Video Article

Monitoring Activation of the Antiviral Pattern Recognition Receptors RIG-I And PKR By Limited Protease Digestion and Native PAGE

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Abstract

Host defenses to virus infection are dependent on a rapid detection by pattern recognition receptors (PRRs) of the innate immune system. In the cytoplasm, the PRRs RIG-I and PKR bind to specific viral RNA ligands. This first mediates conformational switching and oligomerization, and then enables activation of an antiviral interferon response. While methods to measure antiviral host gene expression are well established, methods to directly monitor the activation states of RIG-I and PKR are only partially and less well established.

Here, we describe two methods to monitor RIG-I and PKR stimulation upon infection with an established interferon inducer, the Rift Valley fever virus mutant clone 13 (CI 13). Limited trypsin digestion allows to analyze alterations in protease sensitivity, indicating conformational changes of the PRRs. Trypsin digestion of lysates from mock infected cells results in a rapid degradation of RIG-I and PKR, whereas CI 13 infection leads to the emergence of a protease-resistant RIG-I fragment. Also PKR shows a virus-induced partial resistance to trypsin digestion, which coincides with its hallmark phosphorylation at Thr 446. The formation of RIG-I and PKR oligomers was validated by native polyacrylamide gel electrophoresis (PAGE). Upon infection, there is a strong accumulation of RIG-I and PKR oligomeric complexes, whereas these proteins remained as monomers in mock infected samples.

Limited protease digestion and native PAGE, both coupled to western blot analysis, allow a sensitive and direct measurement of two diverse steps of RIG-I and PKR activation. These techniques are relatively easy and quick to perform and do not require expensive equipment.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51415/>

Introduction

A crucial event in antiviral host defense is the rapid detection of the pathogen by the so-called pattern recognition receptors (PRRs)^{1,2}. Intracellular detection of RNA virus infection is dependent on two cytoplasmic RNA helicases, RIG-I (retinoic acid inducible gene I) and MDA5 (melanoma differentiation associated protein 5)³⁻⁵. RIG-I is composed of two N-terminal caspase recruitment domains (CARDs), a central DECH-box type RNA helicase domain, and a C-terminal domain (CTD)^{4,6}. Whereas the CTD and the helicase domain are required for recognition of non-self (viral) RNAs, the CARDs mediate downstream signaling leading to establishment of an antiviral host status.

If RIG-I is in the silent state, *i.e.* in the absence of a specific RNA ligand, the second CARD interacts with the central helicase domain and keeps RIG-I in an auto-inhibitory conformation⁷⁻¹¹. RIG-I binds to short double-strand (ds) RNA bearing a 5'-triphosphate (5'PPP), long dsRNA, and polyU/UC-rich RNA, classic signature structures which are present on the genomes of many RNA viruses¹²⁻¹⁶. Two major characteristics of RIG-I activation are a switch to a closed conformation^{6,17} and the homo-oligomerization^{6,18,19}. The conformational switch enhances RNA binding, exposes the CARDs for downstream signaling, and reconstitutes an active ATPase site^{8,9,11,20}. The formation of oligomeric RIG-I complexes leads to enhanced recruitment of downstream signaling adaptor molecules to form a platform for antiviral signal transduction¹¹. The RIG-I-regulated signaling chain eventually activates the transcription factor IRF-3 for up-regulation of interferon (IFN- α /beta) genes and hence the gene expression of interferon stimulated genes (ISGs) for a full antiviral response^{21,22}. One of the best characterized ISGs is the RNA-activated protein kinase (PKR)²³. PKR belongs to the family of eukaryotic translation initiation factor 2 α (eIF2 α) kinases and is composed of an N-terminal double-stranded RNA binding domain and a C-terminal kinase domain. The kinase domain constitutes the dimerization interface crucial for PKR activation and carries out the catalytic functions of the protein. Binding of PKR to viral dsRNA leads to its conformational change permitting dimerization and auto-phosphorylation at Thr 446 among other residues. PKR then mediates phosphorylation of eIF2 α , thereby blocking the translation of viral mRNAs²³⁻²⁷.

Both RIG-I and PKR undergo major structural rearrangements, form oligomeric complexes and are post-translationally modified by phosphorylation/dephosphorylation and ubiquitination^{10,11,19,23,24,26-29}. For a better understanding of which viral RNA structures are activating RIG-I and PKR (and at what stage viral antagonists could be interfering), it is important to precisely determine the activation status. For both PRRs it was previously described that activation leads to the emergence of trypsin-resistant protein fragments^{6,17,30} and higher-order

oligomers^{6,18,19}. However, given the wealth of literature on these key factors of the antiviral host response^{1,2,24}, application of direct methods seems comparatively rare. In the hope of stimulating broader usage, we provide convenient and sensitive protocols to robustly analyze the activation states of RIG-I and PKR. The IFN competent human cell line A549 is infected with an established activator of RIG-I and PKR, the attenuated Rift Valley fever virus mutant clone 13 (CI 13)^{31,32}. After a simple lysing procedure, the extracts of infected cells are tested by limited trypsin digestion/western blot analysis to evaluate conformational switching, and by blue native polyacrylamide gel electrophoresis (PAGE) / Western blot analysis to measure formation of oligomers.

Protocol

1. Seeding of A549 Cells for Infection

1. Cultivate a T75 flask of A549 cells at 37 °C and 5% CO₂ in cell culture medium (DMEM supplemented with 10% FCS, 526.6 mg/l L-glutamine, 50.000 U/l penicillin, and 50 mg/l streptomycin).
2. Before starting to harvest the cells, warm up cell culture medium, PBS and 0.05% trypsin-EDTA in a waterbath heated to 37 °C.
3. Remove the medium and wash the cells with 10 ml PBS. Remove the PBS again.
4. Add 3 ml of trypsin-EDTA and distribute equally in the flask. Transfer the flask in an incubator with 37 °C and 5% CO₂.
5. When all cells are detached, add 7 ml of cell culture medium, resuspend the cells, and transfer the cell suspension into a 15 ml Falcon tube.
6. Centrifuge the cells at 800 x g for 5 min at RT, remove the supernatant and resuspend the pellet in 10 ml fresh cell culture medium.
7. Count the cells with a counting chamber.
8. Add 2.5 x 10⁶ cells in 5 ml of cell culture medium in two T25 flasks each. Incubate for 16 hr at 37 °C and 5% CO₂. One flask serves for the mock control and one for CI 13 infection.

2. Infection with Rift Valley Fever Virus Clone 13 (CI 13)

1. NOTE: CI 13 is an attenuated virus mutant which in Germany can be handled under BSL-2 conditions. Please refer to relevant national guidelines. Other typical IFN inducers would be Sendai virus (strain Cantell) or Newcastle disease virus.
2. Pre-warm PBS, serum-free medium, and cell culture medium containing 5% FCS.
3. Prepare 1.25 x 10⁷ PFU/ml of CI 13 in serum-free medium to infect 2.5 x 10⁶ A549 cells with a multiplicity of infection (MOI) of 5. Prepare a slightly (roughly 10%) greater amount than needed to account for pipette errors.
4. Wash the cells with PBS as described under 1.3.
5. Add 1 ml of the CI 13 dilution or of serum-free medium (uninfected control, mock) to the cells, and incubate for 1 hr at 37 °C and 5% CO₂. Move the flask carefully every 15 min to ensure equal distribution of CI 13 dilution and serum-free medium, respectively.
6. After 1 hr of infection, remove the inocula, add 5 ml of pre-warmed cell culture medium with 5% FCS, and incubate for 5 hr at 37 °C and 5% CO₂.

3. Preparation of Cell Lysates

1. Prepare PBS / 0.5% Triton X-100 at 4 °C. Do not add serine protease inhibitors.
2. Wash the cells with cold PBS and add 10 ml of fresh PBS.
3. Scrape the cells off, transfer the cell suspension in a falcon tube, and centrifuge at 800 x g for 5 min at RT.
4. Remove the supernatant and resuspend the cell pellet in 30 µl PBS / 0.5% Triton X-100. Transfer the lysate into a fresh 1.5 ml tube and incubate for at least 10 min at 4 °C.
5. Centrifuge the lysate at 10.000 x g for 10 min at 4 °C and transfer the clarified cell lysate (supernatant) into a fresh tube.
6. Determine the protein concentration by Bradford assay as described elsewhere³³.
7. Store at -20 °C or proceed to trypsin digestion (4.1) or native PAGE (5.1).

4. Determination of Conformational Changes of Pattern Recognition Receptors

1. TPCK-trypsin Treatment of Cell Lysates
 1. Dilute L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated (TPCK) trypsin in PBS to a final working concentration of 2 µg/µl.
 2. Adjust in two new tubes a final protein concentration of 25 µg of each protein lysate (mock or CI 13) in a final volume of 9 µl with PBS. Hence, it should be four tubes with 25 µg lysate each, two times mock and two times CI 13 infection. One set as input control (untreated) and one set for treatment with TPCK-trypsin.
 3. Add 1 µl of PBS (untreated) or 1 µl of 2 µg/µl TPCK-trypsin (final concentration: 0.2 µg/µl) to the cell lysates and mix the reactions by pipetting. DO NOT freeze and thaw TPCK-trypsin aliquots, because it will compromise the efficiency of digestion.
 4. Incubate the lysates at 37 °C for 25 min. Stop the reaction by adding 5x denaturing sample buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.5% bromophenol blue) and by boiling for 5 min at 95 °C. It is important to NOT extend the trypsin incubation time. In case no protease-resistant fragments are detectable, the time of trypsin digestion must be shortened.
 5. After boiling, the samples can be stored at -20 °C.
2. SDS Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting
 1. Load the samples on a sodium dodecyl sulfate (SDS) polyacrylamide gel containing a 5% stacking over a 12% resolving gel. Separate the proteins at 25 mA per gel until the bromophenol blue runs out.
 2. Activate a polyvinylidene fluoride (PVDF) membrane for 30 sec with methanol and put it into transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol).

3. Prepare the blotting with a semidry blotting system and allow the transfer of the proteins at 10 V for 1 hr. Take the membrane out, rinse it briefly with water, and let it dry.
 4. Reactivate the membrane by shortly transferring into methanol. Wash for 5 min with TBS. Block with 10% skim milk in TBS for 1 hr at RT or at 4 °C O/N. Wash the membrane 3x for 5 min each with TBS.
 5. Prepare antibody dilution as recommended in table 1 and incubate the membrane for 1 hr at RT or at 4 °C O/N.
 6. Wash the membrane 3x for 10 min each with TBS-T. Add the appropriate secondary antibody coupled with horseradish peroxidase at a 1:20,000 dilution in 1% skim milk in TBS. Incubate 45 min at RT.
 7. Wash the membrane 3x for 10 min each with TBS-T, and one additional time with TBS.
 8. For signal detection use a commercial chemiluminescence kit and a digital gel imaging system.
3. Coomassie Brilliant Blue G-250 Staining
 1. Perform SDS-PAGE as described in 4.2.1. Load samples on an SDS polyacrylamide gel and run the gel at 25 mA per gel until bromophenol blue runs out.
 2. Perform Coomassie Brilliant Blue G-250 staining at RT. Do all incubations under constant shaking.
 3. Transfer the gel to the fixation solution containing 40% methanol and 10% acetic acid for 30 min.
 4. Exchange the buffer to destaining solution (25% ethanol and 8% acetic acid) and incubate for 5 min.
 5. Stain the gel with 0.2% Coomassie Brilliant Blue G-250 in 40% methanol and 10% acetic acid for 1 hr.
 6. Destain the gel with the destaining solution and exchange the buffer after 10 min, 30 min, and 60 min.
 7. Store the gel in 25% ethanol, 8% acetic acid, and 4% glycerol at 4 °C.
 8. Perform imaging and analysis as described under 4.2.8.

5. Analysis of Oligomeric States of Pattern Recognition Receptors

1. Native PAGE
 1. Prepare 50 µg of cell lysate in a final volume of 10 µl with PBS and add 5× native sample buffer (250 mM Tris-HCl pH 6.8, 1% sodium deoxycholate, 50% glycerol, 0.5% bromophenol blue) to a final concentration of 1x.
 2. Load the samples IMMEDIATELY on a native polyacrylamide gel with 5% as stacking and 8% as resolving gel. Any delay will result in a loss of native complexes³⁴.
 3. Run the gel at 20 mA per gel at 4 °C with 50 mM Tris-NaOH pH 9.0, 384 mM glycine as anode and 50 mM Tris pH 8.3, 384 mM glycine, 1% sodium deoxycholate as cathode buffer. After 1.5-2 hr (bromophenol blue band has left the gel approximately 45 min earlier) the electrophoresis is finished.
2. Western Blotting
 1. Activate a polyvinylidene fluoride (PVDF) membrane for 30 sec with methanol and put it into Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol).
 2. Assemble a wet blot chamber according to the manufacturer's instructions and fill the tank with Towin buffer.
 3. Perform the blotting with 250 mA for 1.5 hr at 4 °C.
 4. When blotting is finished proceed as described from 4.2.3 on.

Representative Results

Recognition of a viral agonist by RIG-I or PKR triggers conformational switching^{6,17,30} and oligomerization^{6,18,27}. We assayed these two activation markers by limited protease digestion and native polyacrylamide gel electrophoresis (PAGE), respectively.

Human A549 cells were infected with Rift Valley fever virus clone 13 (Cl 13), which is characterized by a mutation of the IFN antagonist NSs^{35,36}. Due to the absence of functional NSs, Clone 13 strongly induces RIG-I and PKR, leading to the establishment of a robust antiviral state in the cells^{12,31,32,37}.

Trypsin digestion of mock infected cell lysates results in a rapid degradation of RIG-I, whereas Cl 13 infection leads to the generation of a 30 kDa resistant RIG-I fragment **Figure 1A**. Also PKR shows partial resistance to trypsin digestion in infected samples, which coincides with its phosphorylation **Figure 1A**. To monitor efficiency and specificity of trypsin digestion, gels were stained with Coomassie Brilliant Blue G-250. Untreated samples show equal amounts of loaded proteins **Figure 1B**. Subjecting mock and Cl 13 cell lysates to trypsin digestion leads to a comparable decrease of global protein amounts. This demonstrates that trypsin treatment has the same efficiency for mock and Cl 13-infected samples.

The formation of oligomeric complexes was assayed by native PAGE. In uninfected cells, only monomers of RIG-I and PKR were detected **Figure 2**. As additional control we included the transcription factor IRF-3, which is present as a monomer but known to dimerize upon activation via e.g., RIG-I³⁸. Cl 13 infection leads to a strong accumulation of RIG-I oligomeric complexes in form of a smear and of PKR and IRF-3 dimers/oligomers as a defined protein band.

These results demonstrate that limited trypsin digestion and native PAGE are useful tools to monitor conformational changes and oligomer formation of RIG-I and PKR upon infection.

Figure 1. Conformational switch of RIG-I and PKR. A549 cells were mock infected or infected with CI 13 at an MOI of 5. After 5 hr, cells were lysed in PBS supplemented with 0.5% Triton X-100 and cleared cell lysates were either left untreated or treated with trypsin. Samples were subjected to SDS PAGE followed by Western blotting (**A**) or Coomassie staining (**B**). Blots were stained against RIG-I, PKR, phosphorylated PKR (Thr 446) and against RVFV nucleoprotein (RVFV N) and beta-actin as infection and loading control, respectively. [Please click here to view a larger version of this figure.](#)

Figure 2. Oligomerization of RIG-I, PKR, and IRF-3. Cell lysates of mock and CI 13-infected cell lysates were subjected to native PAGE followed by Western blot analysis. Staining was performed with antibodies against RIG-I, PKR, IRF-3, and beta-actin as loading control. PKR oligomers most likely represent dimers²⁷. [Please click here to view a larger version of this figure.](#)

Discussion

Sensing the presence of viruses and activation of the antiviral type I IFN system are crucial for successful innate immune responses²². Virus detection is thereby mediated by pathogen recognition receptors (PRRs) like RIG-I and PKR, enabling a rapid response and activation of antiviral defense mechanisms. Here, we describe two methods to directly evaluate the activation status of RIG-I and PKR.

Limited protease digestion as a tool to monitor conformational changes of RIG-I and PKR was first described by the groups of M. Gale Jr. and T. Fujita^{6,17}, and J. L. Cole³⁰, respectively. It represents a sensitive method to evaluate sensitivity alterations to trypsin treatment caused by conformational changes. Applying trypsin digestion, we detected rapid degradation of RIG-I and PKR in mock infected samples, whereas trypsin treatment of virus-infected cell lysates led to trypsin resistant RIG-I fragments. Similarly, a trypsin resistant PKR fragment was detected upon CI 13 infection. This was accompanied by PKR phosphorylation at Thr 446, a widely used marker of PKR activation. Comparing trypsin digestion of

mock and CI 13 cell lysates by Coomassie staining demonstrates a comparable decrease of global protein levels. This indicates that formation of resistant fragments is specific for proteins like RIG-I and PKR.

The formation of oligomeric complexes of RIG-I, PKR and IRF-3 was monitored by native PAGE. Subjecting CI 13-infected cell lysates to native PAGE, we detected RIG-I, PKR and IRF-3 oligomers, whereas these proteins remained as monomers in mock infected samples. RIG-I needs to form oligomers to activate downstream pathways. It was hypothesized that RIG-I oligomerization supports recruitment of cofactors to form a signaling platform for antiviral response mechanisms¹¹. The function of PKR dimerization is not entirely understood. Most likely, the PKR subunits in the dimer phosphorylate each other²⁵. The type I IFN system is tightly regulated on a transcriptional level, and IRF-3 represents one central transcription factor for the induction of IFN and ISGs³⁸. Central hallmarks of activation are phosphorylation, dimerization, and translocation to the nucleus, where it recruits the transcriptional co-activators p300 and CREB-binding protein (CBP) to initiate IFN mRNA synthesis^{21,39}. Analysis of IRF-3 dimerization is a widely used tool to monitor activation of the type I IFN response³⁸. Therefore, we used the detection of IRF-3 oligomeric complexes as a proof of principle for the oligomerization assays of RIG-I and PKR oligomerization. Indeed, allowing separation of protein lysates under non-denaturing conditions, we were able to detect IRF-3 dimerization in CI 13 infected samples.

Undoubtedly, each lab will need to optimize the protocols of limited protease digestion and native PAGE. If confronted with no detection of any resistant proteins or too many resistant fragments, one might shorten or prolong the time of digestion, respectively. Differences can also be due to the specific TPCK-trypsin stock, as preparations slightly differ. Therefore, various TPCK-trypsin concentrations should be tested for optimization. Cell lysates can be prepared from other cell lines than A549, but adaptations of the protocol might be required. It is recommended to adjust the amount of total proteins for trypsin digestion and native PAGE according to the expression level of the protein of interest in this specific cell type. Moreover, limited detection or weak separation of oligomeric complexes by native PAGE can have several reasons and can be addressed as followed: make sure that the equipment is cleaned to remove remaining denaturing agents, keep all samples and the gel during the PAGE at 4 °C, and do not extend the time between sample preparation and loading onto the gel. Limited protease digestion and native PAGE have also been employed to monitor the activation of another important, closely related PRR, MDA5⁴⁰⁻⁴². Monitoring of MDA5 activation required different experimental conditions compared to RIG-I and PKR, and was therefore not included here.

In summary, point-by-point protocols for two useful and sensitive methods to measure activation of RIG-I and PKR are presented. Limited protease digestion and native PAGE, both coupled to Western blot analysis, permit monitoring of conformational changes and oligomerization, respectively. Using these methods, we had previously shown that RIG-I can be activated by nucleocapsids of various viruses directly after their entry into the cells³².

The exact nature and origin of the RNA species relevant for RIG-I and PKR activation in infected cells are still not entirely solved. Furthermore, many viruses interfere with the functions of PRRs by a wide variety of strategies^{12,43-46}. The presented techniques enlarge the spectrum of methods by allowing an easy and direct measurement of RIG-I and PKR activation, are quick to perform, and do not require expensive equipment.

Disclosures

No conflicts of interest declared.

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