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RIP Kinases at the Crossroads of Cell Death and Survival

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Protein kinases of the receptor interacting protein (RIP) family collaborate with death receptor proteins to regulate cell death. Recent studies (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) reveal that the RIP3 kinase functions with RIP1 at the crossroads of apoptosis, necroptosis, and cell survival.

A cell can die in many different ways, but caspase-dependent apoptosis is thought to be the predominant pathway for cell death. However, in the absence of caspase activation, a process of programmed necrosis, called necroptosis, prevails. Little is known about the proteins involved in necroptotic signaling, but recent reports indicate that it shares some regulatory mechanisms with apoptosis. Indeed, the kinase receptor interacting protein 1 (RIP1) has been implicated in the regulation of apoptosis (Wang et al., 2008) as well as necroptosis (Degterev et al., 2008; Holler et al., 2000). Findings recently reported in *Cell* (Cho et al., 2009; He et al., 2009) and *Science* (Zhang et al., 2009) now make an important contribution to the understanding of necroptotic signaling by describing an essential role for the RIP3 kinase in the decision between tumor necrosis factor (TNF)-induced necroptosis and survival when apoptotic signaling is blocked.

RIP1 in Life and Death

RIP serine/threonine kinase family members are essential sensors of cellular stress. They share a homologous N-terminal kinase domain but have different recruitment domains. The death domain of the RIP1 kinase binds to death receptors such as TNF receptor 1 (TNFR1), Fas, tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAILR1), and TRAILR2. It also associates with death domain-containing adaptor proteins, such as TNF-receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), that are required for caspase-8 activation and apoptosis (Schutze et al., 2008; Wilson et al., 2009). The intermediate domain of RIP1 contains a RIP homotypic interaction motif that enables it to interact with RIP3. Consistent with a role for RIP1 in regulating apoptosis, mice lacking RIP1 display extensive apoptosis and die at 1 to 3 days of age (Kelliher et al., 1998). Moreover, cultured cells lacking RIP1 are highly sensitive to TNF-induced cell death, possibly because they are unable to activate the prosurvival transcription factor NF-κB.

In most cell types, the death receptors TNFR1, Fas, and TRAILR mediate apoptosis. Activated TNFR1 triggers the polyubiquitination of RIP1 by inhibitors of apoptosis proteins (IAPs) cIAP1 and cIAP2 (Mahoney et al., 2008; Varfolomeev et al., 2008). The ubiquitination state of RIP1 determines whether it functions as a prosurvival scaffold molecule or a kinase that promotes cell death. RIP1 decorated with ubiquitin chains linked through lysine 63 (K63) of ubiquitin promotes the downstream activation of mitogen-activated protein kinases (MAPKs) and NF-κB (Figure 1, complex I), which govern the expression of prosurvival genes. The ubiquitin chains can be removed by the deubiquitinases cylindromatosis (CYLD) and A20 (Wilson et al., 2009). When K63 ubiquitination of RIP1 and TNF-induced NF-κB activation are reduced through cIAP1 and cIAP2 depletion by genetic ablation or Smac mimetics (small molecules that induce IAP degradation) (Mahoney et al., 2008; Varfolomeev et al., 2008), RIP1 switches its function to that of promoting cell death (Wang et al., 2008) (Figure 1).

When apoptotic cell death is blocked by pan-caspase inhibitors such as benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD), the cell uses necroptosis as an alternative cell death pathway (Festjens et al., 2007; Degterev et al., 2008; Hitomi et al., 2008). RIP1 kinase activity is crucial for this alternative pathway induced by the FasL, TNF, and TRAIL death receptors (Holler et al., 2000). Indeed, necrostatin-1, an allosteric RIP1 kinase inhibitor, inhibits death receptor-induced necroptosis in different cellular models, indicating that necroptotic signaling is mediated by a common mechanism (Degterev et al., 2008). Knockdown of CYLD expression inhibits TNF-induced necroptosis in cultured human Jurkat cells that lack the apoptotic factor FADD (Hitomi et al., 2008). In the presence of Smac mimetics, unubiquitinated RIP1 forms a complex with FADD and caspase-8 independently of the TRADD-dependent death-inducing signaling complex (Figure 1, complex IIB) (Wang et al., 2008). Under these conditions, RIP1 kinase activity and CYLD are critical for caspase-8 activation and apoptosis, suggesting that deubiquitinated RIP1 promotes the formation of apoptotic or necrotic signaling complexes (Hitomi et al., 2008; Wang et al., 2008). However, in the absence of Smac mimetics, RIP1 kinase activity is not required for death receptor-induced activation of caspase-8 (Wilson et al., 2009).

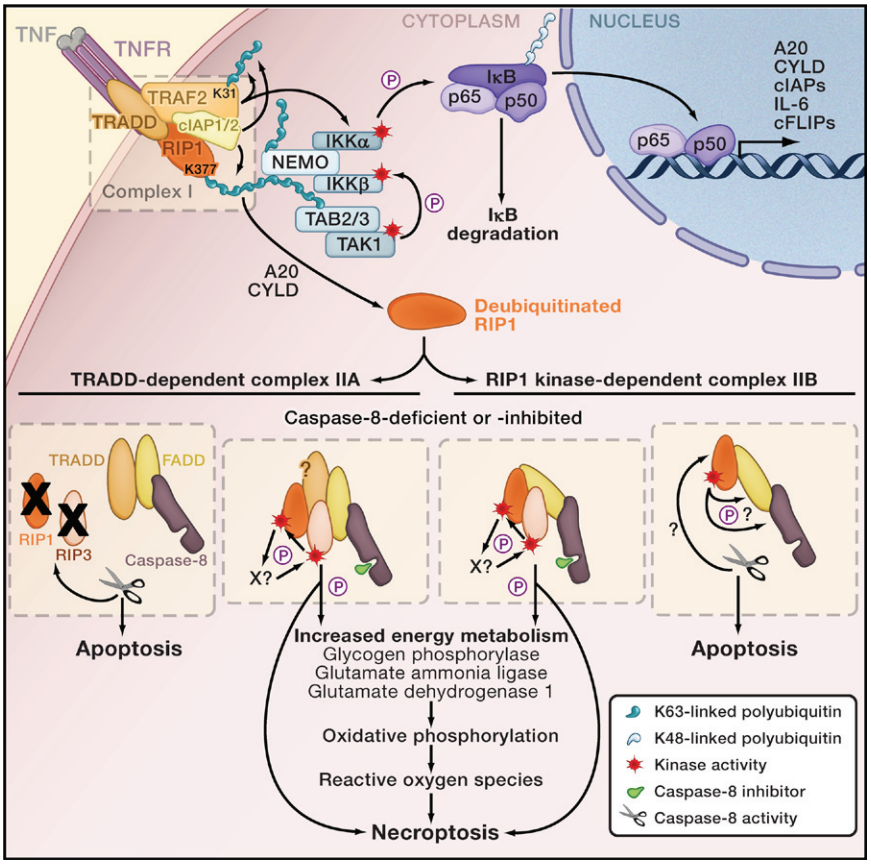

Figure 1. The Necrosome, a New Player in Death Receptor-Induced Necroptosis

Upon stimulation of the tumor necrosis factor receptor 1 (TNFR1), TNF-receptor-associated death domain (TRADD) provides a scaffold for the assembly of complex I at the plasma membrane by binding receptor interacting protein 1 (RIP1), TNF-receptor-associated factor 2 (TRAF2) or TRAF5, and the inhibitor of apoptosis proteins (IAPs) cIAP1 and cIAP2. This complex is crucial for activating NF-κB and mitogen-activated protein kinase (MAPK) pathways. cIAPs direct the formation of polyubiquitin chains linked through lysine 63 (K63) of ubiquitin on RIP1, thereby allowing it to interact with the TAK1/TAB2/3 (transforming growth factor-β-activated kinase 1/TAK1-binding protein 2/3) complex. K63-linked polyubiquitination on TRAF2 can also recruit the TAK1/TAB2/3 complex. TAK1 activates the IκB kinase (IKK) complex (composed of IKKα, IKKβ, and NEMO/IKKγ) that in turn phosphorylates the NF-κB inhibitor IκB to induce its K48 polyubiquitination and proteasomal degradation. Once freed from its inhibitor, NF-κB translocates to the nucleus to activate transcription. In a negative feedback loop, NF-κB upregulates A20 and CYLD, which remove K63-linked polyubiquitin chains from RIP1 and abolish its ability to activate NF-κB. After internalization of the TNFR1 receptor, secondary cytosolic complexes dependent on either TRADD (complex IIA) or RIP1 (complex IIB) are formed to initiate apoptosis. Complex IIA formation involves Fas-associated death domain (FADD)-mediated recruitment and activation of caspase-8 for RIP1 and RIP3 cleavage. Complex IIB is formed in the presence of Smac mimetics and acts independently of TRADD through a RIP1-FADD scaffold to activate caspase-8 in a RIP1 kinase-dependent way. It is assumed that these secondary complexes primarily contain the unubiquitinated form of RIP1 (generated by CYLD or A20 deubiquitination activity). Smac mimetics facilitate cIAP autoubiquitination and proteasomal degradation, thereby promoting the accumulation of unubiquitinated RIP1 that can be incorporated into complex IIB. When apoptosis is blocked by experimental (e.g., zVAD treatment) or physiological (e.g., viral proteins) conditions, RIP1 and RIP3 assemble a complex containing FADD and caspase-8 (TRADD may also be a component of this complex). Interdependent phosphorylation of RIP1 and RIP3 activates necroptotic signaling. RIP3 increases the activities of glycogen phosphorylase, glutamate ammonia ligase, and glutamate dehydrogenase 1. This stimulates the production of reactive oxygen species (ROS) due to increased oxidative phosphorylation. In some cells, ROS production as a consequence of RIP3 signaling is required for necroptosis.

RIP3, a Molecular Switch for Necroptosis?

Many cultured cell lines are protected against TNF-induced apoptosis by zVAD treatment, whereas others will undergo TNF-induced necroptosis when caspases are inhibited by the drug (Festjens et al., 2007). In addition to RIP1, RIP3 kinase activity has been implicated in this caspase-independent mode of cell death (Feng et al., 2007). However, although ectopic expression of RIP3 induces apoptosis, mice lacking RIP3 do not exhibit defects in development, NF-κB activation, or apoptosis (Newton et al., 2004). From the work of Cho et al. (2009), He et al. (2009), and Zhang et al. (2009), it is now clear that the expression of RIP3 renders cells permissive to necroptosis upon TNF treatment. Zhang et al. show through microarray analysis that RIP3 is not expressed in A-type NIH 3T3 cells protected from TNF-induced apoptosis in the presence of zVAD, whereas it is present in N-type NIH 3T3 cells that show enhanced TNF-induced necroptosis in the presence of zVAD (Zhang et al., 2009). Knockdown of RIP3 expression in the N-type cells prevents necroptosis in the presence of zVAD. Cho et al. and He et al. also demonstrate that the kinase activity and RIP homotypic interaction motif of RIP3 are required for necroptotic signaling. Together, these data implicate RIP3 as a key switch between TNF-induced necroptosis and survival and suggest that the complex containing RIP3 could function as a "necrosome" distinct from other complexes that induce apoptosis or NF-κB activation.

He and colleagues further implicate both RIP1 and RIP3 as essential mediators of TNF-induced necroptosis in cultured mouse L929 cells, macrophages in the presence of zVAD, and cultured human HT-29 cells in the presence of Smac mimetic and zVAD. In cultured cell lines where RIP3 is not expressed, a combination treatment of TNF and Smac mimetic results in apoptosis (which can be inhibited by zVAD) and does not induce a switch to necroptosis. Examining FADD-deficient cultured human Jurkat cells, Cho and colleagues identify RIP1 and RIP3 among 10 kinases that are required for TNF-induced necroptosis. Although they only address the involvement in necroptosis of the RIP kinases, several of the other identified kinases, such as TAO kinase 3 and protein kinase N1, are known to be involved in MAPK signaling or to interact with components of the TNF signaling pathway. For example, knockdown of the membrane-associated tyrosine/threonine kinase 1, which interacts with the antiapoptotic protein Bcl-2, protects FADD-deficient Jurkat cells against TNF-induced

necroptosis (Cho et al., 2009). Intriguingly, Bcl-2 modifying factor (Bmf), a proapoptotic Bcl-2 protein family member, is also required for death receptor-induced necroptosis (Hitomi et al., 2008), suggesting that Bcl-2 protein family members function in necroptosis as well as apoptosis.

Although RIP3 is not a component of the TNFR1-induced complex I (Cho et al., 2009; He et al., 2009), it associates with TRADD-dependent complex IIA or, in the presence of Smac mimetics, with RIP1-dependent complex IIB (Figure 1) (Cho et al., 2009; He et al., 2009). It is not known whether RIP3 and RIP1 form heterodimers within these complexes or interact as homodimers. RIP3 is likely activated and autophosphorylated at serine 199 within either complex IIA or -B (He et al., 2009), but the sequence of events for activation is not yet clear. In vivo, phosphorylation of RIP1 and RIP3 is clearly mutually dependent (Cho et al., 2009; He et al., 2009). Similar to the recruitment of RIP1 and RIP3 to FADD, the interaction between RIP1 and RIP3 is abolished by the RIP1 kinase inhibitor necrostatin-1 (Cho et al., 2009), indicating that the kinase activity of RIP1 is also required for stable RIP1 and RIP3 association. Necrostatin-1 also abolishes necroptosis-specific RIP3 phosphorylation. However, RIP1 does not phosphorylate RIP3 in vitro, whereas RIP3 weakly phosphorylates RIP1 (Cho et al., 2009). Taken together, these observations suggest the existence of a phosphorylation-dependent regulatory loop that involves RIP1, RIP3, and an unknown kinase (Figure 1).

Cho and colleagues show that FADD is required for RIP3 recruitment and activation in TNF-treated Jurkat cells. However, it is not clear how to reconcile these observations with the fact that FADD-deficient Jurkat cells treated with TNF can undergo necroptosis in a RIP1- and RIP3-dependent manner (Cho et al., 2009; Hitomi et al., 2008). Additionally, RIP1 phosphorylation still occurs in TNF-treated FADD-deficient Jurkat cells in the presence of the RIP1 kinase inhibitor necrostatin (Cho et al., 2009). This implies that RIP1 and RIP3 can be activated in the absence of FADD, possibly as components of TRADD-containing complex IIA (Figure 1) (Pobezinskaya et al., 2008). However, it has not yet been formally proven that TRADD is present in complex IIA when cells are treated with caspase inhibitors.

Cho et al. show that RIP1 in complex IIA is cleaved during apoptosis in a manner that can be inhibited by zVAD. Cleavage of RIP1 under apoptotic conditions could be a mechanism to ensure that the cell undergoes apoptotic death by inactivating the protein, thereby preventing RIP1-mediated gene activation and necroptosis. It seems likely that RIP3 is similarly cleaved in complexes containing active caspase-8 (Feng et al., 2007). Cho et al. conclude that although RIP1 cleavage by caspase-8 at aspartic acid 324 might inactivate the kinase, zVAD likely sensitizes cells to necroptosis via regulatory events other than preventing RIP cleavage because full-length RIP1 can still be detected in complex IIA in the absence of caspase inhibitors. In view of its known off-target effects, it is possible that zVAD has additional necrosis-sensitizing functions (Festjens et al., 2007). However, in T cells where limited caspase-8 activation is required for proliferation, loss of caspase-8 results in antigen- or mitogen-activated necroptosis that requires RIP1 (Ch'en et al., 2008) and likely also RIP3 (Cho et al., 2009). Caspase cleavage of RIP3 abolishes its ability to activate caspase-independent cell death (Feng et al., 2007), supporting the idea that zVAD functions by blocking RIP kinase cleavage to allow necroptosis to proceed (Zhang et al., 2009). Nonetheless, zVAD could also prevent capsase 8 from cleaving as yet unknown necroptotic factors; additional work will be required to resolve zVAD's precise mechanism of action.

RIP3 Governs the Cell's Metabolic State

Reactive oxygen species (ROS) derived from mitochondria are required for TNF- and double-strand RNA-induced necroptosis in L929 cells (Festjens et al., 2007). Cho et al. and Zhang et al. now show that RIP3 is required for TNF-induced ROS production during necroptosis in L929 cells, N-type NIH 3T3 cells, macrophages, and mouse embryonic fibroblasts. However, ROS quenching does not prevent necroptosis in HT-19 cells treated with a combination of TNF, Smac mimetic, and zVAD, indicating that ROS production is only required for necroptosis in some cell types or conditions.

Zhang et al. identify seven metabolic enzymes in the RIP3 complex immunoprecipitated from N-type NIH 3T3 cells that are treated with TNF/zVAD. Of these enzymes, RIP3 directly interacts with glycogen phosphorylase (PYGL), glutamate ammonia ligase (GLUL), and glutamate dehydrogenase 1 (GLUD1). RIP kinase activity is required to enhance the activities of all three enzymes both in vivo and in vitro. PYGL catalyzes the degradation of glycogen by releasing glucose-1-phosphate and so plays a key role in utilizing glycogen reserves as an energy source. GLUL is a cytosolic enzyme that catalyzes the condensation of glutamate and ammonia to form glutamine, whereas GLUD1 is found in the mitochondrial matrix and converts glutamate to α-ketoglutarate. Both GLUL and GLUD1 are essential for the use of glutamate or glutamine as substrates for ATP production in oxidative phosphorylation. Importantly, knockdown of PYGL, GLUL, or GLUD1 partially inhibits TNF/zVAD-induced ROS levels and necroptosis in N-type NIH 3T3 cells. Because breaking down glycogen and using glutamate and glutamine as energy substrates increases energy metabolism, the role of RIP3 in rendering cells more permissive to TNF-induced necroptosis could, at least partly, occur through increasing ROS production as a consequence of energy metabolism (Festjens et al., 2007) (Figure 1). Glutamine metabolism also indirectly feeds the Krebs cycle and the subsequent respiratory chain, which contributes to ROS production. Indeed, cells that are adapted to grow without glutamine and those that are pretreated with inhibitors of key enzymes of glutaminolysis or glycolysis show decreased TNF-induced ROS production, oxidative stress, and necroptosis relative to control cells (Festjens et al., 2007).

Physiological Roles for RIP3-Mediated Necroptosis

Necrostatins have recently been shown to prevent tissue damage in mouse models of cerebral ischemia and myocardial infarction, implicating necroptosis in pathological cell death (Vandenabeele et al., 2008). This form of cell death may occur only when caspase activation is impaired or when caspases are inhibited. Notably, viruses often express antiapoptotic proteins that prevent recruitment or activation of caspase-8 in the death-inducing complex to prolong cell viability and to facilitate

viral replication. Cho et al. show that when T cells lacking RIP3 are infected with vaccinia virus expressing the viral caspase inhibitor B13R/Spi2, they exhibit markedly reduced necrotic activation-induced cell death (AICD) in response to anti-CD3 antibody in comparison to wild-type T cells. Moreover, treatment of wild-type T cells expressing RIP3 with a combination of anti-CD3, zVAD (to mimic the effects of vaccinia virus infection), and necrostatin-1 inhibits AICD. The requirement for RIP3 in the induction of cell death in vaccina virus-infected cells suggests that in vivo, the protection against vaccina virus infection mediated by TNF could act through RIP3-dependent necroptosis. Indeed, mice lacking RIP3 have increased viral titers and succumb to vaccina virus infection, whereas wild-type mice do not (Cho et al., 2009). Necroptotic cell death likely serves two functions in vivo. First, it acts as a backup mechanism to kill virus-infected cells if the apoptotic pathway is blocked. Second, necroptosis may promote the release of intracellular danger-associated molecular patterns, which act as endogenous adjuvants that boost the innate immune response.

Interestingly, the pancreatitis-inducing effects of the chemical cerulein (including pancreas acinar cell loss and necrosis) are largely absent in mice lacking RIP3 in comparison to their wild-type littermates (He et al., 2009; Zhang et al., 2009). In view of the role of RIP3 in metabolic regulation, it is possible that the primary purpose of RIP3 signaling is to increase the cellular energy levels to promote cell survival or repair after toxic insult, injury, or infection. Thus, necroptosis may be a byproduct of overstimulation or dysregulation of this prosurvival pathway, similar to the role played by excess autophagy in promoting cell death instead of survival.

Conclusions and Perspectives

Death receptor-induced necroptosis is a physiologically relevant form of cell death that occurs in the absence of sufficient caspase-8 activity. The work of Cho et al., He et al., and Zhang et al. indicates that the RIP1/RIP3 necrosome regulates the decision between necroptosis and survival. Furthermore, apoptosis actively prevents necroptotic signaling through the cleavage of RIP1 and RIP3 by caspases. Thus, apoptotic pathways appear to be mainly controlled by the proteolytic caspase network, whereas necroptotic signaling is controlled by a kinase cascade. The RIP3 signaling cascade further connects necroptosis with energy metabolism, suggesting a role for necrotic cell death in metabolic diseases such as diabetes and obesity. The potential involvement of necroptosis in disease could lead to the development of kinase inhibitors (in addition to the necrostatins) that prevent pathological cell death resulting from ischemia-reperfusion injury as occurs following organ transplantation, cardiac infarction, stroke, or traumatic brain injury. The findings of Cho et al. also establish an in vivo role for RIP1/RIP3-mediated necroptosis in antiviral responses, suggesting that inhibition of necroptosis, like inhibition of apoptosis, might be a strategy used by viruses to evade the immune system. A better understanding of alternative cell death pathways could also be exploited therapeutically in the treatment of cancers, which often inactivate apoptotic pathways. In the coming years, our view of this alternative cell death pathway will be broadened by further identification of RIP1 and RIP3 substrates, molecular signal transducers that lead to necroptosis, and physiological and pathological conditions involving necroptosis.

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