Visualisation of direct interaction of MDA5 and the dsRNA replicative intermediate form of positive strand RNA viruses

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Running title: MDA5 recognises the dsRNA replicative intermediate
Summary

The innate immune system is a vital part of the body’s defences against viral pathogens. RIG-I and MDA5 function as cytoplasmic PRRs that are involved in the elimination of actively replicating RNA viruses. Their location and their differential responses to RNA viruses emphasises the complexity of the innate detection system. Despite the wealth of information on the types of RNA that trigger RIG-I, much less is known about the nature of the RNAs that act as agonists for MDA5. In order to identify which RNA species triggers MDA5 activation during infection, we isolated viral ssRNA and replicative intermediates of RNA from positive sense ssRNA viruses. We reveal that MDA5 recognises not the genomic ssRNA but the dsRNA generated by the replication of these viruses. Furthermore, using fluorescent imaging we present the first report of the visualization of dsRNA and MDA5, which provides unique evidence between the relationship of viral dsRNA and MDA5 and proves without a doubt that MDA5 is the key sensor for the dsRNA replicative intermediate form of positive sense ssRNA viruses.

Keywords: RIG-like helicases / MDA5 / RIG-I / RNA viruses
Introduction

Viruses are obligate intracellular pathogens that need to hijack the host’s machinery in order to produce progeny of viruses, but this is often limited by the host’s antiviral response. Successful sensing of the viruses by the host leads to a rapid innate immune response. Viral RNA seems to be a molecular signature that the host recognises and is a potent inducer of the innate host response. Viral RNA is recognised by Toll-like receptors (TLRs), or retinoic acid-inducible gene-I (RIG-I)–like receptors (RLRs), which are the two main families of pattern recognition receptors (PRRs) that play a key role in sensing viral RNA and activate inflammatory cytokines and type I interferons (IFNs) (Akira, 2001; Takeuchi and Akira, 2008).

RLRs have been recently discovered and play a key role in sensing RNA virus invasion. They recognise viral RNA independently of TLRs and unlike TLRs, which are found either on the cell surface or endosomes, RLRs are found in the cytoplasm (Takeuchi and Akira, 2009). The two main RNA helicases are RIG-I (retinoic acid inducible protein I) and MDA5 (melanoma differentiation associated gene 5). Both proteins consist of: two caspase recruitment domains (CARD-like domains) at the N-terminus, which are required for the initiation of downstream signalling following their activation; a middle sector where a DExD box RNA helicase domain is found, which is responsible for the detection of PAMPs; and a repressor domain at the C-terminus which is functional in RIG-I but may not be in MDA5 (Takeuchi and Akira, 2009; Takeuchi and Akira, 2008).

RLRs were initially thought to recognise the same ligand, dsRNA, but it has since become apparent that the two receptors possess distinct virus specificities. It has been shown that
RIG-I and MDA5 recognize different viruses and different viral RNAs (Kato et al., 2006; Loo et al., 2008). RIG-I recognizes single-stranded RNA (ssRNA) containing a terminal 5'-triphosphate (ppp) (Pichlmair et al., 2007), as well as linear dsRNA no longer than 23 nucleotides (Kato et al., 2008). MDA5 recognizes long strands of dsRNA but the mechanism by which this occurs is less clear (Kato et al., 2008).

RIG-I recognizes negative sense ssRNA viruses including influenza virus, Newcastle disease virus, Sendai virus as well as Hepatitis C virus, while positive sense ssRNA viruses such as Picornaviruses, and more specifically encephalomyocarditis virus and Coxsackievirus B3 have been shown to activate MDA5 and trigger IFN response (Kato et al., 2006; Loo et al., 2008; Wang et al., 2010).

Despite the wealth of information on the types of viruses and RNA that can activate RIG-I and MDA5, the natural ligand responsible for triggering the response remains unclear. RNA recognition by RIG-I seems to require the presence of a free 5'-triphasphate structure (Pichlmair et al., 2007; Hornung et al., 2006; Rehwinkel et al., 2010), but much less is known about the nature of the RNAs that act as ligands for MDA5. It has been suggested that higher order RNA viral structures are required in order to activate MDA5 (Pichlmair et al., 2009), but the specific agonist has not been identified. Using biochemical methods, Pichlmair et al (Pichlmair et al., 2009) concluded that MDA5 does not recognize the replicative intermediate dsRNA but higher order RNA structures. Their results suggest that MDA5 activation requires an RNA web rather than simply long molecules of dsRNA, however that fails to explain why MDA5 can sense Poly:I:C, which is an equivalent of long dsRNA or dsRNA viruses such as reoviruses (Kato et al., 2008) or why it cannot sense picornavirus genomic ssRNA that contains highly ordered secondary structures and tertiary RNA structures in the 3' and 5'UTR regions. Since not all MDA5 agonists seem to fit the model of an RNA web, or branches of
RNA, there is a pressing need to identify what is the exact MDA5 ligand. Revealing how MDA5 becomes activated during infection will lead to the future development of new therapies to combat viral disease.

In order to identify the specific ligand for MDA5, we chose to utilise enteroviruses, a genus of positive sense ssRNA viruses which belong to the Picornavirus family. Although members of the family have been shown to trigger the innate immune response via TLRs (Triantafilou and Triantafilou, 2004; Richer et al., 2006; Triantafilou et al., 2005) and by MDA5 (Kato et al., 2006; Loo et al., 2008; Wang et al., 2010), little is known about the exact ligand. Total RNA extracted from enteroviruses triggers MDA5, however the specific ligand within such pools has not been identified. Candidates include viral genomes, viral transcripts, and replication intermediates. Since enteroviruses are (+) ssRNA viruses their replication follows a strategy common for all positive-sense RNA viruses. Their viral genome is transcribed into complementary RNA (-strand) which in turn is used as a template to synthesize new strands forming double-stranded RNA (dsRNA) replicative intermediate forms (RF). In order to identify which RNA species triggers MDA5 activation during infection by enteroviruses, we modified purification procedures which have been used in the past for the isolation of viral ssRNA and replicative intermediates of RNA, (Spector and Baltimore, 1975) (Miller et al., 1975; Flanagan et al., 1977). We successfully isolated the replicative intermediates and utilised them for our experiments. Our experiments revealed that MDA5 recognises not the genomic ssRNA but the dsRNA generated by the replication of these viruses. Using fluorescent imaging methods, we were able to visualise the association of MDA5 with the replicative form of enteroviruses. This is the first report of the visualization of dsRNA and MDA5, which provides unique evidence between the relationship of viral dsRNA and MDA5 and proves that MDA5 is the key sensor for the dsRNA replicative intermediate form of positive sense ssRNA viruses.
Results

Studies with different types of RNA

Enteroviruses are (+) ssRNA viruses thus their replication follows a strategy common for all positive-sense RNA viruses. The viral genome is transcribed into complementary RNA (−strand) which in turn is used as a template to synthesize new strands forming double-stranded RNA (dsRNA) replicative intermediate forms (RF) and a complex partially double stranded RNA intermediate (RI) which has double stranded core to which attached is ssRNA. Therefore during an enteroviral infection, the host’s innate immune system will be presented with a pool of foreign RNA, such pools would contain whole viral genomes, viral transcripts as well as replication intermediates. Since our data suggests that when the hosts is challenged by these pools of foreign RNA, there is MDA5 activation leading to IFN production, the question that remains is what is the exact ligand for MDA5? Which particular constituent of the viral genomic pool is responsible for triggering MDA5 activation?

In order to determine whether viral replication is essential for MDA5 recognition and to characterize the RNA species responsible for MDA5 activation in cells infected with positive stand RNA viruses such as Picornaviruses, we infected cells and isolated the full length genomic ssRNA as well as the viral dsRNA replicative form (RF) and the high molecular weight RI-RNA which is a complex molecule composed of double stranded core and nascent positive single-stranded RNA branches, from the cytoplasm of these cells using modified purification procedures for the isolation of viral ssRNA and poliovirus replicative intermediates (RF) and (RI) similar to Richards and Ehrenfeld (Richards et al., 1987a; Spector and Baltimore, 1975) and purified by Fast performance liquid chromatography (FPLC) (Figure 1A). The purified ssRNA was resistant to DNase I and digested by pancreatic
RNAse into two bands of 6,000 bases and 1,500 bases (Figure 1C). RF RNA was resistant to DNAse I and over 99% resistant to pancreatic RNAse indicating that there was no contamination by DNA or sensitive ssRNA (Figure 1D). The purified RI RNA was resistant to DNAse I and over 50% resistant to pancreatic RNAse (Figure 1E).

To verify the specificity of the RI and RF RNA, it was incubated at 95°C for 5min to denature dsRNA to ssRNA, reversed transcribed and amplified by PCR. A 380-bp amplicon generated with the OL26-OL27 primer pair, which has been used extensively in the past to identify picornaviruses was generated and verified by sequencing. We thus proceeded to mimic a live infection and determine whether the accumulation of viral RF RNA, RI RNA or genomic ssRNA has a stimulatory effect in cells.

**RLR involvement in recognition of enteroviruses**

In order to investigate the involvement of RIG-I and MDA5 in the innate recognition of enteroviruses, we used HEK-RIG-I and HEK-MDA5 cells. These cells were infected with different positive-sense RNA viruses such as Coxsackievirus A9, Enterovirus 70 and Echovirus 9 as well as influenza A virus (a negative-sense RNA virus) as a control. We tested MDA5 and RIG-I expression levels at different time points after infection (Figure 2A, B). Our data demonstrated that MDA5 was up-regulated in HEK-MDA5 cells infected with either Coxsackievirus A9 (CAV-9), Enterovirus 70 or Echovirus 9 (ECHO-9) but not influenza A virus while as expected in HEK-RIG-I cells RIG-I was only up-regulated in cells infected with influenza A virus.

In order to determine the immuno-stimulatory effect of the viruses, IFNβ secretion in response to these viruses was also examined (Figure 2C,D). Our data demonstrated that all
viruses triggered a significant IFN response (Figure 2C,D), confirming the involvement of RLRs in the sensing of enteroviruses.

**MDA5 senses the viral replicative intermediate dsRNA**

In order to determine which RNA species triggers MDA5 activation and induction of IFNα/β we isolated CAV-9, Enterovirus 70 and ECHO-9 genomic ssRNA as well as the dsRNA replicative intermediates (RF) and the (RI) which is dsRNA with a nascent viral strand, and transfected into cardiac cells. The data showed a small increase in IFNβ production when ssRNA was used (Figure 3A) possibly due to TLR7/8 detection and a very high IFNβ production in response to RF (Figure 3B). RI-RNA induced IFNβ response as well (Figure 3C), however it was lower than the response induced with RF-RNA.

In order to determine whether MDA5 was responsible for the IFNα/β response and to exclude other pattern recognition receptors such as TLR7 and TLR8 which recognise ssRNA or TLR3 which recognises dsRNA but trigger mainly IL6 and TNFα secretion, we used RNA interference to knock down expression of RIG-I, MDA5, TLR3, TLR7 and TLR8 in cardiac cells (Figure 3D). Following confirmation of knockdown we stimulated the cells with ssRNA, RF-RNA (dsRNA) and RI-RNA which contains both dsRNA and ssRNA and measured IFNβ production (Figure 3E, 3F, 3G). Our results showed that by knocking down TLRs there was no significant decrease in type I IFN production, only a very small decrease when TLR7 or TLR8 were silenced and the cells were stimulated with ssRNA (Figure 3E) which is consistent with their role in ssRNA recognition (Diebold et al., 2004; Triantafilou et al., 2005). When we knocked down RIG-I there was no down-regulation on IFNβ response, while knocking down MDA5 almost abrogated IFNβ production. These results confirmed that MDA5 senses enteroviruses and more specifically (since it doesn’t sense the genomic
ssRNA but mainly the RF as well as the RI) the dsRNA formed during their replication in the cell cytoplasm.

Furthermore HEK-MDA5, HEK-RIG-I and HEK-TLR3 cells were used, they were stimulated with ssRNA, RF-RNA and RI-RNA and the IFNβ produced was measured (Figure 3H, 3I and 3J). The data confirmed that MDA5 recognises RF-RNA and to a lesser extent RI-RNA and triggers IFNβ production, however RIG-I didn’t play a role in their recognition. TLR3 didn’t trigger IFNα/β production in the presence of ssRNA, RF-RNA or RI-RNA. Our results demonstrated that TLR3 induced the production of IL-6 and TNFα in the presence of RF-RNA (data not shown) thus confirming that it plays a role in viral RF-RNA recognition but elicits different signalling cascades to MDA5.

**Visualisation of MDA5 and viral RNA**

Our experiments revealed that MDA5 recognises not the genomic ssRNA but the dsRNA generated by the replication of these viruses. In order to confirm MDA5 and viral RNA interactions we used confocal cell imaging. The ssRNA or dsRNA, or RI-RNA were delivered directly into the cells’ cytoplasm using streptolysin O (SLO), which is a pore-forming bacterial toxin as a non endocytic delivery method in order to avoid TLR detection. This is a simple and rapid means of introducing nucleic acid into the cytoplasm of eukaryotic cells (Bao et al., 2009; Giles et al., 1998). The ssRNA, dsRNA and RI-RNA were labelled with Alexa 488 Alexa 488. MDA5 was labelled with Alexa 633-Fab goat specific Ig, while MAVs was labelled with Alexa 546-Fab rabbit specific Ig. RIG-I was labelled with Alexa 633-Fab goat specific Ig.

When we investigated MDA5-viral RNA interactions using confocal microscopy, a high level of colocalisation of dsRNA with MAVs and MDA5 was observed, whereas we saw no colocalisation of dsRNA with RIG-I (Figure 4). Furthermore when we used RI-RNA
colocalisation of RI-RNA with MAVS and MDA5 was also shown (Figure 4). Interestingly, when we used enteroviral ssRNA, we found no colocalisation neither with RIG-I nor with MDA5 (Figure 5), suggesting that MDA5 does not engage the ssRNA form of the viruses but only interacts and recognises the dsRNA intermediate that is formed during the replication phase. As expected, when Influenza genomic 5’ triphosphate ssRNA was used, we found colocalisation with RIG-I but not with MDA5 (Figure 6).

**Fluorescence resonance energy transfer (FRET).**

Confocal microscopy had revealed that MDA5 colocalised with dsRNA RF, as well as RNA-RI suggesting their association. Although colocalisation studies demonstrate proximity within microscopic structures, fluorescence resonance energy transfer (FRET) is a biophysical method that can determine associations between molecules less than 10 nm apart. FRET can occur over 1-10 nm distances, and effectively increases the resolution of light microscopy to the molecular level. In order to verify the direct association of MDA5 with dsRNA RF and RI-RNA we utilised FRET. FRET was measured in terms of dequenching of donor fluorescence after complete photo-bleaching of the acceptor fluorophore. Increased donor fluorescence after complete destruction of the acceptor indicated association between the two molecules of interest. Prior to our experiments, the energy transfer efficiency in our system was measured using a positive control (MHC-class I and β2-m) as well as a negative control for the energy transfer (Figure 7). Maximum energy transfer efficiency (E) was 28±1.5%.

In order to visualise the viral ssRNA, we labelled purified viral ssRNA, dsRNA and RI-RNA with Alexa 488 using the ULYSIS nucleic acid labelling kit. Therefore we measured FRET on cardiac cells between MDA5, RIG-I or MAVs (using Alexa 543-conjugated specific Fab mAb) and viral RNAs. A positive control using different epitopes on MHC-class I was also used to examine the maximum efficiency of our system for these fluorophores as well as a
negative control. Similarly to our confocal data, we saw no association of MDA5 or RIG-I with enteroviral ssRNA while we found increased association between RIG-I and Influenza ssRNA. MDA5 was shown to interact with viral dsRNA whereas RIG-I did not. Furthermore MDA5 also interacted with RI-RNA although to a lesser degree than with dsRNA (Figure 7), visualising for the first time the interaction of MDA5 with its specific ligand. Therefore confirming that MDA5 recognises the dsRNA replicative form (RF) of positive ssRNA viruses.

**Picornavirus replication essential for MDA5 sensing**

To gain more insight as to whether picornavirus replication and the generation of dsRNA is a prerequisite for MDA5 sensing, confocal microscopy was once again used. This method helped us visualise dsRNA formed de novo during virus infection. Cardiac cells were infected with different picornaviruses (EV70, EV9, CAV-9, CBV3) for different time points during infection and the dsRNA was visualised using the monoclonal dsRNA-specific mouse antibody J2 Fab directly conjugated to Alexa 488. MDA5 was labelled with Alexa 546-Fab goat specific Ig, while MAVs was labelled with Alexa 546-Fab rabbit specific Ig. To verify that the dsRNA detected was indeed of viral not cellular origin, cells were also treated with the DNA dependent RNA polymerase inhibitor actinomycin D and the dsRNA signal was still detected verifying that it was specific for viral dsRNA.

The experiments were consistent with our previous studies and highlighted that dsRNA colocalises with MDA5 and MAVs but not RIG-I (figure 8).

**Discussion**
RNA viruses comprise approximately 80% of all viruses in animals and humans and are etiologic agents of infectious diseases that pose major public health concerns. In the last decades we have seen the emergence of many new viruses such as Human immunodeficiency virus, avian influenza viruses, haemorrhagic fever viruses that cause severe morbidity and death worldwide. Thus there is a pressing need to develop new strategies to combat viral disease but in order to achieve this more must be learned about virus-host interactions and the mechanisms of antiviral responses exerted by the host’s immune system in response to viral-derived RNA.

The RNA helicases, RIG-I and MDA5, have been defined as essential PRRs for host detection of a variety of RNA viruses and activation of type I interferons. It has been widely known that RIG-I is a key sensor for negative strand ssRNA viruses such as Hepatitis C virus and influenza viruses whereas induction of type I IFN by positive strand Picornaviruses, including encephalomyocarditis virus, Theiler’s virus and Mengo virus, is mediated by MDA5 and not RIG-I (Kato et al., 2006; Loo et al., 2008; Meylan et al., 2005).

A large research effort has focused on understanding the ligands that are recognized by each of these RLRs since these new insights will help in the development of improved vaccines and new antiviral therapeutics to control virus infections.

Despite the wealth of information on the types of RNA that can activate RIG-I and induce IFN production there is not a lot of information on the viral RNA required for MDA5 activation. It has been shown that RIG-I recognizes single-stranded RNA (ssRNA) containing a terminal 5’-triphosphate (ppp), as well as linear dsRNA no longer than 23 nucleotides. MDA5 recognition is less clear, since it does not sense viral genomic ssRNA. It seems to recognise long strands of dsRNA but the mechanism by which this occurs has not been revealed. Pichlmair et al (Pichlmair et al., 2009) using mainly biochemical methods had suggested that MDA5 senses high order structured RNA containing ssRNA and dsRNA,
however that fails to explain why MDA5 can sense Poly:I:C, which is a synthetic long dsRNA or dsRNA viruses such as reoviruses (Kato et al., 2008) or why it cannot sense picornavirus genomic ssRNA that contains highly ordered secondary structures and tertiary RNA structures in the 3’ and 5’UTR regions. The inherent problem with the study by Pichlmair et al is that they rely on immunoprecipitations with a dsRNA-specific antibody in order to isolate RNA/MDA5 complexes. Using this method they demonstrated that infected cells contained not only dsRNA but also ssRNA of high molecular weight, which was found to be immunostimulatory. The problem is that such biochemical isolations rely exclusively on the specificity of the antibody used and they are prone to artefactual associations. Under these conditions, weak or transient receptor-ligand associations might be lost and artefactual associations might be retained. Therefore although Pichlmair et al have given us a clue on what the MDA5 ligand might be, by saying that it is most likely an RNA web rather than simply long molecules of dsRNA, they have left us with a lot of questions regarding the exact MDA5 ligand.

The goal of the present study was to determine whether RIG-I or MDA5 participate in the induction of type I IFN in response to enterovirus infection and to determine the specific RNA ligand that was recognized and activated these sensors using non-invasive methods. Enteroviruses are positive ssRNA viruses therefore in addition to their ssRNA genome there are also substantial amounts of cytosolic dsRNA produced during the replicative life cycle of these positive ssRNA viruses. Thus during an enteroviral infection, there is a pool of viral RNA that trigger the host’s innate immune response, raising the question of which viral RNA species is responsible for MDA5 activation. In this study, we have used three different positive sense viruses and for the first time, we have isolated viral ssRNA as well as the replicative intermediate dsRNA (RF) and the high order replicative intermediate RI which is basically a dsRNA RF with a nascent ssRNA viral strand. We have confirmed that although
RIG-I acts as a sensor triggering interferon production in the presence of negative ssRNA virus such as Influenza A virus it cannot recognise the replicative intermediate dsRNA of positive strand viruses.

Our experiments have shown that there was no interaction of MDA5 with the positive sense genomic ssRNA. However MDA5 recognised the dsRNA produced during the enteroviral replication as well as RI-RNA which has a dsRNA core, highlighting the importance of dsRNA in MDA5 recognition. Using fluorescent imaging methods, we were able to visualise the association of MDA5 with the replicative form of enteroviruses. This is the first report of the visualization of dsRNA and MDA5, which provides unique evidence between the relationship of viral dsRNA and MDA5 and proves without a doubt that the dsRNA replicative intermediate form of positive sense ssRNA viruses is the effective agonist recognised by MDA5.

These findings have not only identified the dsRNA replicative intermediate form as the specific ligand for MDA5, but have given us an insight into MDA5 ligand recognition. This model is probably true for other RNA viruses sensed by MDA5 as well.

It seems that MDA5 is an essential sensor of the innate response that unlike TLRs or RIG-I is not involved in the initial steps of ssRNA viral infection but detects and activates the interferon response only when the RNA viruses begin to replicate generating replicative intermediates thus posing a threat for the host cell.

A very recent study by Peisley et. al. supports our findings of dsRNA viral recognition by MDA5. This study revealed the MDA5 mechanism of ligand recognition. They have shown MDA5 assembly into oligomers composed of segmental arrangement of MDA5 dimers along the length of dsRNA (Peisley et al., 2011). They propose a role of ATP hydrolysis as a conformational switch for MDA5 activation and signaling.
These new insights should pave the way for the development of future antiviral therapeutics. An increased understanding of the complex mechanisms of viral host defence will eventually helps us design new targets for viral infections.

Author contribution

MT contributed in the conception, design, acquisition and interpretation of data as well as in the preparation of the manuscript. EV contributed in the acquisition and interpretation of data, SK contributed in the acquisition and interpretation of data, ER contributed in the acquisition and interpretation of data, GLE contributed in the acquisition and interpretation of data. KT contributed in the conception, design, acquisition and interpretation of data as well as in the preparation of the manuscript.

Materials and methods

Cells

Primary human aortic muscle cells isolated from a healthy, 35 year old male were purchased from (Promocell, Heidelberg, Germany) and maintained in muscle cell growth medium. The cell cultures are produced at PromoCell’s cell culture facility from normal human tissue obtained from surgical operations. The cells are isolated according to referenced procedures. Each isolate undergoes extensive testing for the presence of specific cardiac cell markers as well as the absence of specific cell markers.

Human embryonic Kidney 293 (HEK-239) cells transfected with TLR3 were maintained in DMEM containing 4.6 g/L glucose with 10% FCS, 10 µg/ml ciprofloxacin, and 0.5 mg/ml G418-sulfate. Transfections of HEK/MDA5 and HEK/RIG-I cells with puno-hMDA-5, or puno-RIG-I (Invivogen) were maintained in DMEM containing 4.6 g/L glucose with 10% FCS and 10 µg/ml blasticidin (Triantafilou et al., 2011).
Viruses

Prototype strains of Coxsackievirus A9 (CAV9), Echovirus 9 (EV9), Enterovirus 70 (EV70) and influenza A virus (IVA) were obtained from the American Type culture collection (ATCC).

The viruses were purified by sucrose gradient purification (Spector and Baltimore, J virol 1975). Viral genomic ssRNA was isolated from purified virus using TRIzol Reagents (Invitrogen) according to manufacturer’s instructions.

In addition the replicative intermediate dsRNA and the high order RI-RNA containing dsRNA with nascent ssRNA strands of CAV-9, EV70 and EV9 was isolated from infected cells, where the infection had proceeded for 5 hrs and dsRNA was purified from the cytoplasm of these cells using a protocol similar to Richards and Ehrenfeld (Richards et al., 1981; Richards et al., 1987b; Spector and Baltimore, 1975). Girardi cardiac cells 5x10^8 were infected with virus at a multiplicity of 50 PFU/cell. Cells were lysed with lysis buffer (0.5% Nonidet P-40, 10mMTris-Hcl, 10mMNacl, 1mM MgCl₂, 0.5 mM CaCl₂). Cell debris was removed and the supernatant was extracted with an equal volume of buffer A (0.15 M Nacl, 0.01M Tris-Hcl pH 8.3, 5mM EDTA in saturated phenol). After centrifugation the aqueous phase was removed and again treated with buffer A. The aqueous layer was removed and precipitated with 0.2M sodium acetate and 95% ethanol at -20 °C. The ethanol precipitate was dissolved in 10mM EDTA-1% SDS and fractionated in 2M LiCl at -20°C. The suspension was centrifuged at 16,000g and the LiCl soluble fraction containing replicative dsRNA and RI-RNA was chromatographed through a CF11 cellulose column. The material eluted was ethanol precipitated and further purified by analytical zonal sedimentation in a 15% to 30% sucrose gradient (On 15 to 30% sucrose gradients RF RNA sedimented at 20S and banded at a density of 1.63g/ml. Whereas viral ssRNA sedimented at 35S and banded at a
density of 1.68g/ml). The precipitated RNA from appropriate fractions was further fractioned on a FPLC Superdex 200 column. The fractions containing dsRNA were pooled and ethanol precipitated.

Reversed transciption

The RF RNA and RI-RNA was denatured then mixed with oligonucleotide pairs and RT-PCRs were carried out by using a One-Step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. Briefly, 5µl RNA, 100pmol of each primer, 1µl RT-Taq mix and 25µl 2× reaction mix were mixed together in a total volume of 50µl (made up with sterile ultrapure water). Amplicons were isolated by agarose gel electrophoresis and purified using PCR DNA and gel band purification kit (Amersham Pharmacia) according to the manufacturer's instructions. The purified fragments were then sequenced commercially

Primers used OL26 (GCACCTTCTGTTTCCCC) to antisense DNA position 182-197 and OL27 (CGGACACCCAAAGTAG) coplementary to sense DNA position 547-562.

Chemicals

All fine chemicals were obtained from Sigma (UK). MDA5, RIG-I and MAVs specific polyclonal antibodies were obtained from SantaCruz antibodies Inc (Germany). The mAb J2 used for dsRNA detection recognises double-stranded RNA (dsRNA) provided that the length of the helix is greater than 40 bp. dsRNA-recognition is independent of the sequence and nucleotide composition of the antigen (Schönborn J et al., 1991).The J2 antibody was obtained from English & Scientific Consulting Bt (Hungary). Alexa 488-Ulysis reagent was obtained from Molecular Probes Inc (Cambridge Biosciences, Cambridge, UK). Viral RNAs were labelled with Alexa-488-Ulysis reagent according to the manufacturer’s instructions.

Flourescence Resonance energy transfer
FRET is a non-invasive imaging technique used to determine molecular proximity. FRET can occur over 1-10 nm distances, and effectively increases the resolution of light microscopy to the molecular level. It involves non-radiative transfer of energy from the excited state of a donor molecule to an appropriate acceptor. The rate of energy transfer is inversely proportional to the sixth power of the distance, between donor and acceptor. The efficiency of energy transfer (E) is defined with respect to r and R₀, the characteristic Forster distance by:

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E = \frac{1}{1 + (r/R₀)^6} \tag{1}
\]

FRET will be measured in terms of dequenching of donor fluorescence after complete photo-bleaching of the acceptor fluorophore. Increased donor fluorescence after complete destruction of the acceptor indicates association between the two molecules of interest.

**RNA interference**

RNA interference was used in order to silence the RiG-I and MDA5 genes. Different psiRNA clones were generated using the psiRNA-h7SK vector from Invitrogen the most efficient was against the sequence: for RiG-I, GGAAGAGGTGCAGTATATT, for MDA-5, GGTGAAGGAGCAGATTCAG, for TLR7 GGGTATCAGCGTCTAATATCA, for TLR8 GACCAACTTGCATACCTAAA, TLR3 GAGTTAGATATGCCTTTAAAT.

Human primary cardiac cells (1 x 10⁵) were seeded in six well plates and transfected with 0.5 µg of psiRNA for RiG-I or MDA5 or scrambled siRNA as a control using Lipofectamine 2000 (Invitrogen). After 48h the level of silencing was determined by western blotting. Transfections with the specific siRNAs resulted in an approximately 75% decrease in receptor expression as determined by western blotting whereas transfection of cells with the scrambled siRNA did not show any decrease in RiG-I and MDA-5 expression.
Confocal microscopy

Human primary cardiac cells on microchamber culture slides (Lab-tek, Gibco), were incubated with Alexa 488- ssRNA (20 µg/ml) or dsRNA for different time points, and were subsequently rinsed twice in PBS/0.02% BSA, prior to fixation with 4% formaldehyde for 15 min. The cells were fixed in order to prevent potential re-organisation of the proteins during the course of the experiment. Cells were permeabilised using PBS/0.02% BSA/0.02% Saponin and labelled with antibodies for RIG-I, MDA5 and MAVs directly labelled with the appropriate fluorophore.

Cells were imaged on a Carl Zeiss, Inc. LSM510 META confocal microscope (with an Axiovert 200 fluorescent microscope) using a 1.4 NA 63x Zeiss objective. The images were analysed using LSM 2.5 image analysis software (Carl Zeiss, Inc.).

IFN assays

Cell supernatants were collected after each stimulation and frozen until the IFN assays were performed. The BectonDickinson bead array flex system was used in order to determine IFNα/IFNβ level.
Figure legends

Figure 1. Purification of RNA by size exclusion chromatography.

(A) The standard elution curves for RNA oligonucleotides of various lengths on the Superdex 200 column size exclusion column was used for calibration. The elution profile of viral ssRNA and viral RF and viral RI obtained from the size exclusion chromatography step on the Superdex 200 column is shown. FPLC was performed at 3mL/min. CAV-9 ssRNA (lane 1) and replicative intermediate RNA RF (lane 2) and high order replicative intermediate RI-RNA (lane 3) were isolated and analysed by agarose gel electrophoresis (B). The RNA samples ssRNA (lane 1) and RF-RNA (lane 2) were treated with pancreatic RNAse (1µg/ml) (C), ssRNA (lane 1) and RF-RNA (lane 2) were treated with DNAse I (20µg/ml) (D) and RI-RNA (E) were treated with with DNAse I (20µg/ml) (lane 1) or pancreatic RNAse (1µg/ml) (lane 2) and analysed by agarose gel electrophoresis

Figure 2. RIG-I/MDA5 expression in HEK cells

HEK-MDA5 and HEK-RIG-I cells were either not stimulated (0h) or stimulated at different time points with different viruses (5 moi).The cells were fixed and permeabilised, followed by antibody staining against MDA5 in HEK-MDA5 cells(A) and RIG-I in HEK-RIG-I cells (B) followed by incubation with the appropriate secondary antibody conjugated to FITC. The supernatants were harvested and assayed for IFNβ secretion using the Flex set system (Becton Dickinson). In HEK-MDA5 cells (C) and HEK-RIG-I cells (D). Fluorescence was detected using a FACSCalibur (BectonDickinson) The data presented is the mean of three independent experiments.

Figure 3. IFN upregulation induced by viruses

Human cardiac cells were stimulated with different viral RNAs, genomic ssRNA (A), replicative intermediate dsRNA (RF) (B) and dsRNA-ssRNA replicative intermediate (RI) (C). The supernatants were harvested and assayed for cytokine secretion using the Flex set system (Becton Dickinson). Fluorescence was detected using a FACSCalibur (BectonDickinson). IFNβ secretion is depicted in graph A, B and C. RNA interference for MDA5, RIG-I, TLR3, TLR7 and TLR8 in cardiac cells is also depicted (receptor expression levels were reduced by 85% by RNA interference panel D). A loading control for B-actin is also shown (before siRNA and after siRNA panel D ). Human cardiac cells were silenced for either RIG-I, MDA5, TLR3, TLR7 or TLR8 and either not stimulated (white bar charts), or incubated with ssRNA from different viruses (E), or incubated with RF-RNA (F) or RI-RNA (G). The supernatants were harvested and assayed for IFNβ secretion in 4hr using the Flex set system (Becton Dickinson). HEK-MDA5 cells (H), HEK-RIG-I cells (I) and HEK-TLR3 cells (J) were also used and stimulated with ssRNA, RF-RNA or RI-RNA. The supernatants
were harvested and assayed for IFNβ secretion. The data represents the mean of three independent experiments.

**Figure 4. Localisation of enteroviral dsRNA RF or RI-RNA and MDA5 in cardiac cells**

Human cardiac cells containing CAV-9 ssRNA, dsRNA RF or RI-RNA directly conjugated to Alexa 488. MDA5 was labelled with Alexa 633-Fab goat specific Ig (A), while MAVs was labelled with Alexa 546-Fab rabbit specific Ig (C, H). RIG-I was labelled with Alexa 633-Fab goat specific Ig (F). The indicated regions in merged images are enlarged in panels A, B, C and D. Yellow regions indicate localisation of MAVs with dsRNA RF and RI-RNA. White regions indicate localisation of MDA5, MAVs with dsRNA RF and RI-RNA. Bars 10 µm.

**Figure 5. There is no localisation of enteroviral ssRNA neither with RIG-I nor with MDA5**

Human cardiac cells containing CAV-9 ssRNA conjugated to Alexa 488. MDA5 was labelled with Alexa 546-Fab goat specific Ig. RIG-I was labelled with Alexa 546 Fab goat specific Ig. We see no degree of localisation in the displayed images. Bars 10 µm.

**Figure 6. Localisation of influenza ssRNA and RIG-I in cardiac cells**

Human cardiac cells containing influenza ssRNA conjugated to Alexa 488 (B, F). MDA5 was labelled with Alexa 633-Fab goat specific Ig. while MAVs was labelled with Alexa 546-Fab rabbit specific Ig. RIG-I was labelled with Alexa 633-Fab goat specific Ig. Yellow regions indicate localisation of MAVs and ssRNA. White regions indicate localisation of RIG-I, MAVs and ssRNA. Bars 10 µm.

**Figure 7. FRET measurements between RLRs and viral RNA**

Energy transfer between RLRs (RIG-I or MDA5) and viral RNA (ssRNA or dsRNARF or RI-RNA) was detected from the increase in donor fluorescence after acceptor photobleaching. Results are shown as means of % energy transfer ± SD, calculated from three independent experiments. The maximum and minimum energy transfer efficiencies in the system were 38 ± 1.2 and 4 ± 0.8, respectively (not shown), and were determined as the energy transfer between two different epitopes on the same molecule (MDA5) (maximum), or between molecules that do not engage in heterotypic associations (MDA5 and MHC Class I) (minimum).

**Figure 8 Localisation of viral dsRNA and MDA5 in virus infected cardiac cells.**
Human cardiac cells infected with CAV-9 were stained with J2 Fab specific for dsRNA directly conjugated to Alexa 488. MDA5 was labelled with Alexa 546-Fab goat specific Ig while MAVs was labelled with Alexa 546-Fab rabbit specific Ig, RIG-I was labelled with Alexa 546 Fab goat specific Ig. Nuclear stain TOPRO was also used to label the nucleus. Yellow regions indicate localisation of MAVs or MDA5 with viral dsRNA. Bars 10 μm.
References


AIFN-β (pg/ml)

Concentration (μg)

CAV9 RFRNA
EV9 RFRNA
EV70 RFRNA

IFN-β (pg/ml)

Concentration (μg)

CAV9 RFRNA
EV9 RFRNA
EV70 RFRNA

IFN-β (pg/ml)

Concentration (μg)

CAV9 RFRNA
EV9 RFRNA
EV70 RFRNA

IFN-β (pg/ml)

Concentration (μg)

CAV9 RFRNA
EV9 RFRNA
EV70 RFRNA

Unstim
Ev70 RI-RNA
EV9 RI-RNA
CAV9 RI-RNA

B-actin

MDA5 siRNA MDA5
RIG-I siRNA RIG-I
TLR3 siRNA TLR3
TLR7 siRNA TLR7
TLR8 siRNA TLR8

B-actin

Cardiac cells
siRNA RIG-I
siRNA MDA5
siRNA TLR3
siRNA TLR7
siRNA TLR8

B-actin

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