dsRNA-Activation of TLR3 and RLR Signaling: Gene Induction-Dependent and Independent Effects

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Double-stranded (ds) RNA has diverse roles in host defense and disease prevention. dsRNA, produced by viral replication, elicits strong antiviral responses in host; similar protective responses can also be triggered by cellular dsRNA produced by necrotic, apoptotic, or otherwise stressed, uninfected cells. dsRNA is recognized in the cell by a large family of dsRNA-binding proteins, among which are the pattern recognition receptors (PRRs), toll-like receptor 3 (TLR3), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). TLR3 signals from the endosomal membrane where it senses extracellular dsRNA that has been endocytosed, whereas RLRs signal from the cytoplasm using a mitochondrial adaptor protein. In this review, we will summarize the signaling pathways used by these 2 PRRs, which lead to the activation of specific transcription factors and the induction of many proinflammatory and antiviral genes. However, it is becoming increasingly clear that all host responses are not mediated by the products of these induced genes; signal-dependent post-translational modifications of existing proteins can also profoundly change cellular properties. We will discuss how Src activation by TLR3 changes cell migration, adhesion, and proliferation rates and how IRF-3 activation by RLR triggers a gene induction-independent pro-apoptotic pathway that provides strong antiviral protection.

Introduction

DOUBLE-STRANDED (ds) RNA, generated as a byproduct of viral replication or necrotic cells, is a potent danger signal for the host cells to trigger innate and adaptive immune responses. For dsRNA viruses, the genome of infecting viruses can directly generate dsRNA inside the cells. For single-stranded (ss) RNA viruses, dsRNA RNA formed as replication intermediates, ssRNAs with extensive secondary structures, and loop-back dsRNA defective genomes serve as sources of dsRNA. RNA polymerase III has been shown to generate dsRNA from dsDNA, which are produced by DNA viruses or intracellular bacteria. Alternatively, viral mRNAs encoded by opposite strands of DNA viral genomes can form dsRNA. In addition to viral infections, cellular RNA, generated by tissue damage or necrotic cells, contains substantial ds structures to serve as potential sources of dsRNA. A synthetic dsRNA, poly(I:C), is often used as an experimental mimic to trigger host's response to virus infection. Cellular proteins, which specifically recognize dsRNA, known as dsRNA-binding proteins, often share similar structural motifs for dsRNA-binding. Although cellular functions of many dsRNA-binding proteins are not fully known, they are of broad biological significance. Among the dsRNA-binding proteins of known functions, 1 family comprises of enzymes, such as dsRNA-dependent protein kinase (PKR), 2'-5' oligoadenylate synthetase (OAS), and adenosine deaminases acting on RNA, all of

which mediate different cellular antiviral responses (Saunders and Barber 2003; Sadler and Williams 2008; Samuel 2011; Chattopadhyay and others 2012). The second family constitutes pattern recognition receptors (PRRs), for example, the toll-like receptor 3 (TLR3) and RNA helicases such as, the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and Nod-like receptors (Kawai and Akira 2008, 2010; Nakhaei and others 2009; Loo and Gale 2011; Yu and Levine 2011; Dixit and Kagan 2013). Extracellular dsRNA is endocytosed and transported to endosomal lumen for presentation to TLR3, whereas cytosolic dsRNA generated during viral replication is directly recognized by cytosolic RLRs. These receptors initiate cascades of signaling pathways leading to the transcriptional upregulation of dsRNA-induced genes, many of which encode cytokines, such as interferon, and other antiviral, proinflammatory, and antitumor genes. All cellular functions of these signaling cascades are not mediated by the induced genes; some effects do not require new gene expression. In this review, we will discuss how dsRNA signals through TLR3 and RLR and what are the gene induction-dependent and independent functional effects of these signaling pathways on cellular physiology.

dsRNA as a Regulator of Gene Expression

dsRNA is a potent regulator of multiple cellular functions; many of these functions of dsRNA are mediated by

transcriptional regulation of an array of cellular genes, including the interferon genes. Externally added or transfected dsRNA can activate multiple transcription factors, for example, NF- κ B, IRF-3, c-JUN, and ATF-2, via engagement of distinct signaling pathways (Sen and Sarkar 2005; Kawai and Akira 2008, 2010; Yu and Levine 2011). These transcription factors can individually transcribe their target genes, whereas the coordinated action of all 4 transcription factors is required for the transcription of other genes, for example, IFN- β . Many dsRNA-inducible genes can also be induced by virus infection or IFN. Multiple studies have investigated the repertoire of genes induced by these agents and the results revealed both overlapping and unique sets of genes (Der and others 1998; Geiss and others 2001; Sen and Sarkar 2005). We have performed a microarray analysis of external dsRNA-regulated genes in IFN-deficient GRE cells and identified a vast array of genes that are not only induced, but also repressed by dsRNA (Geiss and others 2001). The genes induced by dsRNA include interferon-stimulated genes (ISGs), genes involved in apoptosis, genes for cytokines and growth factors, RNA synthesis, protein synthesis and degradation, metabolism and biosynthesis, transporters, cytoskeletal components, and extracellular matrix. The dsRNA-repressed genes include those involved in metabolism, cell cycle regulation, and cell adhesion. There is a striking difference between the groups of genes induced by extracellular dsRNA and Sendai virus infection (Elco and others 2005). Sendai virus infection activates cytoplasmic RNA helicases and, therefore, is independent of TLR3 (Strahle and others 2007; Rehwinkel and others 2010; Martinez-Gil and others 2013). Thus, we could conclude that the repertoires of genes induced by the 2 dsRNA receptors, TLR3 and RLR, are only partially overlapping.

Structural Basis of dsRNA Recognition by Cellular Proteins

dsRNA is recognized by a variety of cellular proteins, called dsRNA-binding proteins (DRBPs). Some of these proteins comprise of 1 or more evolutionary conserved dsRNA-binding domains (DRBDs). DRBDs consist of a conserved set of 65–70 amino acids, which bind to the A-form of double-helical RNA in sequence-independent manner, and regulate dsRNA-induced gene expression. The DRBD from human PKR forms a compact $\alpha\beta\beta\alpha$ structure with conserved hydrophobic residues. Mutation of the most conserved residues in DRBDs prevents dsRNA-binding and consequently dsRNA functions. Many biologically important DRBPs, for example, OAS1, TLR3, and RIG-I, do not contain well-defined DRBDs. Structural and biochemical studies of these proteins revealed key aspects of their dsRNA interaction and subsequent functions. We have used crystallographic and mutagenesis approaches to identify dsRNA-binding region of OAS1 (Hartmann and others 2003). OAS1 forms a positively charged groove at the interface of the N- and C-terminal domains; mutation of solvent-exposed positively charged amino acids in this groove reduced dsRNA-binding. The structural study also helps explain some of our biochemical studies, which led to propose a 2-step activation model for OAS1 (Hartmann and others 2003). The ectodomain of TLR3 consists of leucine-rich repeats (LRRs), which form a horseshoe-like structure (Bell and others 2005; Choe and others 2005). The N-terminal LRRs (1–3) and C-terminal LRRs (19–21) interact with the dsRNA

molecule. Structural studies further revealed that the dsRNA acts as a bridge for TLR3 homodimerization, which is critical for its biological functions. Similar structural studies indicate that the C-terminal domain (CTD) of RIG-I is critical for dsRNA-binding (Cui and others 2008). Mutation of critical residues that form a basic cleft in the CTD, abolishes the dsRNA-binding and function of RIG-I.

Role of TLR3 in Viral and Nonviral Pathogenesis

TLR3 recognizes extracellular dsRNA released from damaged tissues or virus-infected cells. TLR3 activation by dsRNA leads to direct induction of antiviral genes in virus-infected cells. Although TLR3 signaling is activated by a variety of virus infection, the role of TLR3 in viral pathogenesis is complex, with both pro- and anti-viral effects. In addition to the cell-intrinsic antiviral function, TLR3 signaling also regulates the innate and adaptive immune responses: dsRNA regulates maturation of dendritic cells (DCs), which in turn promotes antigen-specific T-cell responses (Kumar and others 2008). *In vivo* studies showed mice lacking TLR3 or a lethal mutation in TRIF are highly susceptible to the infection by mouse cytomegalovirus, with 1,000-fold higher viral titers in spleen, compared to the WT mice (Tabeta and others 2004). The most convincing antiviral role of TLR3 is reflected by its ability to control HSV-1 replication in CNS (Zhang and others 2007, 2013). A dominant negative mutant of TLR3 has been identified in a subset of HSV-1 patients, indicating protective TLR3 functions. TLR3^{-/-} mice are highly susceptible to HSV-2-induced CNS infection (Reinert and others 2012). Contrary to the anti-HSV activity, TLR3 has been shown to promote West Nile virus (WNV) pathogenesis (Wang and others 2004). TLR3 deficiency causes resistance to WNV-induced encephalitis in mice; TLR3^{-/-} mice show reduced viral load and inflammation in the WNV-infected brain, compared with the WT mice. Genetic evidence shows that TLR3 plays a critical role in a number of nonviral diseases. A major anti-angiogenic function of TLR3 has been described in clinical study and animal models (Kleinman and others 2008). Subsequent genetic studies revealed that the anti-angiogenic effect of TLR3 is independent of IRF-3, but dependent on NF- κ B activity. Age-related macular degeneration (AMD) is a common cause of irreversible visual impairment and TLR3 signaling is causally related to the disease progression. A variant of TLR3 (L412F) protects against AMD in human patients, probably by suppressing the death of retinal pigmented epithelial cells (Allikmets and others 2009). TLR3 signaling contributes to the protection against dextran sulfate sodium (DSS)-induced colitis, an experimental model for inflammatory bowel disease and Crohn's disease. Subcutaneous, but not intragastric, injection of dsRNA protects the mice against DSS-induced colitis; the protective role of dsRNA is ablated in TLR3^{-/-} mice (Vijay-Kumar and others 2007). A recent study further demonstrates that TLR3 signaling induced by dsRNA of commensal bacteria can be protective against DSS-induced colitis. The observed protective function is mediated by TLR3-induced IFN- β production by intestinal DCs (Kawashima and others 2013). TLR3 also plays a protective role in type I diabetes mellitus, which is caused by selective destruction of islet beta cells secreting insulin (Castano and Eisenbarth 1990). Viral infection can also induce type I

diabetes and TLR3 in hematopoietic cells can limit beta cell infection. Chimeric mice containing TLR3^{-/-} hematopoietic cells and WT stroma cells are sensitive to encephalomyocarditis virus strain D (EMCV-D)-induced diabetes, while those with WT hematopoietic cells and TLR3^{-/-} stroma cells were resistant to the disease (McCartney and others 2011). TLR3 signaling has also been shown to have antitumor activities via induction of cellular apoptosis using both extrinsic and intrinsic apoptotic pathways (Sun and others 2011).

TLR3 Signaling

TLRs represent a major group of PRRs, which are transmembrane proteins located in either the plasma membrane or internal membranes. The ectodomain of TLR3, containing 23 LRRs, is located in the endosomal lumen, whereas the cytoplasmic domain of TLR3 consists of the linker region (LR) and the toll/interleukin-1 receptor (TIR) domain. The LR regulates the subcellular localization of TLR3, whereas the TIR domain serves as a platform for the assembly of the signaling complex. TLR3 is the only TLR that does not use MyD88 as an adaptor protein; it uses TRIF as its downstream adaptor molecule (Yamamoto and others 2003). TRIF contains a TIR domain that interacts with the TIR domain of TLR3. Recruitment of TRIF to TLR3 is considered to be the key event that dictates the downstream signaling process. Upon interacting with TLR3, TRIF undergoes oligomerization and recruits TBK1 or IKKε, which phosphorylates IRF-3 (Fitzgerald and others 2003; Sharma and others 2003). The NF-κB branch of TLR3 is activated by TRIF-dependent recruitment of RIP1, TAK1, and IKK. Activation of these transcription factors leads to their nuclear translocation, where they bind to their respective promoter elements of the target genes. We have been investigating the steps of TLR3 signaling that follow dsRNA-binding, but precede TRIF recruitment. In the process, we discovered that, unlike other TLRs, TLR3 needs to be tyrosine-phosphorylated after dsRNA-binding but before TRIF recruitment (Fig. 1). The cytoplasmic domain of TLR3 contains 5 tyrosine residues, and phosphorylation of at least Tyr759 and Tyr858 is essential for TLR3 signaling (Sarkar and others 2004); mutation of either of these 2 tyrosine residues produces a protein that cannot signal. We have identi-

fied 2 protein tyrosine kinases, EGFR and Src, as the enzymes responsible for phosphorylating them (Yamashita and others 2012a). In growing cell cultures, there is abundant EGFR in the endosomal membrane, from where TLR3 signals. Binding of dsRNA to the ectodomain of TLR3 in the endosomal lumen, changes the conformation of its cytoplasmic domain and exposes the EGFR-binding site in its LR. EGFR binding to TLR3, but not its enzymatic activity, is required to recruit Src to the complex. The 2 kinases phosphorylate the 2 Tyr residues of TLR3 with high specificity; EGFR phosphorylates Tyr858 and Src phosphorylates Tyr759. TRIF can bind only to the dual phosphorylated TLR3 and trigger transcriptional signaling. Another kinase, PI3K kinase (PI3K), binds to activated TLR3 and is required for the full activation of IRF-3 and NF-κB (Sarkar and others 2004, 2007). The above observations have revealed an unexpected connection between TLR3-mediated innate immunity and EGFR and Src, which are cell growth regulators and potential oncoproteins.

Gene Induction-Independent Cellular Effects of TLR3 Signaling

dsRNA-activated TLR3 signaling changes many properties of the stimulated cell; they were all thought to be mediated by the products of the TLR3-induced genes. However, our detailed study of TLR3 signaling has uncovered a novel adaptor-independent branch of TLR3 signaling, which does not lead to gene induction, but affects multiple cellular properties, for example, cell migration, adhesion, and proliferation (Yamashita and others 2012b) (Fig. 1). The new branch of TLR3 signaling is triggered by activation of Src, by auto-phosphorylation, upon its binding to the TLR3-EGFR complex. This step precedes TRIF-recruitment and the resultant gene induction; hence, the Src-branch of TLR3 signaling is active in TRIF^{-/-} cells, treated with dsRNA, without any accompanying gene induction. Thus, the 2 branches of TLR3 signaling require several common early steps but then bifurcate before the TRIF-TLR3 interaction. Activated Src can change many properties of the cell. We have demonstrated that the migration of multiple cell types, both primary cells and cell lines, is affected upon TLR3 activation by dsRNA-treatment or influenza virus infection. The cell migration effect is biphasic: dsRNA-treatment

FIG. 1. Activation of TLR3 by extracellular dsRNA and its functions. Model showing the steps of TLR3 activation upon dsRNA-treatment and various branches of downstream signaling. dsRNA, double-stranded RNA; TLR3, toll-like receptor 3.

causes immediate increase in cell motility followed by its strong inhibition. The first phase is mediated by dsRNA-induced phosphorylation and activation of Src, whereas the second phase results from the sequestration of activated Src in lipid rafts, thus decreasing its active cytoplasmic pool. As expected, in triple knockout cells, restoration of expression of Src, but not 2 other closely related Tyr kinases, Yes and Fyn, restored the TLR3-mediated effect of dsRNA on cell migration. A physiological effect of the Src branch of TLR3 signaling was tested in primary human vascular endothelial cells which, in culture, form microtubule networks by a process that needs cell migration. TLR3 activation caused a strong inhibition of microtubule formation, thus providing a possible basis for the observed genetic connection between TLR3 and angiogenesis. Two other effects of Src, cell adhesion and cell proliferation, are also modulated by TLR3 activation. A clinical study reported that treatment with dsRNA reduces the risk of metastatic relapse, suggesting a suppression of cell migration, in TLR3-positive but not in TLR3-negative breast cancers (Salaun and others 2011). These results demonstrate that activated TLR3 can engage Src to trigger multiple cellular effects and reveal a link between innate immune response and cell growth regulation.

RIG-I-Like Receptors

RLRs mediate their cellular functions by a variety of mechanisms. Mice, with gene knockout of various components of the RLR signaling pathway, are susceptible to infection by a vast number of viruses (Gitlin and others 2006; Kumar and others 2006; Gitlin and others 2010). Although, RLRs are primarily activated during RNA virus infections, replication of various DNA viruses can also be inhibited by RLRs (Rehwinkel and Reis e Sousa 2010). The downstream effector molecules in RLR signaling are the induced IFNs and the ISGs, which inhibit specific steps of viral replication. RLR signaling can induce cell death in various cell types (Rintahaka and others 2008; Garcia and others 2009) and triggering dsRNA-induced apoptosis is being considered as a therapy against cancer. The apoptotic effects of RLR signaling are often independent of IFN; other induced pro-apoptotic proteins play critical roles. In addition to the apoptotic caspases, RIG-I signaling can activate caspase-12 in WNV pathogenesis (Wang and others 2010). RIG-I-activated caspase-12 is required for protection against WNV; caspase-12-deficient mice show increased viral load and greater morbidity upon WNV challenge. RIG-I signaling activates inflammasome by 2 signaling complexes: one involving MAVS/CARD9/Bcl-10 to activate NF- κ B responses and the second complex containing RIG-I/ASC/caspase-1 for the activation of inflammasome (Poeck and others 2010). MDA-5, in addition to its pro-apoptotic role, activates anti-proliferative autophagic response upon cytosolic dsRNA signaling in melanoma cells; the mechanism behind this function remains unclear (Tormo and others 2009).

RNA Recognition and Activation of RLRs

The RLR family comprises 3 closely related members of RNA helicases, RIG-I, MDA-5, and LGP2, which can detect cytoplasmic dsRNA, generated during replication of RNA viruses (Kawai and Akira 2008; Loo and Gale 2011; Dixit and Gagan 2013). All 3 members contain centrally located

RNA helicase domains, whereas RIG-I and MDA-5, but not LGP2, have 2 N-terminal caspase-recruitment domains (CARDs). The helicase domain is responsible for the recognition of RNA, whereas the CARD domains are required for downstream signaling. LGP2, which lacks the CARD domains, was thought to be a repressor of the cytosolic RNA signaling; however, recent studies suggest that LGP2 co-operates with RIG-I and MDA-5 functions (Moresco and Beutler 2010; Satoh and others 2010; Childs and others 2013). RLRs are known to recognize dsRNA and RNA with 5'-triphosphate ends, generated during replication of RNA viruses (Hornung and others 2006; Pichlmair and others 2006; Goulet and others 2013). Studies have shown that the length of dsRNA is a determinant for the recognition by specific RLRs; RIG-I and MDA-5 can preferentially detect short and long dsRNA species, respectively (Kato and others 2008). Poly(I:C), an artificial analog of dsRNA, is a mixture of various lengths of RNA species, and, therefore, can be recognized by both RIG-I and MDA-5. Normally, cellular RNAs do not contain dsRNA structures and their 5'-ends are typically capped and, therefore, escape recognition by the RLRs. However, RNase L-derived self RNAs can be recognized by RIG-I and MDA-5 to amplify the antiviral signaling of RLRs (Malathi and others 2007). RIG-I and MDA-5 exhibit selectivity toward the recognition of RNA viruses: RIG-I is the predominant cytoplasmic sensor for the members of Paramyxoviridae, Orthomyxoviridae, and Rhabdoviridae, whereas MDA-5 is primarily responsible for the recognition of Picornaviridae (Kato and others 2006; Le Goffic and others 2007; Feng and others 2012; Kuo and others 2013). WNV and dengue virus, from Flaviviridae, and Reoviridae, can be recognized by both RIG-I and MDA-5 (Loo and others 2008; Sherry 2009; Errett and others 2013; Lazear and others 2013). DNA viruses and bacteria can also be sensed by RIG-I after their DNA is transcribed into uncapped RNA by cytosolic RNA polymerase III (Chiu and others 2009). RIG-I and MDA-5 are present in low levels in uninfected cells; viral infection causes significant increase in the cellular levels of these receptors via IFN-signaling. Activation of RLRs is a topic of intense current investigation; a simplified view is that the RLRs are present in a closed conformation in uninfected cells and dsRNA-binding induces conformational changes that activate the downstream signaling. Recent studies suggest that PKC-mediated phosphorylation of RIG-I CARDs maintains its closed conformation in uninfected cells (Gack and others 2010). Subsequent studies indicate that upon binding to viral RNA, dephosphorylation by protein phosphatase 1 (PP1) activates RIG-I signaling (Wies and others 2013). The dephosphorylation of RIG-I allows access to TRIM25, an ubiquitin E3 ligase, which K63 ubiquitinates the RIG-I CARD. This step is critical for oligomerization of RIG-I and its interaction with the downstream adaptor, MAVS (Gack and others 2007). A distinct role of TRIM25 has been reported in producing unanchored ubiquitin chains, which noncovalently bind to RIG-I for its activation (Zeng and others 2010). A recent study indicates the role of a deubiquitinating enzyme, USP15, which inhibits TRIM25 degradation, thereby promoting RIG-I activation (Pauli and others 2014). Another ubiquitin E3 ligase, Riplet, has been shown to ubiquitinate the C-terminal domain of RIG-I, which is also necessary for its activation (Oshiumi and others 2010). MDA5, which has structural and functional

similarities with RIG-I, shares some of these activation features.

RLR Signaling

Upon activation by cytoplasmic dsRNA, RIG-I and MDA-5 activate downstream signaling via the CARD-containing adaptor protein MAVS, which binds to them through CARD–CARD interaction. MAVS is located on the mitochondrial outer membrane, and interaction with RIG-I and MDA-5 facilitates its oligomerization (Seth and others 2005; Hou and others 2011). Oligomerization of MAVS is important for the assembly of signalosome complex, containing TRAF3 and 2 I κ B-related kinases, TBK1 and IKK ϵ (Fig. 2). These kinases directly phosphorylate IRF-3 and IRF-7, resulting in their translocation from cytoplasm to the nucleus, which activates transcription of antiviral genes including interferon and ISGs. MAVS also serves as a platform for the activation of NF- κ B via FADD and caspase-8/10-dependent pathway to regulate the expression of pro-inflammatory genes. Recent studies indicate that MAVS is located on 2 additional sub-cellular organelles, peroxisomes and MAMs, both of which are essential for the activation of downstream transcription factors (Dixit and others 2010; Horner and others 2011). Peroxisomal MAVS has been shown to induce a distinct set of ISGs, but not IFN- β ; however, MAMs-associated MAVS has been shown to coordinate the signaling from mitochondrial and peroxisomal MAVS. Although, the RLRs play a prominent role in triggering innate defenses in epithelial cells, myeloid cells and cells of central nervous system, their actions are not essential for induction of IFNs in plasmacytoid DCs, which specifically use the TLR-dependent responses (Loo and Gale 2011).

Activation of IRF-3, as a Transcription Factor, by RLR Signaling

Activated IRF-3 dimerizes and translocates to the nucleus where it recognizes a specific sequence motif, ISRE, present in the regulatory regions of the target genes (Hiscott 2007). However, to promote their transcription, it needs to interact with the co-activator, CREB binding protein (CBP); surprisingly, this interaction requires a bridging protein, β -catenin.

Moreover, β -catenin is an acetylated protein and its ability of to serve this function depends on its state of acetylation (Li and others 2008); only deacetylated β -catenin is active. Acetylated β -catenin can bind to IRF-3, but not CBP. HDAC6 is the cytoplasmic enzyme that deacetylates β -catenin; consequently, its absence, or its inhibition by chemicals, impairs IRF-3-mediated gene induction (Chattopadhyay and others 2013a). Like IRF-3^{-/-} mice, HDAC6^{-/-} mice are more susceptible to virus infection, compared with WT mice (Chattopadhyay and others 2013a). As described below, we have uncovered another mode of RLR-mediated IRF-3 activation converting it to a pro-apoptotic factor, not a transcription factor.

A New Branch of RLR Signaling Through IRF-3: RIPA

We have discovered a new branch of RIG-I signaling, in which IRF-3 is differentially activated to trigger a direct pro-apoptotic effect, which we called RIG-I-induced IRF-3-mediated pathway of apoptosis (RIPA) (Peters and others 2008; Chattopadhyay and Sen 2010; Chattopadhyay and others 2010) (Fig. 2). RIPA is independent of type I IFN signaling and NF- κ B activity, but RIG-I-activated IRF-3 is essential (Peters and others 2008). RNA viruses trigger RIPA by directly activating RIG-I signaling, whereas DNA viruses use the intermediate RNA polymerase III-dependent step to trigger RIPA. In RIPA, RLR activation of IRF-3 triggers its interaction with BAX and the 2 proteins translocate to the mitochondria to trigger cytochrome C release and apoptosis. RIPA requires many of the same proteins that are required for RIG-I signaling to activate IRF-3 as a transcription factor. But it needs a few additional proteins as well; 2 TRAF proteins, TRAF2 and TRAF6, are specifically required for the activation of RIPA. Conversely, although HDAC6 and PKC- β activities are required for the transcriptional activity of IRF-3 (Chattopadhyay and others 2013a), these 2 proteins are not required for RIPA (our unpublished observation), indicating a distinct, transcription-independent apoptotic effect of IRF-3. The 2 activities of IRF-3 can be readily separated by introducing appropriate mutations to the protein. Mutants of IRF-3 that are transcriptionally inactive, due to either deficiency in phosphorylation of critical Ser/Thr

FIG. 2. RIG-I signaling pathways activated by cytoplasmic dsRNA. Cytoplasmic dsRNA, recognized by RIG-I initiates 2 branches of MAVS-dependent signaling, transcriptional activation of genes and an apoptotic response, RIPA, that is temporally regulated. RIG-I, retinoic acid-inducible gene I; RIPA, RIG-I-induced IRF-3-mediated pathway of apoptosis.

residues, or the absence of the DNA-binding domain, are still active in RIPA (Chattopadhyay and Sen 2010; Chattopadhyay and others 2010). Moreover, RIPA activation does not require any new protein or mRNA synthesis. By inducible expression of various levels of IRF-3, we have shown that although the transcriptional activity can be achieved at a low level of IRF-3, a higher level of IRF-3 expression is required for activating RIPA. It is, therefore, possible that a distinct pool of IRF-3 activates RIPA (Chattopadhyay and others 2013b). RIPA requires IRF-3-mediated direct activation of a pro-apoptotic, BH3-only protein, BAX, an activator of intrinsic apoptosis pathway. The activation of BAX is achieved by its direct interaction with IRF-3 and concomitant translocation of the IRF-3:BAX complex to the mitochondrial membrane. Translocation of BAX to the mitochondria triggers its oligomerization, followed by release of cytochrome C into the cytosol, activation of caspase-9, which in turn activates the executioner caspase, caspase-3. Using cell-free system, we have shown that BAX can be activated by direct interaction with IRF-3 to release cytochrome C from the mitochondria. Unexpectedly, our analyses revealed a previously uncharacterized BH3-like domain in IRF-3, which is distinct from its transcriptional-activation domain, and is required for the interaction with, and activation of BAX. Mutation of the critical residues of the BH3-like domain of IRF-3 abolishes the BAX-interaction and RIPA.

Temporal Regulation of RIPA

Viruses, which trigger RLRs, activate both functions of IRF-3 very soon after infection; despite that, Sendai virus-infected cells do not die quickly. This observation led to the realization that RIPA is temporally controlled after virus infection; it is not functional in the early phase of the infection because viruses activate a PI3K/AKT-mediated cellular survival pathway to delay the apoptotic response (Peters and others 2008). If PI3K activity is inhibited, manifestation of RIPA is accelerated. Our detailed analyses revealed that the virus-activated PI3K/AKT signaling stabilizes the cellular pool of XIAP, an inhibitor of cellular apoptosis, to prevent early induction of apoptosis (White and others 2011). XIAP inhibits the activation of caspase-9 by the apoptosome complex, consisting of APAF-1, procaspase-9, and cytochrome c. Although early after infection, cytochrome c is released from the mitochondria by virus-activated RIPA, the PI3K/AKT/XIAP regulatory axis prevents the formation of active apoptosome complex. AKT-induced phosphorylation inhibits the degradation of XIAP; inhibitors of PI3K block AKT activation, causing rapid degradation of XIAP and accelerated RIPA. In the later phase of infection, the level of XIAP goes down and the brakes on the apoptosome complex are released. This temporal regulation of RIPA might help the virus by first keeping the cell alive to replicate and then bursting it open to disseminate.

Biological Significance of RIPA

RIPA provides a defense mechanism by which the virus-infected cells commit suicide and it considerably contributes to the inhibition of viral replication and pathogenesis. By

genetic manipulation of the pathway-specific components, we evaluated the relative contribution of RIPA on viral replication and pathogenesis. IRF-3-deficient cells, where both pathways of IRF-3 are missing, showed enhanced viral replication. IRF-3^{-/-} mice are highly susceptible to pathogenesis caused by multiple viruses, for example, Sendai virus (SeV) (Chattopadhyay and others 2013a) and EMCV (Sato and others 2000). BAX^{-/-} cells, in which only the transcriptional branch of IRF-3, but not RIPA, is active, showed enhanced viral replication compared with WT cells. Moreover, BAX^{-/-} mice exhibit enhanced replication of EMCV in the brain and greater morbidity, compared WT mice (Chattopadhyay and others 2011). These results clearly demonstrate that RIPA is a major antiviral branch of RIG-I/IRF-3 signaling.

In cell cultures, RIPA prevents the establishment of viral persistence; in the absence of RIPA, cells become persistently infected (PI) with SeV (Peters and others 2008). We have studied viral persistence by experimentally eliminating the RIPA branch in human cells upon ablation of IRF-3 or RIG-I signaling. Cells expressing transcriptionally inactive, but RIPA-active IRF-3 mutants are spared from PI. Conversely, PI can be achieved in cells expressing a low level of IRF-3, which can activate the transcriptional, but not the RIPA, branch of IRF-3. Expression of normal levels of IRF-3 restores RIPA and induces apoptosis in PI cells. These results indicate that transcriptional activity of IRF-3 is not sufficient to inhibit SeV-persistence. This notion was further reinforced by studying the mechanism of natural PI establishment from WT cell populations (Chattopadhyay and others 2013b). Many clonal PI isolates from SeV-infected WT MEFs were analyzed for their RIPA status; they all were RIPA-defective. Because SeV infection causes IRF-3 degradation, many of the PI isolates had no or low levels of IRF-3. In others, with normal levels IRF-3, RIPA was inactive because of the degradation of Caspase 3, the executioner caspase. Interestingly, these cells had functional transcriptional activity of IRF-3, which of course was not sufficient to inhibit viral persistence. Our studies clearly established that a novel branch of RIG-I signaling, RIPA, is capable of inhibiting viral replication, persistence, and pathogenesis.

RIPA-like activity has recently been reported in primary human monocytes, which do not support a productive infection by HTLV1 (Sze and others 2013). Detailed analyses revealed the existence of an IRF-3/BAX-mediated apoptotic pathway, which clears the virus-infected cells. The activation signal in HTLV1-induced apoptotic pathway is provided by STING, which detects viral reverse transcriptase intermediates. This study suggests a protective role of STING/IRF-3/BAX-mediated monocyte apoptosis to inhibit HTLV1 replication. In another study, RIPA-like activity has been associated with ethanol-induced liver injury, which leads to Alcoholic Liver Disease (Petrasek and others 2013). Unexpectedly, ethanol activates an apoptotic pathway mediated by STING/IRF-3/BAX, causes apoptosis of hepatocytes, and leads to liver injury. Analysis of the pathway revealed that the STING-activation is provided by ER-stress, induced by ethanol. These studies indicate that, although we have discovered and studied RIPA in the context of antiviral innate immunity, RIPA can be instrumental in regulating pathogenesis in many other diseases.

Conclusions

dsRNA is a broad and potent regulator of cellular functions. Its effects are mediated by dsRNA-binding proteins or receptor, which play important roles in both viral and non-viral diseases. The regulatory mechanisms of TLR3 and RIG-I signaling are only beginning to be uncovered and future studies will reveal cell- and tissue-specific regulatory mechanisms of these signaling pathways and their consequences in disease pathogenesis. dsRNA-binding proteins exhibit additional functions in microRNA biogenesis, thus bridging the fields of microRNA action and dsRNA-response, a connection that remains to be fully explored. Future research on the diverse biological roles of dsRNA will produce exciting and novel results.

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