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Preface

Toll-like receptor and pattern sensing for evoking immune response ☆

Interferon (IFN)-α/β (type I IFNs) are antiviral mediators [1] that induce various effectors required for host immunity. It has been known for 40 years that viruses or nucleotide derivatives including double-stranded RNA (dsRNA) trigger IFN induction in a variety of cell types [2]. In fibroblasts, viruses and polyI:C (a dsRNA analog) induce IFN-β in distinct manners [3]. While it is evident that type I IFN has a central role in anti-microbial immune responses, until recently the mechanisms leading to induction of IFN-α/β were unclear.

Recently, several microbial pattern-recognition receptors (PRRs) and their corresponding signaling pathways have been identified as molecular cascades that results in type I IFN induction. These cascades transmit signals that activate several transcription factors, among them the interferon regulatory factor (IRF) family, which include IRF-1, IRF-3 and IRF-7. The transcription factor IRF-1 and its regulatory factor IRF-2 were first reported to participate in the induction of IFN-α/β [4]. However, later studies revealed that IRF-3 and IRF-7 were more crucial as triggers of IFN-α/β induction [5]. Studies examining how viruses and polyI:C activate members of the IRF family to induce IFN-α/β production are beginning to identify links between microbial PRRs and IRFs.

PRRs consist of several receptor families, including Toll-like receptor (TLR)3/4, TLR7/9, RIG-I-like helicases (RLHs), DNA sensors and RNA-dependent protein kinase (PKR). PRRs contain NOD-like receptors (NLRs) which may additionally participate in microbial nucleotide recognition. These DNA/RNA sensors have their own unique adaptors and signal transduction pathways, which activate a variety of immune responses including cytokines and chemokine production, apoptosis, autophagy, regulatory T cell (Treg) induction, antibody (Ab) production, natural killer (NK) cell activation and cytotoxic T lymphocyte (CTL) induction. This issue of *Advanced Drug Delivery Reviews* (ADDR) reviews these DNA/RNA recognition proteins.and their unique functional profiles. Since elucidation of the functional roles of PRRs has only recently been described, discussion of each PRR has been assigned to authors who have focused on studying these nucleotide sensors.

Studies by Flavell and colleagues used TLR3 knockout mice to demonstrate that TLR3 initiates IFN-β induction in association with NF-κB activation [6]. However, it is still unclear how the IFN-β promoter is activated in this case. By developing a mAb against human TLR3 that blocked polyI:C-mediated IFN-β induction, Matsumoto et al. independently obtained similar results in human fibroblasts [7]. TLR3 recruits a unique adaptor molecule TIR-containing adaptor molecule (TICAM)-1 (or TRIF), which induces IRF-3 activation resulting in activation of the IFN-β promoter [8,9]. Soon after this report, Fitzgerald and Maniatis demonstrated that TICAM-1 links the IRF3-activating kinases TBK (TANK-binding kinase)1 and IKK (iκB kinase)ε [10]. In addition, studies showing that TLR4 recruits TICAM-1 in conjunction with TICAM-2 (TRAM), [11,12] partially explain the LPS-mediated IRF-3 activation previously reported by Akira's group [13]. These seminal studies facilitated identification of a critical link between the activation of the TLR signaling pathway and the IFN-β-inducing pathway.

Yoneyama and Fujita reported that recognition of dsRNA by the cytoplasmic protein RIG-I activates the IFN-β promoter [14]. Furthermore, the RIG-I family proteins MDA5 and LGP2 (later named RLHs) are sensors of RNA derivatives of viral origin [15]. Following these observations, it soon became clear that these cytoplasmic RNA sensors are primarily involved in detection of intrinsic viral replication in mammalian cells. These findings are consistent with the fact that RLHs are ubiquitously distributed in host organs/tissues. Since TLR3 localizes to the endosomes of dendritic cells (DCs) and rarely resides in the cell surface of human fibroblasts and epithelial cells [16], this receptor may be involved in extrinsic recognition of RNA duplex in phagosomes. Although the TLR3-TICAM-1 pathway in myeloid DCs potentially drives the cellular effectors NK and CTL [17], it is unclear whether direct viral RNA recognition by RIG-I/MDA5 is involved in the activation of cellular immunity. Thus, the functional differentiation between TLR3 and cytoplasmic viral sensors such as RIG-I is complex and equivocal.

TLR7 and TLR9 highly expressed by plasmacytoid DCs (pDCs), formerly called IFN-producing cells (IPC), respectively recognize viral RNA and DNA during viral infection and induce high levels of IFN-α/β [18]. Honda and Taniguchi suggested that activation of IRF-7, rather than IRF-3, is required for IFN-α production in pDCs [19]. MyD88 is the adaptor for IRF-7 activation [20]. Functional modulation of pDCs in concert with activation of these TLRs is currently under investigation.

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Some NLRs such as NOD1 and NOD2 have been shown to recognize bacterial cell-wall pattern molecules and induce danger signals to activate NF-κB, although the biology of most mammalian NLRs is still ill-defined. Recent reports suggested that NALP3 forms a caspase-1 activating molecular complex termed the inflammasome. Caspase-1 activation by the inflammasome promotes cytokine maturation, as in IL-1 family cytokines, IL-1β, IL-18 and IL-33 [21]. Multiple stimuli, including pathogen recognition, activate NALP3 to trigger inflammation that is mainly mediated by the IL-1 family cytokines [22]. The NALP3 inflammasome may detect endogenous danger signals such as uric acid and ATP. Further studies on each NLR member will clarify the importance of the biological function of NLRs.

Natural ligands of these RNA/DNA sensors are presumed to be viral genomes/mRNAs, although polyI:C and CpG DNA have been used as analogs of viral RNA and DNA. Since the nucleic acids properties of viral genomes are somewhat different from those of host nucleic acids through cytosolic modification [23–25], hosts must recognize these DNA/RNA differences. Are the recognition strategies of viral DNA/RNA in host cells sufficient to discriminate between the exact differences of virus species in terms of their nucleic acid properties? We should examine in detail the IFN-inducing signaling pathways and cellular responses in a variety of virus infections. Elucidating the signaling cascades and molecules in the IFN-inducing signaling pathways is a primary concern for species-specific viral infection and development of vaccines.

Vertebrates have developed an acquired immune system together with innate TLR/RLH/NLR systems. Although their relationship is unclear, they emerge simultaneously in lower vertebrates including fish [26]. What happens in persistently virus-infected cells in terms of infection versus host immune responses is an intriguing issue that would help to identify the origin of the human immune system.

Adjuvants are essential factors for the development of modern vaccines that promote various types of immune responses. Many studies have suggested that adjuvants are ligands for PRRs. For example, some anti-viral vaccines are effective since they contain sufficient PRR agonists, whereas other vaccines are less effective presumably due to the lack of contaminating PRR agonists. Tumors possess tumor-associated antigens but not PRR agonists, which may explain the minimal immune response against cancer in patients. Periodic fever syndromes with systemic inflammation in human patients are closely associated with a NALP3-mediated IL-1 overproduction. Here, we summarize our current understanding of the microbial pattern-recognition system and immune cellular outputs in association with diseases and pathogenesis.

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Pan-Vertebrate Toll-Like Receptors During Evolution

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Abstract: Human toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) to raise innate immune responses. The human TLR family was discovered because of its sequence similarity to fruit fly (*Drosophila*) Toll, which is involved in an anti-fungal response. In this review, we focus on the origin of the vertebrate TLR family highlighted through functional and phylogenetic analyses of TLRs in non-mammalian vertebrates. Recent extensive genome projects revealed that teleosts contain almost all subsets of TLRs that correspond to human TLRs (TLR1, 2, 3, 4, 5, 7, 8, and 9), whereas the urochordate *Ciona intestinalis* contains only a few TLR genes. Therefore, mammals likely obtained almost all TLR family members at the beginning of vertebrate evolution. This premise is further supported by several functional analyses of non-mammalian TLRs. We have summarized several teleost TLRs with unique properties distinct from mammalian TLRs to outline their specific roles. According to *Takifugu rubripes* genome project, the puffer fish possesses fish-specific TLR21 and 22. Surprisingly, phylogenetic analyses indicate that TLR21 and 22 emerged during an early period of vertebrate evolution in parallel with other TLRs and that the mammalian ancestor lost TLR21 and 22 during evolution. Our laboratory recently revealed that TLR22 recognizes double-strand RNA and induces interferon production through the TICAM-1 adaptor, as in TLR3, but unlike TLR3, TLR22 localizes to the cell surface. Therefore, differential expression of TLR3 and TLR22, rather than simple redundancy of RNA sensors, may explain the effective protection of fish from RNA virus infection in the water. In this review, we summarize the similarities and differences of the TLR family in various vertebrates and introduce these unique TLRs for a possible application to the field of clinical practices for cancer or virus infection.

MAMMALIAN TLR FAMILY

Mammalian toll-like receptors (TLRs) were discovered because of their sequence similarities to fruit fly (*Drosophila*) Toll, which plays a crucial role in both anti-fungal protection and dorsal and ventral pattern establishment in the embryo [1, 2]. The human genome encodes 10 TLRs, each of which recognizes different pathogen-associated molecular patterns (PAMPs); they are not involved in development. Among human TLRs, the function of TLR4 was the first to be revealed *via* the analysis of C3H/HeJ mice, which have a defective response to LPS endotoxin [3, 4]. Inquiry into the genetic basis of LPS resistance revealed a single locus (LPS) at which homozygosity for a codominant allele (LPSd) caused the endotoxin-unresponsive site. The codominant LPSd allele of C3H/HeJ mice corresponds to a missense mutation in the TLR4 ORF [5]. Now the functions of almost all human TLRs are known: TLR3, 5, 7, and 8 or 9 recognize viral double-strand RNA, flagellin protein, single-strand RNA, or CpG, respectively [5-11], and TLR2 and 6 and TLR2 and 1 form a heterodimer to recognize diacyl or triacyl lipopeptides, respectively [12-15]. Their functions were determined mainly by knockout mice analyses. Human patients who harbor mutations of the TLR gene exhibit abnormal innate immune responses. Missense mutations of TLR3 occur in patients with herpes simplex encephalitis, and the TLR3 allele confers dominant hyporesponsiveness to a TLR3 ligand polyI:C in fibroblasts [16]. IRAK4 is a component of TLR signaling, and mutation of IRAK-4 is found in children with recurrent infections with a poor inflammation response whose blood and fibroblasts do not respond to TLR ligands [17].

The innate immune system is composed of Tolls in invertebrates and TLRs in vertebrates, and distinct differences exist between mammalian TLRs and the arthropod Toll family. For example, mammalian TLRs directly recognize PAMPs, whereas *Drosophila* Toll receives the PAMP signal indirectly through endogenous proteins. Recently, crystal structures of mammalian TLRs have been reported, which illustrate the mammalian process. Kim, Lee and their colleagues described the crystal structure of the TLR4 extracellular domain in complex with MD-2 bound to eritoran, an analog of LPS, that antagonizes TLR4 signaling [18]. Jin, Lee and their colleagues reported the crystal structure of the complex of TLR1 and 2 extracellular domains bound to a synthetic lipopeptide agonist Pam3CSK4 [19]. In contrast, the fruit fly utilizes another strategy to recognize PAMPs. For example, the peptidoglycan-recognition protein PGRP-SA is a soluble-receptor that directly recognizes Gram-positive bacteria [20]. Microbial recognition by these receptors triggers the zymogen cascades that lead to the cleavage of the proform Spatzle into an activated form and ultimately to amplifying the Toll responses. The active Spatzle binds

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Toll and activates a signaling pathway that produces antibacterial peptides [21]. Phylogenetic analyses show that no orthologous relationship exists between mammalian and fruit fly Toll family members, and the two families supposedly developed independently during evolution [22, 23]. Thus, the next question is when the current human TLR subsets appeared during evolution.

VERTEBRATE TLR FAMILY

The draft genome sequence of the puffer fish (*Takifugu rubripes*) is firstly reported to be non-mammalian vertebrate genome [24], and its information provided a complete view of the puffer fish TLR family of genes. Interestingly, the puffer fish genome encodes orthologues of human TLR1, 2, 3, 5, 7, 8, and 9. TLR4 does not exist in this genome; however, a TLR4-like gene was reported in the genomes of several other teleosts, such as *Danio rerio* [22], although the functional features of this gene remain undetermined. Thus, the common ancestor of humans and teleosts is predicted to have lived in the Devonian Period and to have had TLR1, 2, 3, 4, 5, 7, 8, and 9 genes. In contrast to the teleost genome, the genome of the urochordate *Ciona intestinalis* contains only a few TLR genes [25], and that of *S. purpuratus*, a sea urchin, possesses ~200 TLRs with uncharacterized functions that are incomparable with the human TLR subsets [26, 27]. Interestingly, the lamprey (*Lamprey japonica*, a jawless fish) possesses TLR14a and 14b genes [28]. TLR14 is a member of the TLR2 subfamily, and the gene is present in the genome of teleosts and amphibians [22, 28]. This suggests that the current TLR subsets emerged before the mammalian ancestor diverged from the jawless fish ancestor. Based on chordate genome information and phylogenetic analyses, the prototype of the current human TLR family likely emerged during the Cambrian period when the vertebrate ancestor emerged [22].

Generally speaking, proteins from orthologous genes do not necessarily share common functions. Do the TLR orthologs conserved in fish and mammals have the same functions? Recent functional analyses revealed that the vertebrate orthologous gene products have the same functions as their human counterparts. For example, the rainbow trout TLR5 gene is up-regulated by stimulation with recombinant flagellin proteins, as shown in teleost IL-1βR. Interestingly, rainbow trout encodes another TLR5 gene, which lacks the transmembrane region and whose products are liberated from cells. The soluble TLR5s promote a teleost membrane type TLR5 chimera protein that mediates NF-κB activation in human cells. Therefore, teleost TLR5 is a receptor for flagellin, as is the case for human TLR5 [29]. Another teleost TLR, *Takifugu rubripes* TLR3, responds to the same ligand as does human TLR3. Recently, our laboratory showed that the *Takifugu rubripes* TLR3 gene is up-regulated by polyI:C stimulation in teleost cell lines, as is true for the human TLR3 gene, whose expression is induced in several cell species and cell lines [30]. TLR3 expression in both human and fish cells provides a responsiveness to the polyI:C and double-strand RNA [30]. Like mammalian TLR3, the teleost TLR3 is localized in the intracellular compartments and is largely merged with an ER marker, Calnexin, in HeLa cells [30]. These data indicate that both human and teleost TLR3 encompass a common RNA-sensing role against virus infection in the innate immune system. Therefore, the common ancestor of humans and teleosts likely contained the double-strand RNA recognition system involving TLR3. These functional conservations are also observed in other non-mammalian vertebrates.

Like human TLR2, chicken (*Gallus gallus*) TLR2 responds to lipoproteins [31]. The chicken likely has the following additional TLR2 subfamily members: TLR2-type1 and 2, TLR1-type1 and 2. Like human TLR2 subfamily members of TLR1, 2, and 6, the avian TLR2 subfamily members form heterodimers and TLR assembly is required for the recognition of PAMPs [32]. These functional similarities support the notion that TLR functions are conserved in vertebrates and that the vertebrate common ancestor had established the innate immune system that detects lipoprotein, peptidoglycan, LPS, flagellin, double- or single strand RNA, and CpG DNA as PAMPs before the vertebrate species diverged.

NON-PRIMATE TLR FAMILY

Interesting differences among vertebrate TLR families also exist. We previously discussed the presence of non-mammalian TLRs (TLR21 and 22) in the puffer fish genome [23], and they are expressed in various tissues, suggesting that they are not pseudogenes but rather functional in the puffer fish [23]. Subsequent analyses revealed presence of other non-mammalian TLRs, such as TLR23 and TLR14 and the non-primate TLRs TLR11, 12, and 13 [22]. Phylogenetic tree analyses showed that some of those TLRs are derived from the TLR2 lineage. The human TLR2 subfamily includes TLR1, 6, and 10, and the TLR2 subfamily is known to be divergent in several avian and teleost species. For instance, the chicken has two TLR2 and two TLR1 genes. The teleost genome contains several non-mammalian TLR2 subfamily members, such as TLR14, which is also found in the lamprey, as described above. Unlike those TLR2 subfamily members, TLR21 and 22 are not included in any clade of human TLRs, and they likely originated around the Cambrian period; this indicates that the human ancestor possessed TLR21 and 22 genes [22, 23].

What are the roles of non-mammalian or non-primate TLRs? Cells expressing TLR11, a non-primate TLR, fail to respond to any primate TLR ligands, but they do respond to uropathogenic bacteria [33]. Analysis of TLR11 knockout mice revealed the importance of TLR11 in IL-12 production from dendritic cells [34]. Recently, we discovered the function of a non-mammalian TLR, TLR22: *Takifugu rubripes* TLR22 expression confers responsiveness to double-strand RNA or polyI:C on transfected cells as human TLR3 responds [30]. This is surprising because teleosts also have TLR3, and indeed the teleost TLR3 protein responds to double-strand RNA in a manner similar to that of teleost TLR22. The question is why teleosts have two double-strand RNA recognition receptors.

We revealed two functional differences between TLR3 and TLR22. The first is that TLR3 and TLR22 discriminate between size-differences of double-strand RNA. TLR3 prefers to recognize short dsRNA (< 1 kbp), whereas TLR22 prefers long dsRNA (< 1 kbp) [30]. TLR3 and TLR22 also differ in their localization profiles. TLR22 is located on the

cell surface membrane [30], whereas the four human TLRs (TLR3, 7, 8, and 9) that recognize nucleic acids are localized in the early endosome or ER in myeloid cells [35-37]. Therefore, TLR22 is the only TLR that can recognize nucleic acids on the cell surface and transmit signals to induce cytokines.

The importance of TLR22 in teleosts is shown by its activity against RNA viruses. Several pathogenic RNA viruses infect teleosts. One of the birnaviruses, IPNV, causes necrosis in the pancreas of teleosts, and its genome is double-strand RNA [38]. When RTG-2 cells (derived from the kidney of rainbow trout) expressing TLR22 are infected with IPNV, TLR22 expression confers resistance to the virus to the RTG-2 cells.

TLR22 is widely conserved among teleosts and amphibians, but extensive genome projects failed to reveal the presence of the TLR22 gene in avian or mammalian genomes. Therefore, it seems likely that TLR22 is required for vertebrates that live in the water [30]. TLR22 is ubiquitously expressed in puffer fish tissues [23], but tissue-specific expression, with strong expression in the head and kidney, mild expression in the trunk, spleen, and gill, and undetectable expression in the intestine, liver, brain, and skin, has been reported for the Japanese founder (*Paralichthys olivaceus*) [39]. This finding illustrates that the expression pattern is different among different teleosts. Interestingly, both the puffer fish and Japanese flounder TLR22 genes are up-regulated by stimulation with polyIC, which is a synthetic analog of double-strand RNA [39]. Therefore, the TLR22 function seems to be conserved among teleosts. Teleosts possess two viral RNA-recognizing TLRs: TLR3 and TLR22. Double-strand RNA derived from RNA virus is recognized by TLR22 on the cell surface and simultaneously by TLR3 in the early endosome (Fig. **1**).

In addition to TLR22, vertebrates can have other non-mammalian TLRs, such as TLR21 [22, 23]. TLR21 is present not only in teleosts but also in *Xenopus tropicalis* and the chicken [22]. Phylogenetic analysis indicates that TLR21 is a member of the TLR11 subfamily, which includes mouse TLR12 and 13 and teleost TLR20 [22, 40]. Like TLR22, TLR21 is also widely expressed in various tissues, such as the liver, spleen, kidney, skin, and gill in both the puffer fish and catfish (*Ictalurus punctatus*) [23, 40]. The function of

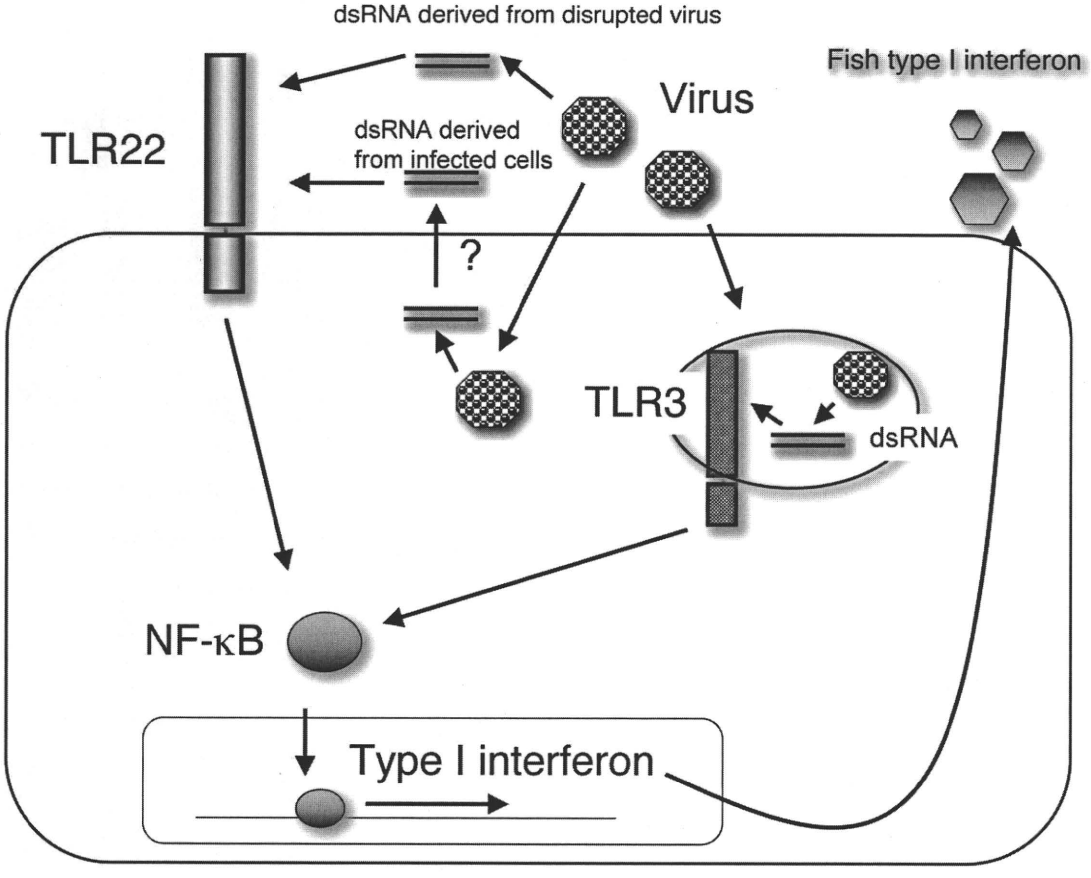

Fig. (1). Two double-strand RNA recognition pathways in teleosts.

TLR22 localizes at the cell surface and recognizes double-strand RNA derived from viruses. Teleost TLR3 proteins reside in the intracellular compartment and are expected to localize at the early endosome, as in human TLR3. When virions are disrupted and their genome RNA flows out to the extracellular space, one might surmise that the viral double-strand RNA is recognized by TLR22. In another case, when the viral RNA in the cytoplasmic region is exported from the cytoplasm to the intercellular space by exocytosis, the double-strand RNA can be recognized by TLR22. Teloeost TLR3 recognizes viral RNA at the early endosome as in human TLR3. Both TLRs can transmit the signal to induce type I interferon, which exerts anti-virus properties.

TLR21 remains to be determined, but we expect that TLR21 responds to the PAMPs that are not recognized by any other TLRs so far described. Considering that the puffer fish does not possess the TLR4 gene, teleosts should have another mode of detecting PAMPs derived from the Gram-negative bacteria because there are many pathogenic Gram-negative bacteria that infect teleosts in the water. Thus, the agonist candidate for TLR21 may be a component of the Gram-negative bacteria that induces a response through this non-mammalian TLR.

EVOLUTION OF THE VERTEBRATE TLR FAMILY

The phylogenetic tree of vertebrate TLR family members strongly supports the notion that the non-mammalian vertebrate TLRs emerged during the Cambrian period together with other mammalian TLRs, and thus the human ancestor should have possessed both current TLR subsets and those of non-mammalian vertebrates. Based on our knowledge of the functional coverage of the vertebrate TLR family members, the expected TLR subsets that the vertebrate common ancestor possessed would include at least the following 10 TLR members: TLR2, 3, 4, 5, 7, 8, 9, 11, 21, and 22. Before the evolution of mammals, gene duplications would have occurred, especially in TLR2 subfamily members. Furthermore, some TLR genes were lost in some lineages, although the reason is as yet unknown. For example, TLR21 was diminished in the mammalian lineage, and TLR22 was lost when the mammalian ancestor began to live on land (Fig. **2**). Why did the human ancestor lose TLR21 and 22 during evolution? We suggest two possible answers to this question. First, mammals obtained another way to detect PAMPs so that non-mammalian TLRs became dispensable in the innate system. This scenario is conceivable because the mammalian acquired system is far more sophisticated than that of teleosts. Second, the mammalian lineage happened to lose

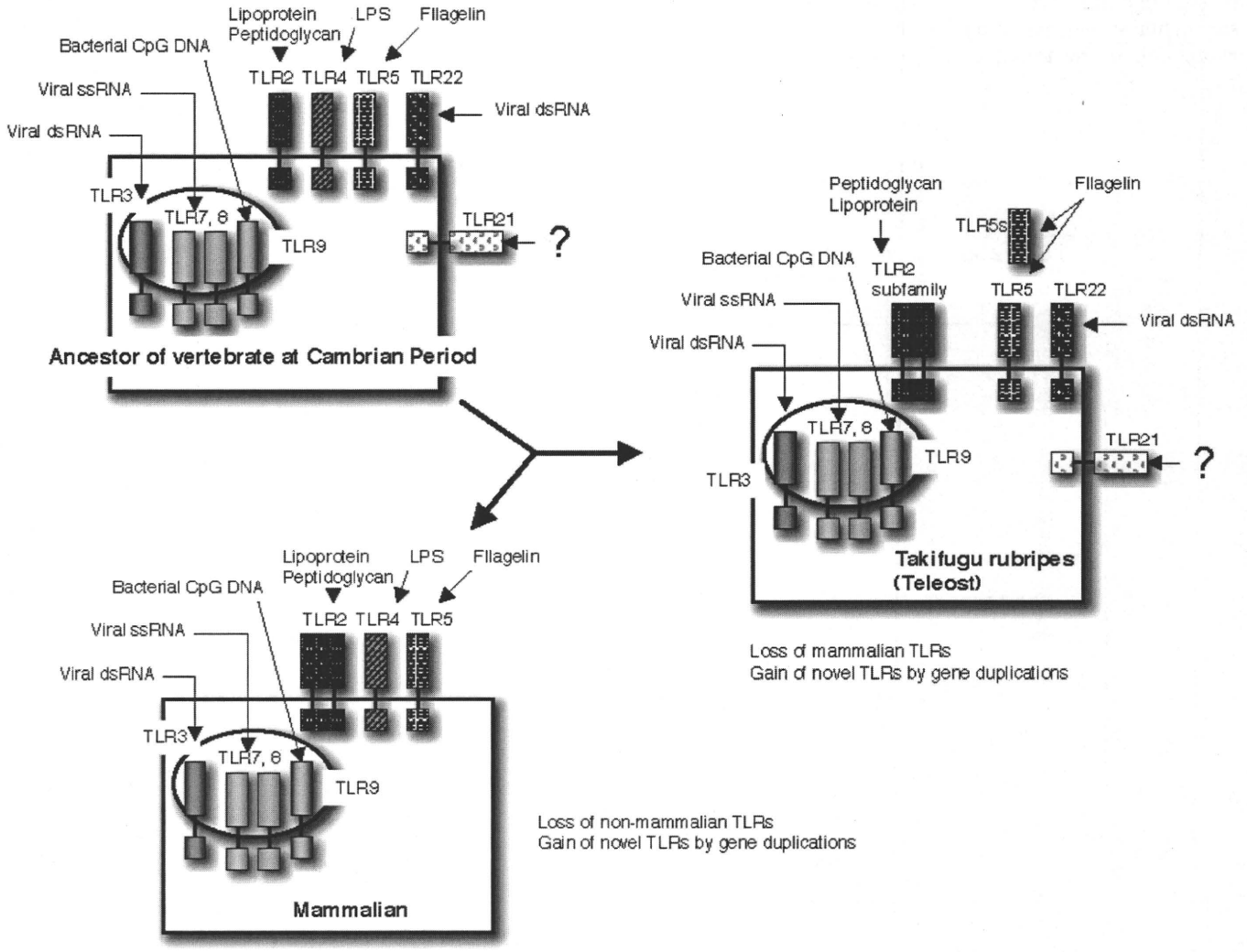

Fig. (2). Evolution of the vertebrate TLR family.

We expect that in the Cambrian period, the vertebrate ancestor possessed at least nine TLR family members: TLR2, 3, 4, 5, 7, 8, 9, 21, and 22. Those TLRs would have responded to PAMPs such as dsRNA, ssRNA, CpG DNA lipoprotein, peptidoglycan, LPS, flagellin, or other unknown PAMPs. Current TLR members in fish and mammals are expected to have been derived from the TLR family members in the Cambrian common ancestor. During evolution, mammalian ancestors obtained novel TLR members by gene duplication, especially in the TLR2 subfamily. On the other hand, both lineages have lost several members for unknown reasons.

the non-mammalian TLRs. This is not surprising because losses of genes, which are useful for their descendant, sometimes occurred during vertebrate evolution. For example, the vertebrate ancestor likely possessed six types of opsin gene for light sensing, but the mammalian ancestor lost three of the pigment genes since their divergence from reptiles [41]; thus, many mammalian species are less sensitive to the difference of light wavelength compared to other non-mammalian vertebrates. If mammals had successfully reproduced TLR22 again in their genomes, human innate immunity would have become better than the current system. Whether this is the case or not seems to reflect the reason why human ancestors lost the genes. If the first hypothesis is true, the addition of TLR22 would not affect mammalian innate immune system. On the other hand, according to the second hypothesis, it is predicted that the addition of TLR22 leads to addition of another mechanism of sensing viral infection on the cell surface to the mammalian innate immune system, and therefore the addition of TLR22 will provide a resistance to some kind of virus infection. Our laboratory currently is conducting research to find a reply to this question: We are trying to produce TLR22 transgenic mice, which will tell us why human lost TLR22 during evolution.

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Riplet/RNF135, a RING Finger Protein, Ubiquitinates RIG-I to Promote Interferon-β Induction during the Early Phase of Viral Infection*[S]

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RIG-I (retinoic acid-inducible gene-I), a cytoplasmic RNA helicase, interacts with IPS-1/MAVS/Cardif/VISA, a protein on the outer membrane of mitochondria, to signal the presence of virus-derived RNA and induce type I interferon production. Activation of RIG-I requires the ubiquitin ligase, TRIM25, which mediates lysine 63-linked polyubiquitination of the RIG-I N-terminal CARD-like region. However, how this modification proceeds for activation of IPS-1 by RIG-I remains unclear. Here we identify an alternative factor, Riplet/RNF135, that promotes RIG-I activation independent of TRIM25. The Riplet/RNF135 protein consists of an N-terminal RING finger domain, C-terminal SPRY and PRY motifs, and shows sequence similarity to TRIM25. Immunoprecipitation analyses demonstrated that the C-terminal helicase and repressor domains of RIG-I interact with the Riplet/RNF135 C-terminal region, whereas the CARD-like region of RIG-I is dispensable for this interaction. Riplet/RNF135 promotes lysine 63-linked polyubiquitination of the C-terminal region of RIG-I, modification of which differs from the N-terminal ubiquitination by TRIM25. Overexpression and knockdown analyses revealed that Riplet/RNF135 promotes RIG-I-mediated interferon-β promoter activation and inhibits propagation of the negative-strand RNA virus, vesicular stomatitis virus. Our data suggest that Riplet/RNF135 is a novel factor of the RIG-I pathway that is involved in the evoking of human innate immunity against RNA virus infection, and activates RIG-I through ubiquitination of its C-terminal region. We infer that a variety of RIG-I-ubiquitinating molecular complexes sustain RIG-I activation to modulate RNA virus replication in the cytoplasm.

Cytoplasmic viral RNA sensors induce production of type I interferon (IFN)[2] (1, 2). Representative cytoplasmic sensors, RIG-I-like receptors (RLRs) of RIG-I, MDA5, and LGP2, belong to the DEA(D/H) box RNA helicase family (3–6). RIG-I recognizes the 5′ end triphosphate of the virus RNA genome or double-stranded RNA (6–8) to sense infection by various RNA viruses (3, 5). The RIG-I protein consists of two N-terminal CARD-like domains, an RNA helicase region and a repressor domain (RD) (9). After recognition of positive or negative single-stranded viral RNA, RIG-I interacts with its adaptor molecule IPS-1/MAVS/Cardif/VISA leading to type I IFN production, thereby protecting host cells from amplified viral replication (10–13). However, only a few copies of viral RNAs usually penetrate the cell membrane to enter the cell at an early infection, and these RLRs are barely present in intact as well as early virus-infected cells (6). The early viral RNA recognition facility should be different from that of the late phase when RIG-I protein is abundant in the cytoplasm and easily re-organizes the virus RNAs. What molecular mechanism is responsible for initial sensing of viral RNA thus remains unknown.

Other RLRs, MDA5 and LGP2, are structurally similar to RIG-I in their having the helicase domain (5, 14). However, MDA5 lacks the RD domain although it possesses CARD-like region at the N terminus like RIG-I. LGP2 does not have a CARD-like region but possesses RD at its C terminus (9). RIG-I and MDA5 recognize different kinds of RNA viruses and in some cases play a redundant role in sensing virus infection, such as influenza B (15). In contrast, LGP2 rather negatively regulates virus replication. LGP2 expression suppressed RIG-I or MDA5 signaling (14, 16), and *lgp2* gene disruption conferred high susceptibility to virus infection on mice (4).

Recently, the majority of proteins involved in the type I IFN-inducing system were found ubiquitinated. For example, the tumor necrosis factor receptor-associated family members, TRAF3 and TRAF6, are ubiquitin ligases to induce ubiquitination of proteins and implicated in activation of IFN regulatory factor (IRF) 3 or nuclear factor (NF) κB (13, 17–19). In contrast, a deubiquitinating enzyme, DUBA or A20, suppresses these signals (19, 20). In addition to ubiquitin, ubiquitin-like protein, ISG15, is also conjugated to proteins involved in the IFN-inducing pathway (21, 22). Recent studies have revealed that viral RNA sensors are also ubiquitinated. TRIM25 (ZNF147 or EFP), a member of the ubiquitin-protein isopeptide ligase family, which possesses a RING finger domain, ubiquitinates the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB470605.

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[2] The abbreviations used are: IFN, interferon; RT, reverse transcription; RLR, RIG-I-like receptor; HA, hemagglutinin; siRNA, small interference; m.o.i., multiplicity of infection; VSV, vesicular stomatitis virus; IRF, IFN regulatory factor; Ub, ubiquitin; ORF, open reading frame; RD, repressor domain.

CARD-like domains of RIG-I thereby facilitating the RIG-I-mediated activation of type I IFN signaling (23, 24), although Shimotohno and co-workers (25) previously reported that TRIM25 (EFP) does not polyubiquitinate the RIG-I CARD-like region as far under their conditions. Expression of TRIM25 increases RIG-I CARD-like region-mediated signaling; however, it remains to be determined whether the activation of full-length RIG-I requires other ubiquitin ligase (23). Another ubiquitin ligase RNF125 mediates lysine 48-linked polyubiquitination of RIG-I, which leads to degradation of RIG-I through the proteasome (25).

Here we examined what molecular complex participates in an early RIG-I-mediated RNA recognition and IFN signaling by yeast two-hybrid screening. Here we detected two novel RING finger proteins that bound to RIG-I, and we found that one, RNF135, facilitated RIG-I-mediated type I IFN induction via ubiquitinating RIG-I. RNF135 plays a crucial role in the RIG-I response to minimal copies of viral RNA, and by binding to the C-terminal helicase and RD regions of RIG-I, RNF135 facilitates RIG-I C-terminal ubiquitination to up-regulate RIG-I-mediated IFN signaling and suppress viral replication. Hence, we renamed it as RNF135 Riplet (RING finger protein leading to RIG-I activation). To our knowledge, this is the first study demonstrating that C-terminal ubiquitination of RIG-I is important for full IFN induction by RIG-I.

EXPERIMENTAL PROCEDURES

Cell Cultures—HEK293 and Vero cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Invitrogen), and HeLa cells were in minimum Eagle's medium with 2 mM L-glutamine and 10% fetal calf serum (JRH Biosciences). HEK293FT cells were maintained in Dulbecco's modified Eagle's high glucose medium containing 10% heat-inactivated fetal calf serum (Invitrogen).

Plasmids—cDNA fragment encoding a C-terminal region of Riplet was isolated by yeast two-hybrid screening using human lung cDNA library. The 5′ region encoding the remaining N-terminal region was amplified by PCR using primers Riplet-F1 and Riplet-R1, and human lung cDNA library was used for its template. Two cDNA fragments, which cover the entire ORF of Riplet, were joined by PCR using primers Riplet-F1, R1, F2, and R2 and then inserted into pCR-blunt vector (Invitrogen). The primers sequences are as follows: F1, GCCTCGAGGCCACCATGGCGGGCCTGGGCCTGGG; R1, CGGCCAGGTCCTGCAGTAGC; F2, GCACCTGCGGAAGAACACGC; and R2, GGGGATCCCACCTTTACTTGCTTTATTATCAGG. The obtained cDNA was cloned into XhoI-NotI restriction sites of pEF-BOS expression vector, and the HA tag was fused at the C-terminal end of Riplet. Riplet-DN (dominant negative) expression vector was constructed by amplifying the relevant Riplet cDNA fragment using the primers Riplet-X-F-C and Riplet-R2 and subcloned into pEF-BOS. The primer sequence of Riplet-X-F-C was as follows: GCTCGAGGCCACCATGCCGCACCTGCGGAAGAACACGC. Riplet-L248fs expression vector was made by deleting 1 base at position 742 by standard PCR-mediated site-directed mutagenesis methods with primers Riplet-L248fs-F and Riplet-L248fs-R as follows: Riplet-L248fs-F, CCAGAGCCACCCTGCATCAGGAGAGCTTCTCGG, and Riplet-L248fs-R, CCGAGAAGCTCTCCTGATGCAGGGTGGCTCTGG. All cloned *RIPLET* cDNA fragments were sequenced, and it was confirmed that there were no mutations. Full-length RIG-I expressing vector, Gal4-IRF-3, Gal4-DBD, and p55 UASG-Luc reporter plasmids were gifts from Dr. T. Fujita (Kyoto University, Kyoto, Japan). p125 luc reporter plasmid was a gift from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). RIG-I RD expressing vector was made with primers RIG-I RD-F and RIG-I RD-R; the RIG-I dRD cDNA fragment, which encodes ORF of RIG-I from the 1- to 754-amino acid region, was made by using primers RIG-I-(1–754)F and RIG-I-(1–754)R. The obtained cDNA fragments were sequenced, and it was confirmed that there were no mutations caused by PCR. The primers sequences are as follows: RIG-I RD-F, GAT GAT AAA GGT ACC ACC GGT AGC AAG TGC TTC CTT CTG; RIG-I RD-R, AAG GAA GCA CTT GCT ACC GGT GGT ACC TTT ATC ATC ATC ATC; RIG-I-(1–754)F, GC AGA GGA AGA GCA AGA TGA TAT CAG GTC CTC AAT CTT C; and RIG-I-(1–754)R, ATT GAG GAC CTG ATA TCA TCT TGC TCT TCC TCT GCC TC.

Northern Blotting—Human *RIPLET* 1092-bp cDNA fragment (208–1299) was used for the probe for Northern blotting. The Northern blot membranes, human 12-lane MTN blot and MTN blot III, were purchased from Clontech. The homology of human *RIPLET* and *TRIM25* in the probe region was 46%. We used a stringent condition for Northern blotting to exclude the cross-hybridization between the *RIPLET* and *TRIM25* genes. Briefly, the probe was labeled with [α-^{32}P]dCTP using Rediprime II Random Prime labeling system (GE Healthcare). The labeled probe was hybridized to the membrane with ExpressHyb hybridization solution (Clontech) at 68 °C for 1 h. The membrane was washed with washing solution I (2× SSC, 0.05% SDS) for 40 min, and then washed with washing solution II (0.1× SSC, 0.1% SDS) for 40 min. Riplet mRNA bands were detected with x-ray film.

Reporter Gene Analysis—HEK293 cells were transiently transfected in 24-well plates using FuGENE HD (Roche Applied Science) with expression vectors, reporter plasmids, and internal control plasmid coding *Renilla* luciferase. The total amounts of plasmids were normalized with empty vector. For poly(I-C) stimulation, 24 h after transfection, cells were stimulated with medium containing poly(I-C) (50 μg/ml) and DEAE-dextran (0.5 mg/ml) for 1 h, and then the medium was exchanged with normal medium and incubated for an additional 3 h. Cells were lysed with lysis buffer (Promega) and luciferase, and *Renilla* luciferase activities were measured by the dual luciferase assay kit (Promega). Relative luciferase activities were calculated by normalizing luciferase activity by *Renilla* luciferase activity, and dividing the normalized value by control in which only empty vector, reporter, and internal control plasmid were transfected. Values are expressed as mean relative stimulations ± S.D. for a representative experiment, and each was performed three times in duplicate (unless otherwise indicated in the legends).

RNA Interference—Reporter and siRNA (20 nM final concentration) for Riplet or control were transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen) by the standard method described in the manufacturer's protocol. Empty vec-

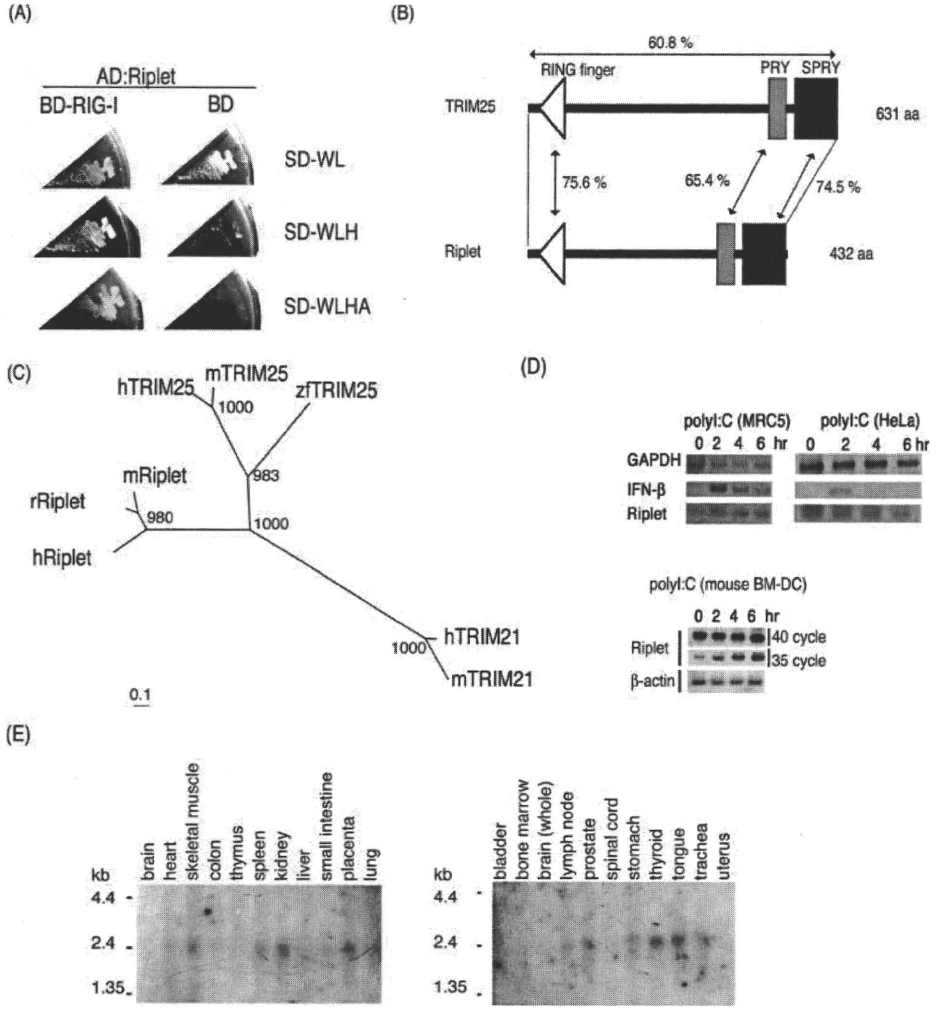


FIGURE 1. **Isolation of Riplet by yeast two-hybrid screening.** *A*, yeast cells carrying both RIG-I and Riplet can grow in selective media (SD-WLH, SD-WLHA), whereas yeast cells carrying RIG-I alone only grow in nonselective media (SD-WL), indicating the physical interaction of RIG-I with Riplet. *B*, human Riplet protein sequence is 60.8% identical to human TRIM25. The RING finger domains and SPRY motifs show higher sequence similarities between the two proteins. *aa*, amino acids. *C*, phylogenetic tree constructed by the Neighbor-Joining method shows that Riplet is similar to TRIM25. *h*, *m*, *r*, or *zf* represent human, mouse, rat, or zebrafish, respectively. The *numbers* on the node are bootstrap probabilities ($n = 1000$). *D*, HeLa cell, human primary-cultured fibroblast cell, MRC5, or bone marrow-derived mouse dendritic cell (*BM-DC*) were stimulated with poly(I-C) (50 μg/ml) for indicated hours. Total RNA was extracted with TRIzol reagent, and then RT-PCR was carried out using primers shown under "Experimental Procedures." *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. *E*, Northern blot membranes containing 1 μg of poly(A)$^+$ RNA per lane from human tissues were blotted with human Riplet probe.

tor was added to normalize the final plasmid amount. 48 h after transfection, cells were stimulated with poly(I-C) for 4 h. For VSV infection, 24 h after transfection, cells were infected with VSV at m.o.i. = 1, and cell lysate was prepared after 12 h for reporter gene assays. The degree of gene silencing was confirmed by RT-PCR using RNA extracted from cells 24 h after transfection. PCR primers used for the RT-PCR were Riplet-F3 (ACTGGGAAGTGGACACTAGG) and Riplet-R3 (ACTCATACAGAAGCTTCTCC). siRNAs were purchased from Funakoshi Co., Ltd. (Tokyo Japan), and the siRNA sequences of Riplet siRNA were GACUAUGGACUCUUGUUGUGU (sense) and ACAACAAGAGUCCAUAGUCCU (antisense). Control siRNA sequences were CUGUUGGUUUAGUAAGCCUGU (sense) and AGGCUUACUAAACCAACAGUC (antisense). Another siRNA, Riplet si-1, and control negative siRNA (silencer negative control 1 siRNA, AM4611) were purchased from Applied Biosystems. siRNA sequences were Riplet si-1 GGGAAAGCUUGCCUUCUAUdTdT (sense) and AUAGAAGGCAAGCUUUCCCdTdC (antisense).

Virus Preparation and Infection—VSV Indiana strain and poliovirus were amplified using Vero cells. HEK293 cells were transfected in 24-well plates with plasmid encoding *RIG-I*, Riplet, or no insert. 24 h after transfection, cells were infected with viruses for 24 h, and the titers of virus in culture supernatant were measured by plaque assay using Vero cells. For RNA interference assay, cells were transfected with siRNA with Lipofectamine 2000. 24 h after transfection, cells were infected with viruses at m.o.i. = 0.001 for 18 h, and the titer in culture supernatant were determined by plaque assay.

Immunoprecipitation—HEK293FT cells were transfected in 6-well plates with plasmids encoding FLAG-tagged RIG-I and/or HA-tagged Riplet. The plasmid amounts were normalized by the addition of empty plasmid. 24 h after transfection, cells were lysed with lysis buffer (20 mM Tris-HCl (pH 7.5), 125 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 30 mM NaF, 5 mM Na_3VO_4, 20 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride), and then proteins were immunoprecipitated with rabbit anti-HA polyclonal (Sigma) or anti-FLAG M2 monoclonal antibody (Sigma). The precipitated samples were analyzed by SDS-PAGE and stained with anti-HA (HA1.1) (Covance) or anti-FLAG M2 monoclonal antibody. For ubiquitination assay of RIG-I, the plasmid encoding two multiple HA-tagged ubiquitins was used. HEK293FT cells were transfected with plasmids encoding FLAG-tagged RIG-I, Riplet, or 2× HA-tagged ubiquitin. 24 h after transfection, cells were lysed, and then RIG-I was immunoprecipitated as described above. The samples were analyzed by SDS-PAGE and stained with anti-HA polyclonal antibody (for detection of ubiquitination) or anti-FLAG monoclonal antibody (for detection of RIG-I). Reproducibility was confirmed with additional experiments (see supplemental figures).

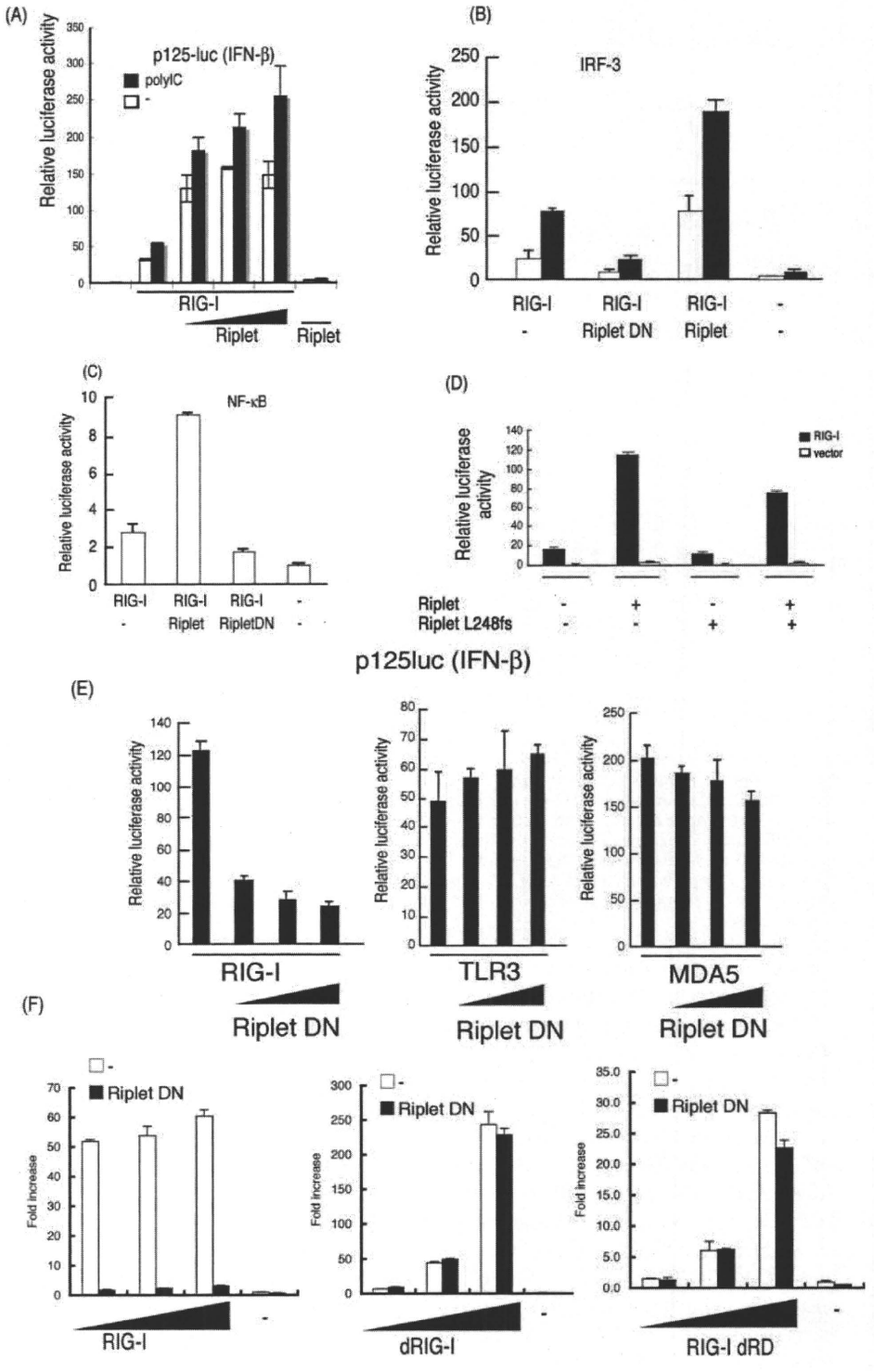
Construction of RIG-I 3KA and 5KA Mutant Genes—The C-terminal three or five lysine residues were mutated into alanines (designated as 3KA and 5KA). RIG-I 3KA has K888A, K907A, and K909A, whereas RIG-I 5KA has K849A, K851A,

K888A, K907A, and K909A. The mutant *rig-I* genes were made by PCR-mediated site-directed mutagenesis. The primers used for the PCR were as follows: K907–909A-forward, GTT CAG ACA CTG TAC TCG GCG TGG GCG GAC TTT CAT TTT GAG AAG, and K907–909A-reverse, CTT CTC AAA ATG AAA GTC CGC CCA CGC CGA GTA CAG TGT CTG AAC; K888A-forward, GAC ATT TGA GAT TCC AGT TAT AGC AAT TGA AAG TTT TGT GGT GGA GG, and K888A-reverse, CCT CCA CCA CAA AAC TTT CAA TTG CTA TAA CTG GAA TCT CAA ATG TC; K849–851A-forward, GAG TAG ACC ACA TCC CGC CCA GCG CAG TTT TCA AGT TTT G, and K849–851A-reverse, CAA AAC TTG AAA ACT GCG CTG GCG CGG GAT GTG GTC TAC TC. PCR was carried with Pyrobest *Taq* polymerase, and the obtained clones were sequenced to exclude the clones harboring PCR error. To construct the plasmid-expressing mutant RIG-I protein, the wild-type *RIG-I* gene on pEF-BOS vector was replaced with the mutant *rig-I* gene.

Real Time PCR—Quantitative PCR analyses were carried out using iCycler iQ real time detection system with Platinum SYBR Green qPCR SuperMix-UDG reagent (Invitrogen). Primer sequences for qPCR were as follows: hGAPDH-qF, GAG TCA ACG GAT TTG GTC GT, and hGAPDH-qR, TTG ATT TTG GAG GGA TCT CG; hIFN-β-qF, TGG GAG GAT TCT GCA TTA CC, and hIFN-β-qR, CAG CAT CTG CTG GTT GAA GA; hMx1-qF, ACC ACA GAG GCT CTC AGC AT, and hMx1-qR, CTC AGC TGG TCC TGG ATC TC; and hIFIT-1-qF, GCA GCC AAG TTT TAC CGA AG, and hIFIT-1-qR, CAC CTC AAA TGT GGG CTT TT. Values were expressed as mean relative stimulations, and for a representative experiment from a minimum of three separate experiments, each was performed in triplicate.

RESULTS

RIG-I-binding Proteins—To isolate the proteins that bind to RIG-I, we performed yeast two-hybrid screening using a human lung cDNA library. Using the RIG-I central region (213–601 amino acids), we isolated a clone that encoded a partial ORF of a gene expressed in a dendritic cell line, DC12, whereas the C-terminal region of RIG-I (557–925 amino acids) resulted in the isolation of two cDNA clones, which encoded partial C-terminal regions of ZNF598 and RNF135 (Fig. 1*A* and data not shown). Preliminary expression studies showed that the RNF135 segment affected the RIG-I IFN-β inducing activity, whereas the other two proteins had no effect (data not shown). We confirmed the



interaction of RIG-I with ZNF598 or RNF135 in HEK293FT cells by immunoprecipitation (data not shown). RNF135 was previously annotated by the genome project and was recently found to be a cause of a genetic disease, neurofibromatosis, although its protein function was unknown. We renamed the protein Riplet (RING finger protein leading to RIG-I activation) based on the following functional analyses. Riplet was most similar to TRIM25 (60.8% sequence homology), in particular between their RING finger domains PRY or SPRY (Fig. 1*B*). Phylogenetic analysis also supported the notion that Riplet was similar to TRIM25 (Fig. 1*C*). Thus, we hypothesized that, like TRIM25, Riplet is a ubiquitin ligase.

Expression of Riplet—RIG-I mRNA is induced by type I IFN or poly(I-C) stimulation in mammalian cells. Unlike RIG-I, however, Riplet mRNA was basally expressed in HeLa and primary-cultured MRC-5 cells irrespective of stimulation (Fig. 1*D* and data not shown). On the other hand, when we treated bone marrow-derived dendritic cells with poly(I-C), the basal level of Riplet mRNA was increased by the stimulation (Fig. 1*D*), suggesting that the regulatory mechanism of Riplet expression somewhat differs among cell types, and that Riplet is expressed before virus infection in some cell types. Next we performed Northern blotting of human tissue RNA. Riplet mRNA was detected as a single band of 2.4 kbp, which is slightly longer than the RNF135 cDNA sequence deposited in GenBank™ (accession number AB470605). Human *RIPLET* is expressed in human skeletal muscle, spleen, kidney, placenta, prostate, stomach, thyroid, and tongue and also weakly expressed in heart thymus, liver, and lung (Fig. 1*E*).

Riplet Enhances RIG-I-mediated IFN-β Induction—At first we characterized the role of Riplet in RIG-I-mediated IFN inducing signaling by reporter gene analyses. When RIG-I was expressed in HEK293 cells, reporter auto-activation was observed even in the absence of exogenous stimulation (Fig. 2*A*) as reported previously (25, 26). Stimulation with poly(I-C) further enhanced the promoter. Co-expression of Riplet with RIG-I potentiated activation of the IFN-β promoter, whereas expression of Riplet alone resulted in only marginal activation (Fig. 2*A*). Detection of endogenous IFN-β mRNA confirmed that Riplet enhanced RIG-I-mediated activation of IFN-β transcription (supplemental Fig. S1). The enhancing role of Riplet in IFN-β promoter activation was also supported by activation of IRF-3 and NF-κB by Riplet (Fig. 2, *B* and *C*). In contrast, expression of a Riplet partial fragment (Riplet-DN) (70–432 amino acids) that lacked the N-terminal RING finger domain reduced promoter activation (Fig. 2*E*). The Riplet-L249fs mutant protein, which was isolated from neurofibromatosis patients (27), did not increase the RIG-I-mediated promoter activation (Fig. 2*D*). These data indicate that Riplet augments RIG-I-mediated IFN-β promoter activation, and that both the RING finger domain and the C-terminal region encoding the SPRY and PRY motifs are important for its function. Riplet (residues 70–432) acted as a dominant-negative form (hereafter called Riplet-DN) (Fig. 2, *E* and *F, left panel*). This functional feature of Riplet-DN was confirmed in Fig. 2, *B* and *C*, and was later confirmed through RIG-I co-precipitation and ubiquitination analyses (see Fig. 5*C* and supplemental Fig. S4*C*). Expression of Riplet-DN did not reduce TLR3 or MDA5 signaling (Fig. 2*E*), suggesting that Riplet-DN is specific for RIG-I signaling. Interestingly, the Riplet-DN only partially suppressed the function of the C-terminal deleted RIG-I (dRD), which is a constitutively active form (Fig. 2*F, right panel*), and RIG-I CARD-like region (dRIG-I)-mediated signaling in high or low dose transfection of dRIG-I was barely inhibited by overexpression of Riplet-DN (Fig. 2*F, center panel*). These data suggest that Riplet requires the RIG-I C-terminal domain (RD) and partial helicase region to activate RIG-I signaling.

Endogenous Riplet Promotes the RIG-I Signaling—We performed Riplet knockdown by siRNA Riplet using Lipofectamine 2000 reagents, instead of FuGENE HD, to reveal the function of endogenous Riplet. Two siRNAs (Riplet siRNA and Riplet si-1) that target different sites of the Riplet mRNA and two control siRNAs were used for knockdown analyses. The two siRNA or control siRNA were co-transfected with HA-tagged Riplet expression vector into HEK293 cells, and after 48 h, cell lysate was prepared and analyzed by Western blotting with anti-HA antibody detecting Riplet. The two siRNAs targeting Riplet abolished exogenously expressed Ripelt-HA, but control siRNA did not (supplemental Fig. S3). Likewise, both Riplet siRNA and Riplet si-1 specifically down-regulate the level of endogenous Riplet mRNA (Fig. 3, *A* and *B*).

Using the siRNA, we examined whether Riplet knockdown reduces RIG-I signaling. As expected, RIG-I-mediated IFN-β promoter activation was reduced by Riplet siRNA or Riplet si-1 compared with control siRNA (Fig. 3, *A* and *B*), indicating that Riplet is required for full activation of the RIG-I signaling. Vesicular stomatitis virus (VSV) is a negative-stranded RNA virus that induces IFN-β production via RIG-I (3). Although the

FIGURE 2. **Riplet enhances IFN-β signaling mediated by RIG-I.** *A*, Riplet enhances the promoter activation by RIG-I. HEK293 cells were transfected with plasmids encoding empty vector, RIG-I (0.1 μg) and Riplet (0.025, 0.05, or 0.1 μg) together with p125-luc (IFN-β promoter) reporter plasmid in 24-well plates. 24 h after transfection, the cells were treated with mock or poly(I-C) (50 μg/ml) for 4 h as described under "Experimental Procedures," and then luciferase activities of cell lysates were measured. *Closed* or *open boxes* represent poly(I-C) or mock stimulation, respectively. *B*, to examine the activation of IRF-3, RIG-I (0.1 μg), Riplet (0.1 μg), and/or Riplet-DN (0.1 μg),expressing vectors were transfected into HEK293 cells with reporter plasmids, GAL4 fused IRF-3 (0.05 μg), and the p55 UASG-luc reporter gene (0.05 μg), in which luciferase reporter gene is fused downstream of GAL4 protein-binding site, and therefore activated IRF-3 promotes the transcription of luciferase reporter gene. The cells were stimulated with poly(I-C) as described above (34). The total amount of transfected DNA (0.5 μg/well) was kept constant by adding empty vector (pEF-BOS). *C*, HEK293 cells were transfected with RIG-I (0.1 μg), Riplet (0.1 μg), and/or Riplet-DN (0.1 μg) expressing vectors together with the NF-κB reporter plasmid (0.1 μg), and 24 h later, the luciferase activities of cell lysates were measured. *D*, Riplet-L248fs, which lacks the C-terminal region, did not enhance the activation at all. HEK293 cells were transfected with the plasmids expressing wild-type Riplet (0.1 μg) or Riplet-L248fs (0.1 μg) together with RIG-I expressing vector (0.1 μg) and p125-luc reporter (0.1 μg). 24 h after transfection, cell were stimulated with poly(I-C), and the luciferase activities of cell lysates were determined as described above. *E*, RIG-I (0.1 μg), MDA5 (0.1 μg), or TLR3 (0.1 μg) expressing vectors were transfected into HEK293 cells with the plasmid encoding the Riplet-DN fragment (0.1, 0.2, or 0.3 μg) in 24-well plates. After 24 h, the cells were stimulated with 50 μg of poly(I-C) for 4 h, and relative luciferase activities were determined. *F*, Riplet-DN (100 ng) was co-transfected with full-length RIG-I (0, 50, 100, or 200 ng), RIG-I CARD-like region (dRIG-I) (0, 50, 100, or 200 ng), or C-terminal deleted RIG-I (RIG-I dRD) (0, 50, 100, or 200 ng) into HEK293 cells in 24-well plate, and reporter gene assays were carried out.

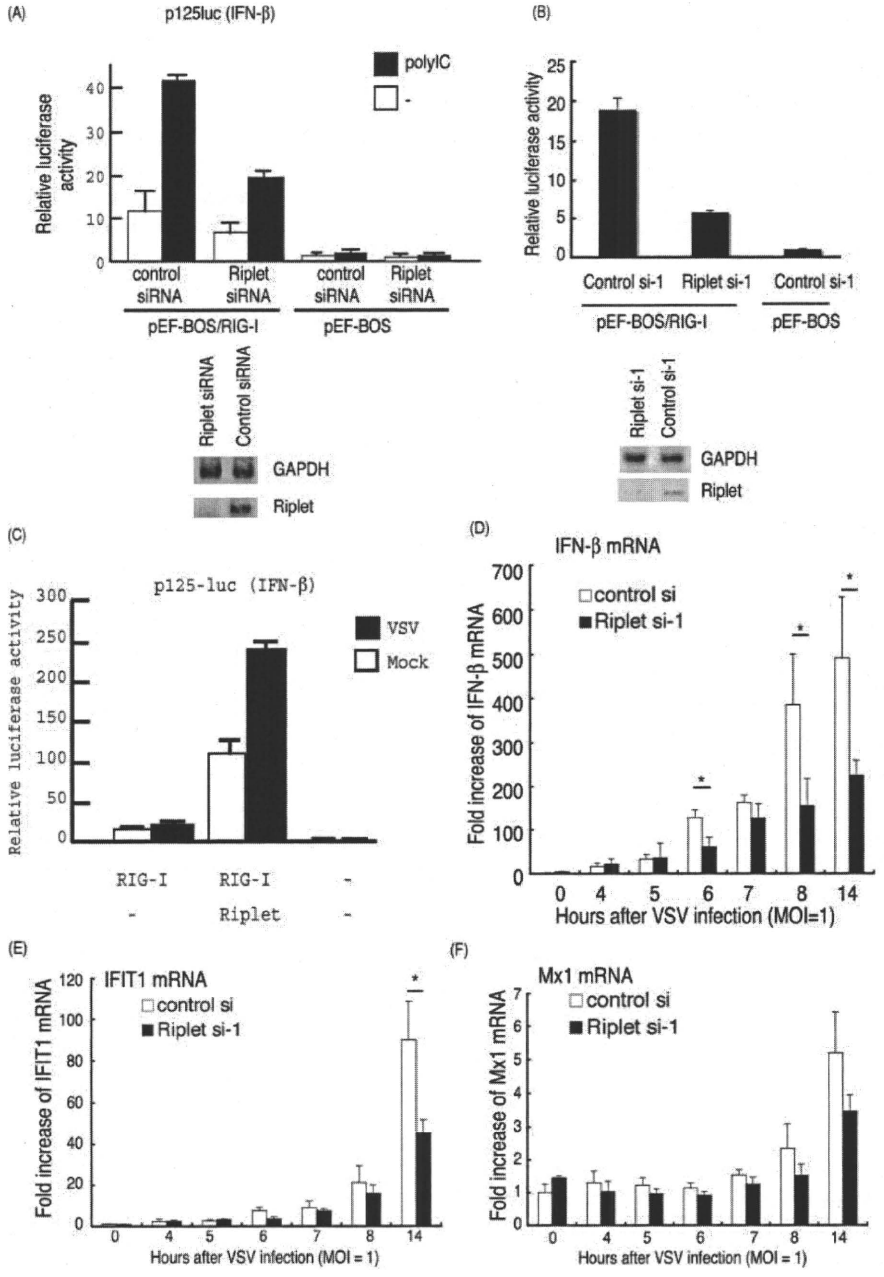


FIGURE 3. **Knockdown analyses of Riplet.** *A*, p125 luc reporter plasmid (0.1 μg), RIG-I expressing vector (0.1 μg), and Riplet siRNA or control siRNA (10 pmol), which were purchased from Funakoshi Co. Ltd., were transfected into HEK293 cells in a 24-well plate with Lipofectamine 2000, and 48 h after transfection, the cells were stimulated with poly(I-C) for 6 h, and the cell lysate was prepared, and luciferase activities were measured. RT-PCR was carried out using total RNA extracted from cells 48 h after transfection. *B*, p125 luc reporter plasmid (0.1 μg), RIG-I expressing vector (0.1 μg), and siRNA, Riplet si-1, or control si-1 (10 pmol), which were purchased from Applied Biosystems, were transfected into HEK293 cells with Lipofectamine 2000. 48 h after transfection, the cells were stimulated with poly(I-C) for 6 h. The cell lysate was prepared, and luciferase activities were measured. RT-PCR was carried out using total RNA extracted from cells 48 h after transfection. *C*, HEK293 cells were transfected with the plasmids expressing RIG-I (0.1 μg) and/or Riplet (0.1 μg) with p125 luc reporter plasmid (0.1 μg) in 24-well plates. After 24 h, the cells were infected with VSV (m.o.i. = 1) for 12 h. The luciferase activities of the cell lysates were measured. Expression of Riplet strongly enhanced IFN-β promoter activation by VSV through RIG-I. *D–F*, siRNA (control si- or Riplet si-1) were transfected into HEK293 cells, and after 48 h, the cells were infected with VSV at m.o.i. = 1. RNA was extracted at the indicated hours, and the quantitative PCR were carried out to detect the expression of IFN-β (*D*), IFIT-1 (*E*), or Mx1 (*F*) mRNA. *, $p < 0.05$. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

IFN-β promoter was only minimally activated by RIG-I in response to VSV (m.o.i. = 1) during the early phase of infection (<12 h), the activity was increased by RIG-I and Riplet (Fig. 3*C*). Riplet was silenced by siRNA and then VSV infected the cells. VSV-derived up-regulation of IFN-β mRNA was started around 6 h post-infection, and Riplet siRNA significantly suppressed the increase of IFN-β mRNA at 6 h (Fig. 3*D*). Because VSV infection is mainly sensed by RIG-I, this is consistent with the notion that Riplet promotes the RIG-I signaling. Other IFN-inducible genes, *IFIT1* and *MX1*, were expressed >8 h post-infection, and their expressions were also suppressed by Riplet siRNA (Fig. 3, *E* and *F*).

Riplet Exerts Protective Activity against Viral Infection—Next we examined the role of Riplet during viral infection. Riplet and/or RIG-I were transiently expressed in the human cells by FuGENE HD reagents, and then the cells were infected with VSV or poliovirus (a positive-stranded RNA virus). The viral titer of the supernatant was determined 24 h post-infection. Under our conditions, expression of RIG-I weakly inhibited VSV propagation. Co-expression of Riplet with RIG-I significantly suppressed VSV replication especially at low m.o.i., whereas Riplet alone did not suppress VSV (Fig. 4, *A* and *B*, *upper panel*). Therefore, a sufficient amount of RIG-I protein is required for Riplet to exert antivirus activity. This requirement of RIG-I is also observed in reporter gene analyses (Fig. 2). Under a similar setting, the antiviral effect of Riplet was marginally observed against poliovirus, which induces IFN-β largely via MDA5 (Fig. 4*B*, *lower panel*). To assess the importance of endogenous Riplet for antivirus effect of human cells, Riplet knockdown cells were infected with viruses. In Riplet knockdown cells, the VSV titer was consistently increased compared with the control ($p < 0.05$) (Fig. 4*C*, *left panel*). In addition, infection of Riplet knockdown cells with poliovirus resulted in only a slight increase in the poliovirus titer compared with the control ($p > 0.05$) (Fig. 4*C*, *right panel*). Because poliovirus is mainly recognized by MDA5 but not RIG-I, this marginal effect of Riplet on poliovirus infection was within expectation (3, 28).

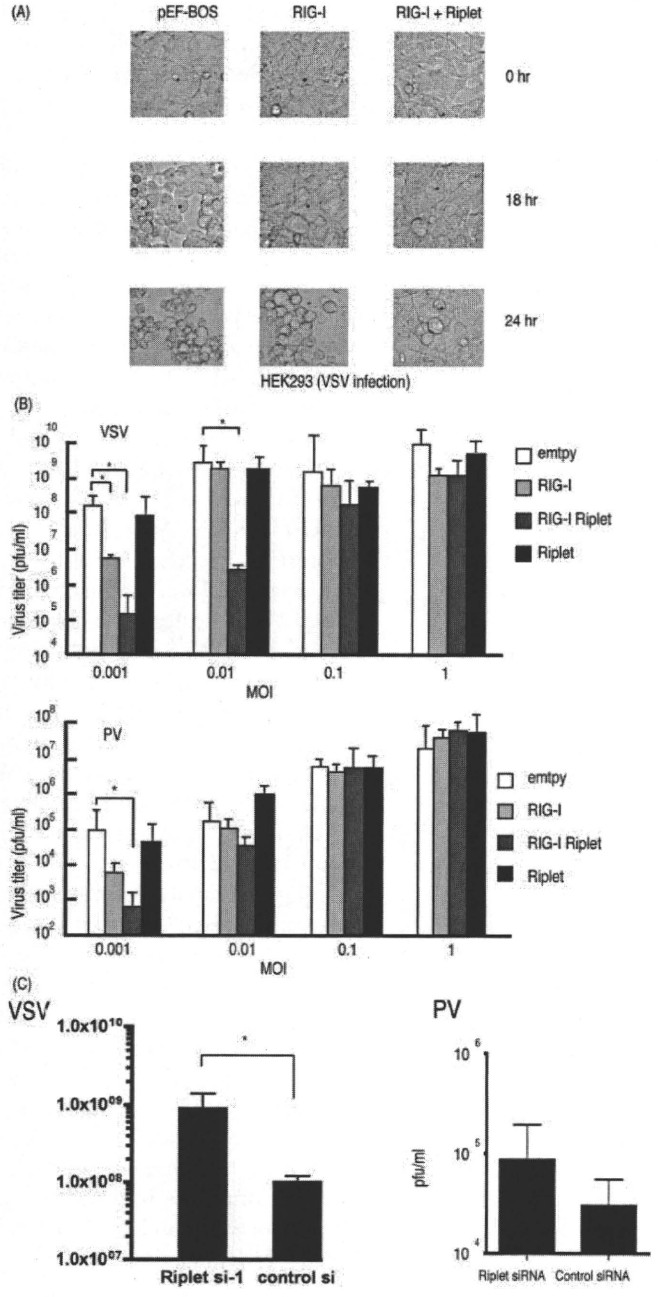


FIGURE 4. **Suppression of RNA viruses by Riplet.** *A*, HEK293 cells were transfected with RIG-I (0.1 μg) and/or Riplet (0.1 μg) expressing vectors. The total amount of transfected DNA (0.5 μg/well) in each well was kept constant by adding empty vector (pEF-BOS). 24 h after transfection, the cells were infected with VSV at m.o.i. = 0.1, and after 0, 18, or 24 h, CPE was observed by microscope. *B*, RIG-I (0.1 μg) and/or Riplet (0.1 μg) expressing plasmids were transfected to HEK293 cells in 24-well plates and incubated for 24 h. The total amount of transfected DNA (0.5 μg/well) in each well was kept constant by adding empty vector (pEF-BOS). The cells were infected with VSV (*upper panel*) or poliovirus (*PV*) (*lower panel*) at the indicated m.o.i. The viral titers in the culture media were measured 24 h after infection by plaque assay. *Error bars* represent standard deviation ($n = 3$). *, $p < 0.05$. *C*, control or Riplet knockdown HEK293 cells were infected with VSV (*left panel*) or poliovirus (*right panel*) at m.o.i. = 0.1. The viral titers in the culture media were measured 26 h after infection by plaque assays. Knockdown of Riplet induced higher VSV titers compared with control ($p < 0.05$), but the increase observed in poliovirus-infected Riplet knockdown cells was not significant ($p > 0.05$).

Riplet and Riplet-DN Bind the Helicase and RD Regions of RIG-I—Yeast two-hybrid analysis showed that a C-terminal region of Riplet bound to the C-terminal region of RIG-I. This cytoplasmic interaction between Riplet and RIG-I was confirmed by confocal microscopy in HeLa cells (supplemental Fig. S2). To further confirm the physical binding of Riplet to RIG-I in human cells, we carried out immunoprecipitation analyses. Full-length Riplet was co-immunoprecipitated with RIG-I (Fig. 5*B*), indicating that Riplet binds directly to RIG-I in human cells.

To determine the region responsible for the RIG-I-Riplet interaction, we constructed a RIG-I and Riplet deletion series as shown in Fig. 5*A*. Riplet-DN also bound to RIG-I (Fig. 5, *B* and *C*), indicating that the RING finger domain is dispensable for the RIG-I-Riplet interaction. This is consistent with the notion that the RING finger domain in ubiquitin ligase proteins is required for their interactions with ubiquitin-conjugating enzymes (29). Unlike TRIM25, Riplet and Riplet-DN failed to co-precipitate the two CARD domains of RIG-I (dRIG-I) (Fig. 5*D*). However, co-precipitation of the RIG-IC or RIG-RD fragments was observed (Fig. 5, *E* and *F*). RD-deleted RIG-I (RIG-I dRD) weakly associated with Riplet (Fig. 5*G*). Taken together, Riplet preferentially binds the RD and also weakly associates with the helicase region of RIG-I with its C terminus. Reporter gene analyses show that Riplet-DN only weakly suppresses RIG-I signaling and barely suppresses dRIG-I, which contains neither helicase nor RD region. Therefore, the physical interaction is correlated with the results of reporter activity.

Riplet Promotes Ubiquitination of RIG-I—Because Riplet shares 60% sequence similarity with TRIM25, we hypothesized that Riplet ubiquitinates RIG-I and that this modification leads to activation of RIG-I signaling. To test this hypothesis, we examined RIG-I ubiquitination. As expected, ubiquitination of RIG-I was increased by co-expression of Riplet under two different conditions (Fig. 6, *A* and *B*). The quantity of RIG-I ubiquitination was significantly high in the presence of Riplet (Fig. 6*C*). RIG-I ubiquitination was suppressed if Riplet was replaced with Riplet-DN (Fig. 6*D* and supplemental Fig. S4*C*). However, unlike TRIM25, Riplet binds to the C-terminal region of RIG-I. Therefore, we examined whether Riplet ubiquitinates the C-terminal region. We found that ubiquitination of RIG-IC was enhanced by Riplet expression (Fig. 6*E*). Both RIG-I dRD and RIG-I RD were also ubiquitinated by expression of Riplet (Fig. 6*F*; supplemental Fig. S4*A* and S5), suggesting that Riplet promotes ubiquitination of the helicase and RD domains of RIG-I in a manner distinct from TRIM25.

Ubiquitin is polymerized through its lysine residue. Lys-63-linked polyubiquitination is frequently observed in signal transduction pathways (30). In contrast, Lys-48-linked polyubiquitination usually leads to the degradation of protein through the proteasome. Indeed, TRIM25-mediated Lys-63-linked polyubiquitination activates the CARD-like region of RIG-I, and RNF125-mediated Lys-48-linked polyubiquitination leads to the degradation of RIG-I (23, 25). We used K48R or K63R mutated ubiquitin and found that K48R was incorporated normally into RIG-IC, whereas polyubiquitination was decreased by K63R (supplemental Fig. S4*B*). K63R mutation abolished RIG-I RD polyubiquitination by Riplet (Fig. 6*F*). These data

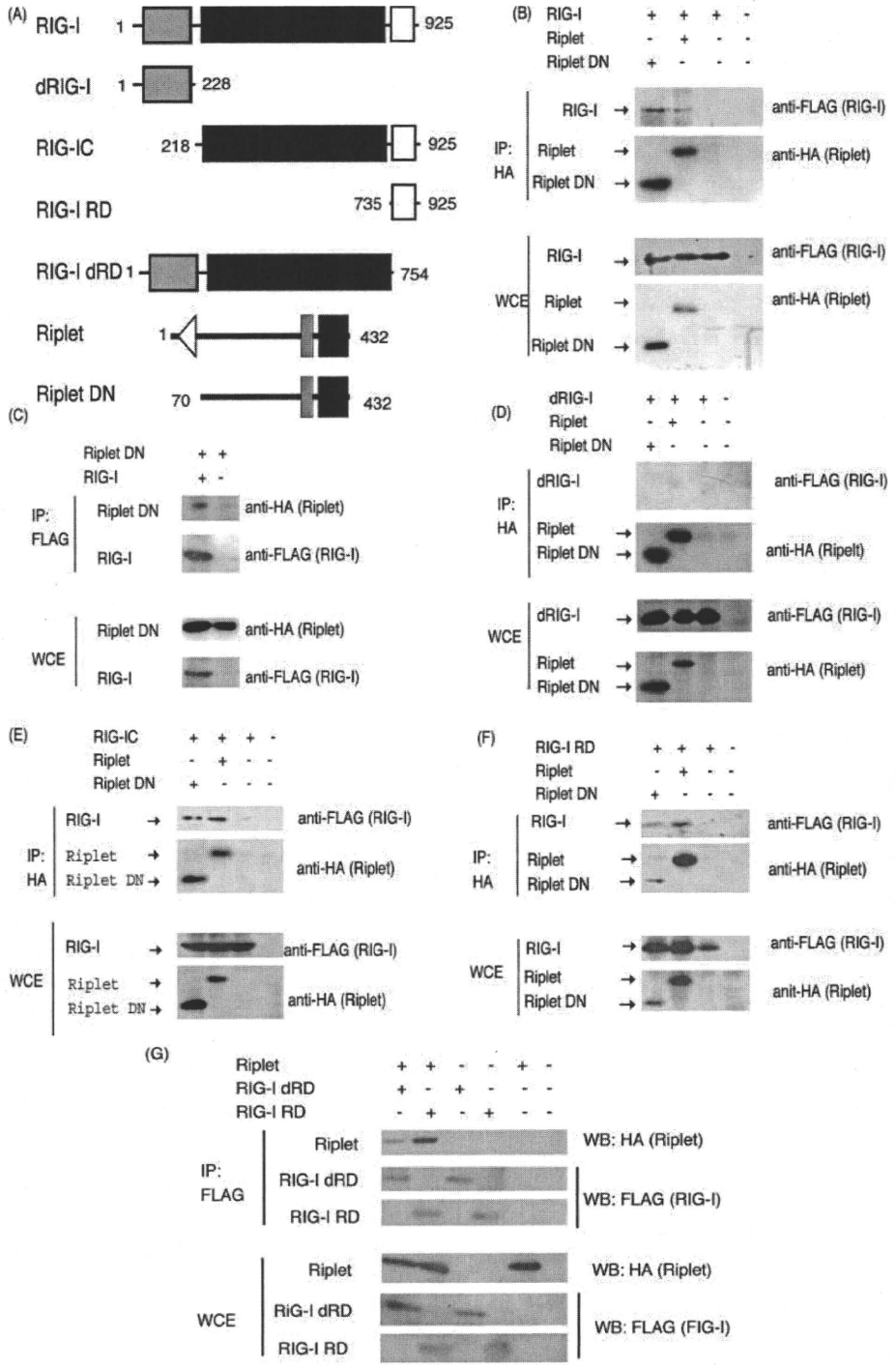


FIGURE 5. **Physical interaction of Riplet with RIG-I.** *A*, schematic representation of RIG-I or Riplet fragments used for immunoprecipitation analyses. *B*, HA-tagged Riplet (0.4 μg) or Riplet-DN (0.4 μg) were transfected into HEK293FT cells in a 6-well plate with FLAG-tagged RIG-I (0.4 μg). HA-tagged Riplet or Riplet-DN were immunoprecipitated (*IP*) with anti-HA antibodies, and samples were analyzed by Western blotting (*WB*) using an anti-FLAG or anti-HA antibody. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). *C*, HA-tagged Riplet-DN (0.4 μg) and FLAG-tagged RIG-I (0.4 μg) were transfected into HEK293FT cells in a 6-well plate. RIG-I was immunoprecipitated with anti-FLAG antibody, and samples were analyzed by Western blotting using an anti-FLAG or -HA antibody. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). *D–F*, interaction of HA-tagged Riplet or Riplet-DN with FLAG-tagged dRIG-I (*D*), RIG-IC (*E*), or RIG-I RD (*F*) was examined using immunoprecipitation assays. The proteins were expressed in HEK293FT cells, and HA-tagged Riplet was immunoprecipitated with anti-HA antibody, and samples were analyzed by Western blotting using an anti-FLAG or -HA antibody. *G*, FLAG-tagged RIG-I RD (0.4 μg) or RIG-I dRD (0.4 μg) was transfected with HA-tagged Riplet (0.4 μg) into HEK293 FT cells in a 6-well plate, and 24 h after transfection, immunoprecipitation was performed with anti-FLAG antibody and analyzed by Western blotting. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). *WCE*, whole cell extract.

indicates that Riplet mediates Lys-63-linked polyubiquitination of the RIG-I C-terminal helicase and RD region. Because Riplet-DN reduced the RIG-I-mediated signaling, we examined whether Riplet-DN reduced the RIG-I ubiquitination. As expected, Riplet-DN reduced RIG-I ubiquitination (Fig. 6*D* and supplemental Fig. S4*C*). These ubiquitination assay data are consistent with the notion that Riplet-mediated Lys-63-linked polyubiquitination of RIG-I is required for full activation of RIG-I signaling.

We tried to determine the ubiquitination sites of RIG-I using Lys-to-Ala (KA)-converting mutants. RIG-I has 25 Lys residues in its C-terminal region. These Lys residues of RIG-I were in turn mutated to Ala, and the degree of ubiquitination and IFN-β-inducing activity were determined with each mutant. RIG-I-mediated IFN-β promoter activation was normally augmented by co-expression of Riplet and 3KA RIG-I. Co-expression of Riplet and 5KA, however, and the ubiquitination level of RIG-I and IFN-β-inducing activity were simultaneously decreased (Fig. 7, *A* and *C*). Riplet-dependent augmentation of IFN-β promoter activation was largely suppressed when RIG-I was replaced with 5KA RIG-I (Fig. 7*B*). Therefore, Lys-849 and Lys-851 of RIG-I were crucial for RIG-I ubiquitination by Riplet. The results confirmed the importance of ubiquitination of specific Lys residues in the C-terminal region of RIG-I and for RIG-I-mediated IFN-β induction.

DISCUSSION

RIG-I plays a central role in the recognition of cytoplasmic viral RNA and is regulated by modification by small modifier ubiquitin or ubiquitin-like protein, ISG15. TRIM25 mediates Lys-63-linked polyubiquitination, which is essential for RIG-I activation (23), and RNF125 mediates Lys-48-linked polyubiquitination (25). RIG-I also harbors ISG15 modification, although the role of ISG15 modification *in vivo* remains to be deter-

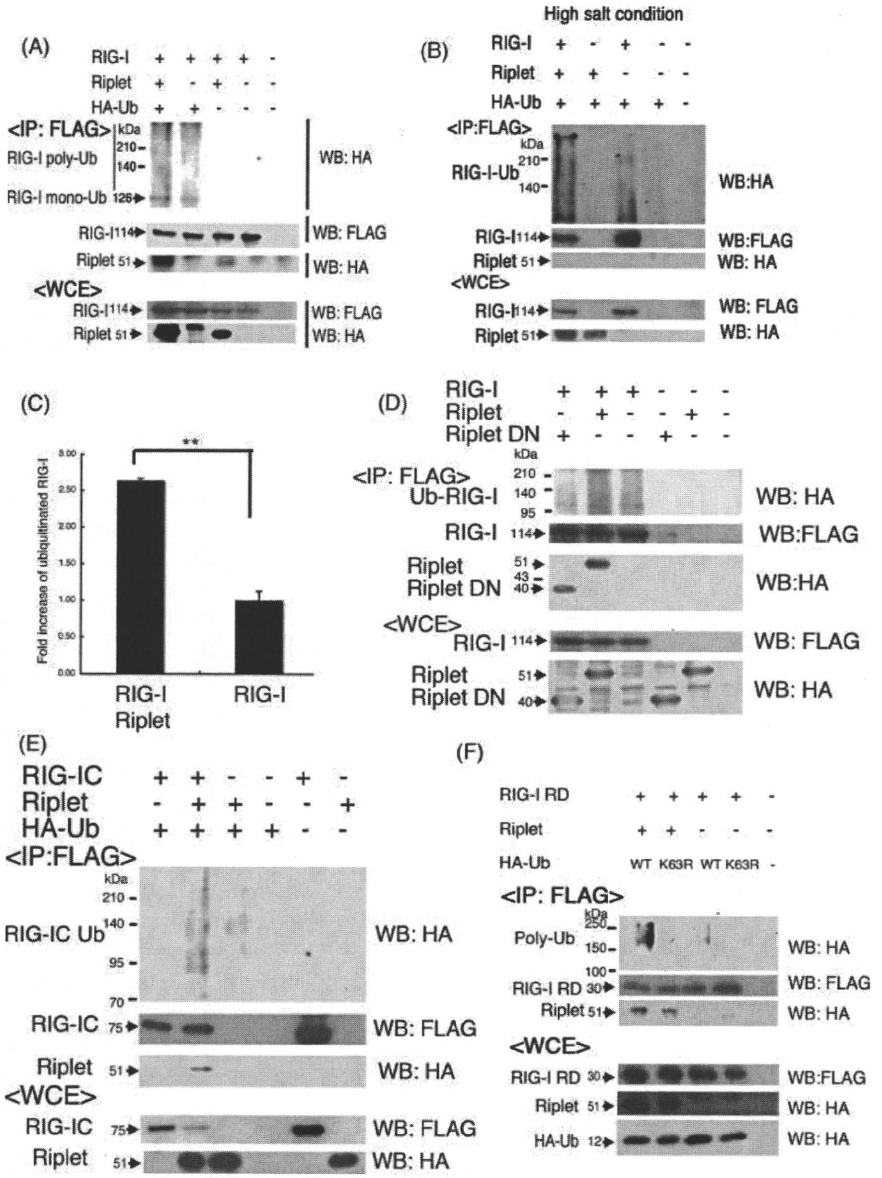


FIGURE 6. **Ubiquitination of RIG-I by Riplet.** *A* and *B*, FLAG-tagged RIG-I (0.4 μg), Riplet (0.4 μg), and HA-tagged ubiquitin (0.4 μg) expressing vectors were transfected into HEK293FT cells in 6-well plates. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). FLAG-tagged RIG-I was immunoprecipitated (*IP*) using an anti-FLAG antibody, and washed with the buffer containing 150 mM NaCl (*A*) or 1 M NaCl (*B*). The immunoprecipitates were separated with 8% acrylamide gel and analyzed by Western blotting (*WB*) using antibodies against HA tag (ubiquitin) or FLAG (RIG-I). Riplet was co-immunoprecipitated with FLAG-tagged RIG-I in *A* but could not co-immunoprecipitate in *B* because of high salt condition. Expression of Riplet enhanced the ubiquitination of RIG-I. Different gel conditions were employed in *A* and *B*. *C*, ubiquitinated RIG-I was quantitated with NIH image software. **, $p < 0.01$. *D*, FLAG-tagged RIG-I (0.4 μg) was transfected into HEK293 FT cells in a 6-well plate with HA-tagged Riplet (0.4 μg) or Riplet-DN (0.4 μg) and HA-tagged ubiquitin, and immunoprecipitation was carried out with anti-FLAG antibody. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). The samples were analyzed with 10% acrylamide gel to clearly separate Riplet from Riplet-DN and stained by Western blotting. *E*, ubiquitination of RIG-IC was also promoted by Riplet expression. HEK293FT cells were transfected with the plasmids encoding RIG-IC (0.4 μg), Riplet (0.4 μg), and/or HA-tagged ubiquitin (0.4 μg) in a 6-well plate, and 24 h after transfection, cell lysates were prepared. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). FLAG-tagged RIG-ICs were immunoprecipitated with anti-FLAG antibodies, and the proteins were analyzed by Western blotting. *F*, Ub-K63R are HA-tagged ubiquitin in which the lysine 3 residues were substituted with arginine. The HA-tagged Ub-K63 expressing vectors (1.2 μg), FLAG-tagged RIG-IC (0.4 μg), and/or Riplet (0.4 μg) were transfected into HEK293FT cells in 6-well plates and analyzed as shown in *A–D*. The total amount of transfected DNA (2 μg/well) was kept constant by adding empty vector (pEF-BOS). Ub-K63R was not incorporated into polyubiquitin chain of RIG-I RD. *WCE*, whole cell extract.

mined (21, 22, 31). Although Riplet and TRIM25 share 60% sequence similarity, the ubiquitination of RIG-I by Riplet is distinct from that by TRIM25; Riplet ubiquitinates the C-terminal region of RIG-I, whereas TRIM25 ubiquitinates its CARD-like region. These findings are also supported by the fact that neither Riplet nor Riplet-DN promoted or inhibited the activation of the IFN-β promoter by expression of the RIG-I CARD-like region (data not shown). It has been reported that ubiquitination of the CARD-like region of RIG-I by TRIM25 is critical for RIG-I-IPS-1 signaling (23). However, how this CARD ubiquitination is essential for activation of IPS-1 by RIG-I remains undetermined. Here we emphasize the importance of RIG-I C-terminal ubiquitination for IFN-β induction and the antiviral response. Because the C-terminal RD region inhibits the IFN inducing activity of the CARD-like region of RIG-I, it is reasonable that RIG-I C-terminal ubiquitination by Riplet inhibits the conversion from the active to inactive form of RIG-I protein after binding to viral RNA. This initial stabilization of RIG-I via ubiquitination by Riplet would provide a sufficient structure for RIG-I to maintain the accessibility to TRIM25 and facilitate TRIM25-mediated ubiquitination of the CARD-like region of RIG-I, which may lead to potential activation of IPS-1.

RIG-I is an IFN-inducible RNA helicase that is expressed at extremely low levels in resting cells (6). Initial penetration of viruses allows generation of 5′-triphosphate RNA and/or double strand RNA followed by induction of IFN-β production. This early response to viral infections triggers up-regulation of RIG-I/MDA5 and TLR3, leading to robust IFN-β production (3, 32, 33). We favor the interpretation of our present findings that during the early stages of viral infection with trace amounts of RIG-I and viral RNAs, Riplet helps host cells rearrange RIG-I conformation to activate IPS-1. This issue will need further proof because it is difficult to

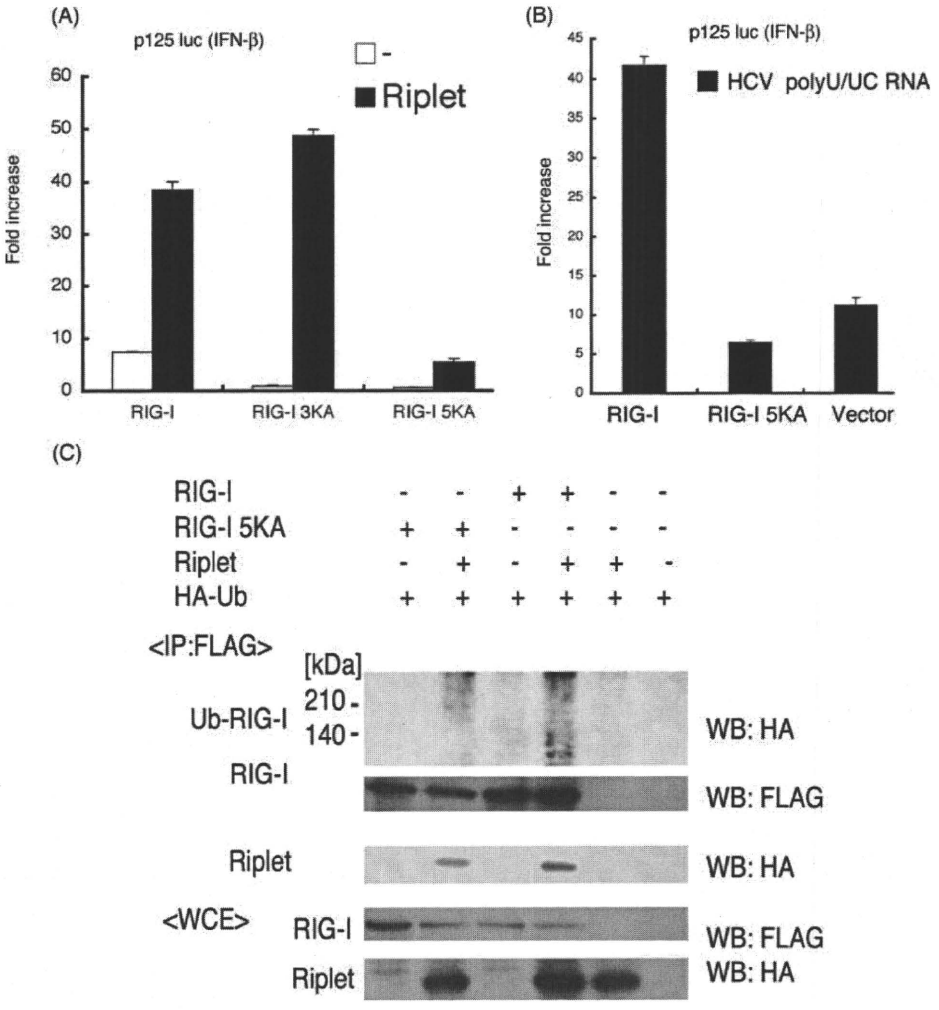


FIGURE 7. **The C-terminal two lysine residues of RIG-I are important for ubiquitination by Riplet.** *A*, RIG-I C-terminal lysine residues were substituted with alanine. RIG-I 3KA mutant protein harbors the triple mutations, K888A, K907A, and K909A. The five lysine residues, Lys-849, Lys-851, Lys-888, Lys-907, and Lys-909, were replaced with alanine in RIG-I 5KA mutant. The plasmid carrying wild-type (100 ng/well), RIG-I 3KA (100 ng/well), RIG-I 5KA (100 ng), or Riplet (100 ng) were transfected into HEK293 cells in a 24-well plate together with p125 luc reporter plasmid (100 ng/well). The amount of transfected DNA was kept constant by adding empty vector. After 24 h, the luciferase activities were measured. *B*, wild-type RIG-I (100 ng), RIG-I 5KA mutant (100 ng), or empty vector (100 ng) was transfected into HEK293 cells in a 24-well plate together with p125 luc reporter plasmids and HCV 3′-untranslated region poly(U/UC) RNA (25 ng), which is synthesized in *in vitro* transcription by T7 RNA polymerase. The amount of transfected DNA was kept constant by adding empty vector. 24 h after transfection, luciferase activities were measured. RIG-I 5KA mutant hardly responded to poly(U/UC) RNA. *C*, to observe the ubiquitinated RIG-I more clearly, we used 800 ng/well of Riplet and HA-Ub expression vector for the following transfection. HEK293FT cells in a 6-well plate were transfected with the plasmids encoding RIG-I (400 ng/well), RIG-I 5KA (400 ng/well), Riplet (800 ng/well), and/or HA-Ub (800 ng/well). The total amount of DNA was kept constant by adding the empty vector. 24 h after the transfection, the cell lysates were prepared, and the immunoprecipitation was carried out using anti-FLAG antibodies. The immunoprecipitates were analyzed by Western blotting with anti-HA or FLAG antibodies.

visualize RNRs and viral RNAs in the early infection stage and to understand the mechanisms that allow viruses to uncoat into naked viral RNA and to replicate.

We have provided several lines of evidence indicating that Riplet complements RIG-I-mediated IFN-β induction upon viral infection by both Riplet siRNA and overexpression analyses. The C-terminal lysines (849 and 851) of RIG-I are critical for Riplet-mediated RIG-I ubiquitination. However, our data indicate that Riplet alone was unable to induce IFN-β production and essentially required RIG-I to confer IFN-β induction. Furthermore, Riplet is not ubiquitously distributed over the organs tested. Ubiquitination of RIG-I induced by poly(I-C) or viruses was accelerated in cells pre-transfected with Riplet. Hence, Riplet works case-sensitive to up-regulate RIG-I antiviral activity predominantly in some organs. The physiological meaning of this response will be clarified by knock-out study.

Unexpectedly, the siRNA experiments were not robust with regard to VSV replication. Possible explanations for this are as follows: 1) the degree of gene silencing is not so profound that the proteins remain in the cells; 2) there are a number of virus-mediated IFN-inducing pathways capable of compensating each other, so that disruption of one factor does not cause a profound effect on VSV replication. Furthermore, in VSV-infected Riplet-knockdown cells, IFN-β levels were reduced even at m.o.i. = 1 (Fig. 3*D*), and accordingly, virus susceptibility was increased at m.o.i. = 0.1 (Fig. 4*C*), whereas in Riplet-overexpressing cells, antiviral activity was observed only at low m.o.i. (Fig. 4*B*). We used different transfection reagents and cell conditions in the knockdown and overexpression experiments to obtain high transfection efficiency in each. These conditional differences in knockdown and overexpression analyses might cause part of the discrepancy between the two results on Riplet antiviral activity. Another possibility to explain the apparent inconsistencies between overexpression and knockdown analyses is that high amounts of Riplet efficiently activate the RIG-I signaling, but low amounts are insufficient for RIG-I activation in high m.o.i.-infecting human cells. High amounts of Riplet with overexpressed RIG-I would confer the ability on cells to respond to very low amounts of VSV as observed in the low m.o.i. experiments. Again, *riplet* knock-out mice would reveal whether it is absolutely required for potential RIG-I activation.

How viral RNAs select RIG-I rather than dicers or the translation machinery is also unknown. During natural infection it is likely that the number of the initial invading virions would be at most several copies/cell. Uncoated viral RNA may assemble a complex consisting of viral and host molecules required for replication. We assume that cells are equipped with various

molecular arms to sensitively detect viral RNA. The molecular complexes sensing viral RNA may not be so simple that we will be able to identify more molecules than Riplet as enhancers for integral RNA recognition. In either case, yeast screening will be a good strategy to pick up such proteins in other RNA recognition systems. A molecular switch selecting IFN induction by virus RNA will then be clarified.

We show that the ubiquitination sites targeted by Riplet are the helicase and RD domains of RIG-I but not its CARD-like domains in contrast to TRIM25. Riplet may be a complement factor of the reported TRIM25 function for RIG-I activation (23). A previous report (25) failed to polyubiquitinate the RIG-I protein by TRIM25 alone. If Riplet were added to TRIM25 for RIG-I ubiquitination in the previous study, Riplet would have enabled TRIM25 to polyubiquitinate the RIG-I CARD-like region. Further studies using TRIM25 and Riplet will be required to clarify this point.

Based on our results, we propose that RIG-I-like receptors form a molecular complex that efficiently recognizes low copy numbers of viral RNA. Riplet is implicated in the RIG-I complex to enhance viral RNA response in some organs. In this context, MDA5-associated molecules might also exist in the cytoplasm to augment IFN output. Although MDA5 possesses the RD domain, it fails to recruit Riplet (data not shown) or augment IFN-β-induction in conjunction with Riplet (Fig. 2*E*). Because RLR-associated molecules naturally reside in cells and facilitate inhibition of low dose viral infection until RLRs become expressed, they may be useful therapeutic targets for an early phase antiviral immunotherapy.

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