

KEY PLAYERS IN INNATE IMMUNE RECOGNITION OF VIRUS INFECTION IN MAMMALS AND FISH

I. Jakovlić, Y. B. Zhang, Q. J. Wu, J. F. Gui

Summary

Viral infection of mammalian cells activates an innate antiviral immune response characterized by production of interferon and subsequent enhanced transcription of interferon-stimulated genes important for antiviral defense. Cells recognize viral infection through various pathogen-associated molecular patterns, of which dsRNA seems to be the most important. In mammals, several gene products are important in recognition of dsRNA: RIG-I, TLR3, PKR and mda-5. Recent research proved that fish possess most of the key elements in recognition of viral infection which indicates that these mechanisms are very similar and evolutionary conserved in vertebrates.

Keywords: interferons; viral infection; dsRNA; PKR; RIG-I; TLR3; mda-5

INTRODUCTION

Viral infection of mammalian cells activates an innate antiviral immune response characterized by production of interferon (IFN) and the subsequent transcriptional upregulation of IFN-stimulated genes (ISGs) mainly by JAK-STAT signaling pathway. Three families of IFNs (type I IFN, type II IFN and IFN- λ) can be distinguished in mammals on the basis of gene structure, protein structure and functional properties (Sen, 2001; Robertsen, 2005). IFN-like activity was identified in fish as early as 1965, and has since been detected in cells and organs of a number of fish species after viral infection or poly I:C treatment. The first fish IFN genes were not reported cloned until 2003, however. To date, IFN genes have been cloned from several fish species and they mostly showed structural and functional properties similar to

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mammalian type I IFNs except that, in contrast to the classical type I IFNs of birds and mammals, fish type I IFN genes contain introns (Robertson, 2005). Since IFNAR-1 (interferon α , β and ω receptor 1) was not found in the putative I IFN receptor gene cluster of pufferfish and may thus be absent in fish, type I IFN possibly use different fish receptors (Mogensen et al., 1999). Before identification of fish IFN genes, some genes involved in IFN response, including IRF, Mx and ISG15, had been cloned. Recently, much more fish IFN system genes, including TLR3, PKR-like, IFN, STAT1, IRF-7, Mx1, Mx2, Viperin, IFI58, ISG15-1, ISG15-2, USP18, Gig1, and Gig2 have been identified from the UV-inactivated *Grass Carp Haemorrhagic Virus* (GCHV)-infected and IFN-producing CAB (crucian carp *Carassius auratus* blastulae embryonic) cells (Zhang et al., 2000; 2003a, b; 2004a, b, c, d) supporting a notion that fish have a complete IFN system with similar antiviral mechanisms to mammals.

Lately there is more and more evidence of multiple signaling pathways for induction of IFN and IFN inducible proteins. Pattern recognition receptors (PRRs) serve as first-line sentinels for innate immune detection of pathogenic infections which, upon recognizing and binding various conserved molecular motifs termed »pathogen-associated molecular patterns« (PAMPs), activate various signaling pathways for mounting an antiviral state in cell and production of IFNs and, subsequently, various IFN-stimulated proteins. Double-stranded RNA (dsRNA), by-product during virus replication, seems to be the most important PAMP to initiate cellular IFN response. An increasing evidence show that fish possess similar mechanisms to trigger innate immune response with some PRRs.

RECENT PROGRESS IN UNDERSTANDING OF INNATE IMMUNE RECOGNITION OF VIRUS INFECTION IN MAMMALS

In mammals, several genes are demonstrated to be important in recognition of dsRNA, including RIG-I, TLR3, PKR and mda-5.

1. PKR

The double-stranded-RNA-dependent protein kinase (PKR) is a 551 amino acid, or 68 kDa, protein encoded from a single gene located on chromosome 2p21 in humans and a 65 kDa protein in mice, which is ubiquitously expressed at a low level, but normally inactive. Functionally, it is a cytoplasmic serine-threonine kinase important in an IFN-mediated antiviral defence pathway (Feng et al., 1992; Gale and Katze, 1998; McCormack et al., 1992; Stark et al., 1998) and structurally, in addition to a very conserved kinase catalytic domain in its C-terminus, PKR contains a N-terminal regulatory domain characteristic for two dsRNA binding motifs (dsRBM) that mediate the binding of its activator, dsRNA (Feng et al., 1992;

McCormack et al., 1992). It belongs to a class of more than 20 dsRNA binding proteins (DRBP), which have been reported to interact with as little as 11 bp of dsRNA, and its interaction with activator RNA is independent of any specific RNA nucleotide motif or sequence. However, *in vitro*, the concentration of activator does play a role in the autophosphorylation of PKR, with low levels of dsRNA potently activating the kinase and higher concentrations of dsRNA being inhibitory. Possibly high concentrations of dsRNA prevent PKR activity by impeding intermolecular interactions and trans-autophosphorylation. Nevertheless, optimal activation requires that both DRBDs of PKR cooperate to form a single binding site that extends to interact with ~ 30–80 bp of dsRNA (Saunders and Barber, 2003).

Interaction with dsRNA causes PKR to become activated through autophosphorylation and dimerisation or phosphorylation by upstream kinases including PKR-activating protein (PACT), another dsRNA-binding kinase. Once activated, PKR phosphorylates the protein synthesis factor eIF-2 α , which induces the formation of a stable inactive complex that involves eIF2-GDP and the recycling factor eIF2B (Gale and Katze, 1998; McCormack et al., 1992; Samuel, 2001), resulting in inhibition of translation initiation and prevention of viral replication. PKR probably plays a role in NF- κ B activation in response to dsRNA and viral infections (Perry et al., 2005).

In addition to role in inhibition of viral replication, purpose of two DRBDs in the C-terminus of PKR is ascribed to recognition of cytoplasmic dsRNA produced by virus infection and, subsequently, activation of IFN expression. Although, the underlying mechanism so far remains unsolved and other, PKR-independent, dsRNA-recognizing mechanisms are probably more important. PKR is also believed to play a role in various signaling pathways leading to gene transcription by modulating the activities of various transcription factors including STATs (signal transducer and activator of transcriptions), and the tumor suppressor p53 for the control of apoptosis, differentiation and cell proliferation (Gale and Katze, 1998). The potency of PKR in host antiviral responses is highlighted by the fact that numerous viruses encode genes to inhibit PKR activity (Oshiumi et al., 2003).

2. *TLR3*

Toll-like receptors (TLRs) are a family of type I membrane receptors characterized by an extracellular leucine-rich repeat (LRR) and a conserved cytoplasmic region called the Toll-interleukin-1 receptor (TIR) domain. LRR participates in ligand recognition and TIR transmits downstream signals. TLR family members are differentially expressed by cells of the immune system and cells involved in first line host defense, including neutrophils, macrophages, dendritic cells, vascular endothelial cells, and intestinal epithelial cells. In mammals, 12 members of TLR family have been identified so far and they act as pathogen-recognition receptors (PRRs), able to differentiate between

chemically diverse pathogens and activate intracellular signal transduction pathways (Akira and Takeda, 2004; Arment and Fenton, 2002). Among them, TLRs3, 7, 8, and 9 recognize nucleic acids, and dsRNA is thought to be the main ligand for TLR3.

Cellular localization of TLR3 depends on cell type. TLR3 is localized in intracellular vesicular compartment in dendritic cells, whereas it is expressed on the cell surface of fibroblasts (Matsumoto et al., 2003; Kawai and Akira, 2006). Thus, it is speculated that recognition of dsRNA, released into the extracellular space by necrotic or virally lysed cells, is achieved through phagocytosis. Unlike all the other TLR-s, which use Myd88-dependent signaling pathway, TLR3 signals in Myd88 independent manner. The main pathway leads through TRIF, TBK1, and IRF-3 to activate type I IFN production, but the whole picture seems to be much more complicated.

3. RIG-I

Retinoic acid-inducible gene-I (RIG-I) was recently identified as a potential viral pattern recognition receptor (PRR) able to recognize cytoplasmic dsRNA (Yoneyama et al., 2004). RIG-I is a member of RNA helicase family that plays diverse roles in regulation of gene expression and cellular functions. Besides sensing cytoplasmic dsRNA, RIG-I also may be involved in the control of cellular differentiation (Imaizumi et al., 2004b). Expression of RIG-I is induced by dsRNA, LPS and by IFN- γ (Imaizumi et al., 2004a; Imaizumi et al., 2004b). Two conserved domains: DExD/H box and helicase C (carboxyl terminus of helicase) were identified (Zhang et al., 2000). RNA helicase domain was found to activate IFN/ production in response to transfected poly I:C and Newcastle disease virus (NDV) infection (Yoneyama et al., 2004). The RNA helicase domain was shown to interact with poly I:C, while overexpression of the caspase recruitment domain (CARD) of RIG-I activated IRF-3 and NF- κ B (Yoneyama et al., 2004).

The main signaling component downstream of RIG-I, as recently was discovered, is IPS-1, a CARD-domain-containing adaptor protein, anchored at the outer membrane of mitochondria, which links RIG-I to the IKK ϵ -TBK1-IRF-3 and the TRAF6-FADD-NF- κ B pathways. Therefore, the signaling pathways activated by TLR3 and RIG-I differ in their initial steps, but converge at the end and activate protein kinases IKK $\alpha\beta$, TBK1 and IKK ϵ . A possible scenario is that binding of dsRNA to the helicase domain results in activation of the ATP-hydrolyzing function and a conformational change of the substrate dsRNA, helicase domain and CARD, which confers the CARD an ability to transmit signals downstream by recruitment of other molecules. Overexpression of RIG-I inhibited replication of vesicular stomatitis virus (VSV) and encephalomyocarditis virus (ECMV) in L929 cells. However, this same study found no defects in IRF-3 activation during NDV infection in fibroblasts deficient in TRIF or TBK1 (Yoneyama et al., 2004). This is in

contrast to a study in which TBK1 was required in fibroblasts for NDV-induced IRF-3 activation and IFN production (McWhirter et al., 2004). Future studies need to address whether or not PKR and RIG-I represent independent pathways of dsRNA recognition leading to type I IFN production.

4. *mda-5*

Melanoma differentiation-associated -5 gene was recently identified as a gene induced during differentiation, cancer reversion, and programmed cell death (apoptosis). It contains, just like RIG-I, a caspase recruitment domain and putative DExH group RNA helicase domains, and is an early response gene, induced without previous protein synthesis, localized in the cytoplasm and inducible by IFN and tumor necrosis factor- α , responding predominantly to IFN- β (Kang et al., 2002). Based on that it can be assumed that it has similar function like RIG-I. It is possible that MDA-5 may be a component of death effector complexes or molecules but by itself lacks the capacity to trigger apoptosis and it is highly probable that, by its ATP-dependent unwinding of dsRNA, might function in inhibiting translation during growth inhibition mediated by IFN. It was also reported that V proteins of a diverse group of paramyxoviruses bind *mda-5* via their highly conserved C-terminal domain and the evidence was presented that links this property to the ability of these viruses to reduce the production of IFN by infected cells (Andrejeva et al., 2004). To reduce, but not completely abolish it, which is consistent with the existence of *mda-5*-independent dsRNA response pathways.

HOMOLOGOUS GENES IDENTIFIED IN FISH

Recently, many genes involved in antiviral response have been identified and characterized from virally infected fish cells and organisms. These include PKR, TLR3, RIG-I and *Mda-5*, indicating that, as the lower vertebrates, fish also possess similar mechanisms for innate immune recognition of virus infection.

A PKR-like gene, *Carassius auratus* PKR-like (CaPKR-like), was first cloned from UV-inactivated GCHV-infected CAB cells. Although CaPKR-like and mammalian PKRs are expressed at a low level in normal cells and upregulated by virus infection and IFNs, CaPKR-like has a specific structure with both Z-DNA binding domains ($Z\alpha$ domains) in its N terminus instead of dsRNA binding domains (dsRBM) positioned in mammalian PKR (Hu et al., 2004). Further study confirmed that $Z\alpha$ domains showed the ability of binding poly I:C in vitro (Hu et al., 2005), the same function as dsRBM (Samuel, 2001). So CaPKR-like might function in the virally infected crucian carp cells as mammalian PKR proteins. Moreover, DrPKZ, a zebrafish gene similar to CaPKR-like, was confirmed to have two $Z\alpha$ -like domains in N terminus, a kinase domain in C terminus and to inhibit translation in transfected cells by

its C-terminal catalytic activity (Rothenburg et al., 2005). But the functions of Z-DNA binding domains and the question why do fish PKR-like activities employ left-handed Z-DNA (or Z-RNA) binding domains, whereas the mammalian enzymes use dsRNA-binding domains, so far remain unanswered. It is likely though, that PKR-like gene is fish-specific, as a PKR orthologue, that showed an identical structure to mammalian PKR, has been found in *Carassius auratus* and *Paralichthys olivaceus* (unpublished data).

To date, TLR family members have been described in various teleosts: *Danio rerio* (Meijer et al., 2004), *Fugu rubripes* (Oshiumi et al., 2003), *Carassius auratus*, *Paralichthys olivaceus* and *Oncorhynchus mykiss* (Bilodeau and Walbieser, 2005). Most have high homology to their mammalian counterparts. However, there is little applied data published regarding the functional role of TLRs in teleosts. So far, the most research has been done on zebrafish (*Danio rerio*) genome, where recently, besides homologues to mammalian TLRs (zfTLR3), TIR domain-containing adaptor proteins IRAK-4 (zfIRAK-4) and TRAF6 (zfTRAF6) were identified. Sequence analysis revealed conserved domains shared with insect and mammalian genes, indicating evolutionary conservation (Jault et al., 2004; Meijer et al., 2004). Interestingly, in mammals TLR3 is strongly associated with the antiviral response via dsRNA intermediates (Jiang et al., 2003; Matsumoto et al., 2004; Oshiumi et al., 2003), yet some results in zebrafish show that zfTLR3 is clearly upregulated following bacterial infection (Phelan et al., 2005). That may represent a unique response, but there is also a possibility that zfTLR3, while structurally similar to mammalian TLR3, could represent a separate TLR that can respond to both viral and bacterial PAMPs. Alternatively, zfTLR3 might form homo or heterodimers with itself and other TLRs in order to broaden the range of ligand-binding possibilities, contributing to the increase in observed mRNA expression (Phelan et al., 2005). In our lab, CaTLR3 (accession number: DQ291158) was cloned from virus infected CAB cells and confirmed to have a virally induced expression.

Very recently, we have also cloned the RIG-I homologue from UV-inactivated GCHV treated CAB cells (unpublished data), but there are still no data related to function of this gene in fish. Although, some results showed that two ESTs, homologous to mammalian RIG-I (CA677) and mda5 (CA666), were highly induced by virus infection and poly I:C, which indicates that fish, just like mammals, in addition to TLR3-dependent antiviral response, also have a RIG-I, mda5 mediated pathway.

CONCLUSION AND QUESTIONS

Relative importance of discussed dsRNA recognition mechanisms during viral infection is still not completely clear. In dendritic cells TLR3 is localized in intracellular vesicular compartments, where it probably encounters dsRNA

released into the extracellular space by necrotic or virally lysed cells and phagocytosed by the cell. In contrast to TLR3, PKR, RIG-I and mda-5 are cytoplasmic and therefore much more suited for the direct recognition of dsRNA produced during viral replication. Another interesting observation is that signaling pathways elicited by TLR3, PKR and RIG-I overlap significantly in that all three activate NF κ -B. RIG-I and TLR3 even seem to depend on the same kinases, IKK- ϵ and TBK1, to phosphorylate IRF3. However, it seems that different gene profiles may be induced through TLR3 and RIG-I (Schroder and Bowie, 2005). Therefore, it is probable that subtle differences exist in the signaling pathways of TLR3, PKR and RIG-I, which, in further investigation, will provide more insights into the specific roles of these dsRNA receptors.

Another question raises from the fact that properties of mda-5 and RIG-I are reminiscent of those exhibited by nucleotide-binding oligomerization domain (NOD)-1 and NOD-2, proteins that recognize intracellular peptidoglycans with the consequent activation of their N-terminal CARD domains and subsequent NF- κ B activation. Thus mda-5, RIG-I, NOD1 and NOD2 may be representative of a wider class of intracellular pattern recognition molecules (Andrejeva et al., 2004).

Although data on fish IFN and IFN signaling pathways are still insufficient for definite breakthrough in research of fish IFN system genes, the identified genes involved in antiviral immune response show high homology to mammalian counterparts, indicating similar functions in fish. In our lab, research on UV-inactivated GCHV infected CAB cells revealed that produced antiviral activity is very similar to mammalian and, in addition to signal genes (*TLR3*, *PKR-like*, *RIG-I*, *Mda5*, *STAT1*, *IRF7*), six known (*Mx1*, *Mx2*, *IFI56*, *viperin*, *ISG15-1*, *ISG15-2*) and possibly two new antiviral genes (*Gig1*, *Gig2*) were discovered. Accordingly, gene expression signature in UV-inactivated GCHV-infected CAB cells revealed an IFN response similar to mammals, from recognition of viral dsRNA to synthesis of cellular IFN and finally to formation of antiviral state. Accordingly, the progress on innate immune recognition of virus infection in fish suggests that in vertebrates these mechanisms are very similar and evolutionary conserved.

Sažetak**KLJUČNI ČIMBENICI U UROĐENOM IMUNOLOŠKOM PREPOZNAVANJU VIRUSNE INFEKCIJE U SISAVACA I RIBA**

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Virusna infekcija u stanicama sisavaca potiče urođeni odgovor karakteriziran proizvodnjom interferona i posljedičnom pojačanom transkripcijom interferonima stimuliranih gena, bitnih u obrani organizma od virusa. Stanice prepoznaju virusnu infekciju preko određenih molekularnih uzoraka povezanih s patogenima, od kojih je najvažnija dvolančana RNK. U organizmu sisavaca ključnu ulogu u prepoznavanju dvolančane RNK ima nekoliko gena: RIG-I, TLR3, PKR i mda-5. Dosadašnja su istraživanja pokazala da ribe posjeduju većinu ključnih elemenata zaduženih za prepoznavanje virusne infekcije, što upućuje na veliku sličnost i evolucijsku očuvanost spomenutih mehanizama.

Ključne riječi: interferoni; virusna infekcija; dsRNK; PKR; RIG-I; TLR3; mda-5

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