 않는 세포사멸 유형으로 오랫동안 알려져 왔다. RIPK1과 RIPK3에 존재하는 RHIM의 고도로 보존된 서열들이 이들의 상호작용을 조절하며 이들은 necrosome이라 불리는 세포질 내 아밀로이드 복합체의 조립을 유도한다. 또한 necrosome은 최근에 하위 신호전달을 조절하는 RIPK3의 기질로 확인된 혼합형 인산화 효소 도메인-유사 단백질(MLKL)을 포함한다. 본 리뷰는 TNF 신호전달에서 RIPK와 MLKL의 기능적, 생리적 특징들에 관한 개요를 제공한다.