Video Article

A High Resolution Method to Monitor Phosphorylation-dependent Activation of IRF3

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Abstract

The IRF3 transcription factor is critical for the first line of defense against pathogens mainly through interferon β and antiviral gene expression. A detailed analysis of IRF3 activation is essential to understand how pathogens induce or evade the innate antiviral response. Distinct activated forms of IRF3 can be distinguished based on their phosphorylation and monomer vs dimer status. *In vivo* discrimination between the different activated species of IRF3 can be achieved through the separation of IRF3 phosphorylated forms based on their mobility shifts on SDS-PAGE. Additionally, the levels of IRF3 monomer and dimer can be monitored using non-denaturing electrophoresis. Here, we detail a procedure to reach the highest resolution to gain the most information regarding IRF3 activation status. This is achieved through the combination of a high resolution SDS-PAGE and a native-PAGE coupled to immunoblots using multiple total and phosphospecific antibodies. This experimental strategy constitutes an affordable and sensitive approach to acquire all the necessary information for a complete analysis of the phosphorylation-mediated activation of IRF3.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53723/>

Introduction

The ubiquitously and constitutively expressed transcription factor Interferon (IFN) Regulatory Factor 3 (IRF3) is critical for the first line of defense against pathogens mainly through the induction of IFN β , but also through the induction of the chemokine (C-C motif) ligand 5 (CCL5) and several antiviral proteins including IFN-induced protein with tetratricopeptide repeats IFIT1/2/3¹⁻³. IRF3 activation has been reported following infection with numerous viruses, or exposure to polyinosinic-polycytidylic acid (poly I:C) or lipopolysaccharide (LPS)⁴. Importantly, most studied viruses have evolved mechanisms to evade the IRF3-mediated response, and thereby escape the host innate immune defense⁵. Thus, monitoring IRF3 activation is of great importance to understand the molecular mechanisms of the innate antiviral host defense, but also to identify the strategy used by viruses to counteract this response.

Many published reports however provide only a limited analysis of IRF3 activation performed by the monitoring of IRF3-target gene induction (*IFNB1* and *IFIT1*) and/or luciferase reporter gene assay coupled to low resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of IRF3. However, numerous biochemical studies, analysis of the behavior of various IRF3 mutants and elucidation of IRF3 crystal structure⁶⁻¹¹ have contributed to establish that IRF3 is subjected to a complex set of sequential post-translational modifications by phosphorylation at multiple sites. The set of phosphorylation involved in IRF3 activation appears to be dependent on the stimulus and most likely on the cell type. In uninfected cells, IRF3 coexists as non-phosphorylated and hypophosphorylated species containing phosphoresidues, including Thr135 and Ser173, in the 1-198 aa N-terminal region^{6,12-14}. Accumulation of this hypophosphorylated form of IRF3 is induced by stress inducers, growth factors and DNA-damaging agents⁶. Phosphorylation of Ser/Thr residues at the C-terminal region of IRF3 containing the transactivation domain is triggered following activation by viruses, poly I:C or LPS in a cell-type dependent manner¹⁵⁻¹⁷. C-terminal phosphorylation of IRF3 involves no less than 7 distinct phosphoacceptor sites organized in two main clusters, Ser385/Ser386 and Ser396/Ser398/Ser402/Thr404/Ser405, that each contribute to IRF3 activation through dimerization, nuclear accumulation, association with the CREB-binding protein (CBP)/p300 coactivators, DNA binding to IFN sensitive response element (ISRE) consensus sequences and transactivation of target genes^{9,10,17-19}. Phosphorylation of Thr390 is also thought to contribute to virus-induced IRF3 activation²⁰. Mass spectrometry analyses of IRF3 have demonstrated that Ser386, Thr390, Ser396 and Ser402 residues are directly phosphorylated by the inhibitor of κ B kinase ϵ (IKK ϵ)/TANK-binding kinase 1 (TBK1) kinases^{9,10}. Phosphorylation at the C-terminal residues is also required for termination of IRF3 activation through polyubiquitination and proteasome-mediated degradation¹⁰. This process is also dependent on the phosphorylation at Ser339, which is necessary for the recruitment of the propyl isomerase Pin1^{10,11}. IRF3 species containing at least phospho-Ser339/386/396 residues are

considered hyperphosphorylated forms. The exact sequence and function of each site remains a matter of discussion^{10,21}. It is now clear that activated IRF3 does not represent a homogeneous state, but that different activated species exhibiting distinct phosphorylation or dimerization characteristics exist^{10,22}.

To provide a complete understanding of IRF3 activation in response to specific pathogens, it is thus necessary to characterize which of the activated species are induced. Induction of IRF3 target genes, *IFNB1* and *IFIT1*, has proven to provide a reliable read-out for IRF3 activation. However, monitoring expression of these genes does not distinguish between different activation states of IRF3. A comprehensive analysis of IRF3 activation states in a particular setting relies on the detailed characterization of its phosphorylation and dimerization status¹⁰. Unphosphorylated (form I), hypophosphorylated (form II) and hyperphosphorylated (forms III and IV) IRF3 forms^{6,18,23} can be successfully resolved by reduced mobility in high-resolution SDS-PAGE analysis. Monomeric and dimeric IRF3 species can be efficiently identified by native-PAGE analysis. These approaches are greatly improved when used in combination with phosphospecific antibodies directed against distinct IRF3 phosphoacceptor sites.

Standard protocols allow a poor resolution of proteins that does not permit efficient separation of distinct IRF3 phosphorylated forms. Here, we describe in detail a procedure to achieve the highest resolution to monitor the induction of distinct virus-activated IRF3 species using SDS-PAGE coupled to native-PAGE in combination with immunoblot using total and phosphospecific antibodies. *In vivo* discrimination between the different activated forms of IRF3 is performed based on their mobility shifts observed on SDS-PAGE. Additionally, IRF3 monomer and dimer can be distinguished by non-denaturing electrophoresis. The combination of these two complementary techniques with immunoblot proves to be an affordable and sensitive approach to acquire all the necessary information for a complete analysis of phosphorylation-mediated activation of IRF3.

Protocol

NOTE: The protocol is described here using A549 cells infected with Sendai virus (SeV). However, the protocol for SDS-PAGE and native PAGE also works with all human and murine cell types tested so far, particularly myeloid cells stimulated with various IRF3-activating stimuli^{9,15,19,24,25}.

1. Infection of A549 Cells

- Maintain A549 cells in culture in a 15 cm plate at 37 °C/5% CO₂ in 20 ml F12K/Ham medium containing 10% heat-inactivated fetal bovine serum (HI-FBS) and 1% L-glutamine (complete F12K/Ham medium).
NOTE: All the solutions used for cell culture and treatments must be sterile.
- At 24 hr before the infection, aspirate the medium and wash the cells with 10 ml of distilled phosphate buffered saline (D-PBS) at RT.
- Add 1 ml of 0.25% trypsin-EDTA solution to each plate to cover the cells and incubate at 37 °C for 3 min.
- Stop the incubation as soon as the cells start to detach from the plate by softly tapping the plate with one hand. Inactivate the trypsin by adding 10 ml of pre-heated complete F12K/Ham medium per plate and transfer the cells to a 15 ml conical tube.
- Centrifuge at 350 x g for 3 min at RT. Remove the supernatant and resuspend the cell pellet in 8 ml of complete F12K/Ham medium to obtain a homogeneous single cell suspension.
- Count the cells using a hemocytometer. Seed the cells at a density of 1 x 10⁶ cells per 60 mm plate in 4 ml of pre-heated complete F12K/Ham medium. Incubate for 20 - 24 hr at 37 °C/5% CO₂. After 20 - 24 hr, the cells form a 90% confluent monolayer (this corresponds to 1.5 x 10⁶ cells).
- Remove the medium and wash the cells with 2 ml of serum-free F12K/Ham medium (SFM). Add 2 ml of fresh SFM per 60 mm plate.
- Thaw an aliquot of Sendai virus (SeV) (stored aliquoted at -80 °C) on ice and vortex briefly.
- Dilute the virus in pre-heated SFM to obtain 60 HAU/100 µl. Mix by pipetting up and down softly and add 100 µl of diluted virus per plate to perform the infection at 40 HAU/10⁶ cells. Do not add virus in the non-infected control plate.
- Incubate the cells in the incubator at 37 °C/5% CO₂. Agitate the plates by hand 3 or 4 times, or using an automatic orbital or rocking shaker placed directly in the incubator, during the first hour of infection.
- At 2 hr post-infection, add 2 ml of F12K/Ham medium containing 20% HI-FBS to obtain a final concentration of 10% HI-FBS.
- Incubate the cells in the incubator at 37 °C/5% CO₂ for an additional 1, 4 and 7 hr to reach total infection times of 3, 6, and 9 hr, respectively. At each of these time points, proceed to step 2.1.

2. Preparation of Whole Cell Extracts (WCE)

- Remove the infection medium. Harvest the cells by scraping in 1 ml of ice-cold D-PBS and transfer the cell suspension to a pre-chilled 1.5 ml centrifuge tube.
- Pellet the cells by centrifugation at 16,000 x g at 4 °C for 20 sec and carefully decant all traces of D-PBS.
NOTE: At this step, the cell pellet can be directly subjected to protein extraction or flash-frozen in liquid nitrogen or dry ice/ethanol bath and stored at -80 °C until lysis.
- Prepare the lysis buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol and 1% Nonidet P-40 in deionized water (ddH₂O). Extemporarily add protease (1 µg/ml leupeptin and 2 µg/ml aprotinin) and phosphatase inhibitors (5 mM sodium fluoride, 1 mM activated sodium orthovanadate, 2 mM p-nitrophenyl phosphate and 10 mM β-glycerophosphate pH 7.5).
NOTE: The lysis buffer without inhibitors can be stored at 4 °C. A specific protocol for the activation of sodium orthovanadate is described in the Table of Specific Reagents/Equipment.
- Resuspend the cell pellet in 70 µl of lysis buffer. Typically the lysate concentration will be around 2 µg/µl.
- Incubate on ice for 20 min. Flash-freeze the lysate by incubation in a liquid nitrogen bath for 15 sec. Thaw the lysate at RT until it is completely melted and vortex for 10 sec. Repeat the freeze/thaw/vortex cycle 3 times.
 - Alternatively, perform the freezing step in an ethanol/dry ice bath for 1 min.

6. Centrifuge at 16,000 x g at 4 °C for 20 min. Transfer the supernatant (corresponding to the WCE) to a new pre-chilled 1.5 ml centrifuge tube. Keep the WCE on ice at all times.
7. Quantify proteins using any protein quantification procedure compatible with the lysis buffer such as Bradford-based protein assay²⁶.

3. Resolution of WCE by High Resolution SDS-PAGE

1. Prepare three denaturing electrophoresis gels.
 1. For the detection of IRF3 forms, pour two gels of a minimum of 16 cm length with a separation gel composed of 7.5% acrylamide/bis-acrylamide (37.5:1), 375 mM Tris-HCl pH 8.8 (RT), 0.1% sodium dodecyl sulfate (SDS), 1% ammonium persulfate and 0.1% TEMED in ddH₂O and a stacking gel composed of 4% acrylamide, 62.5 mM Tris-HCl pH 6.8 (RT), 0.05% SDS, 1% ammonium persulfate and 0.1% TEMED in ddH₂O.
Caution! Acrylamide, TEMED and SDS are toxic and/or irritant. Wear protective gloves and manipulate under a fume hood.
 2. For the detection of SeV proteins, pour one gel of a minimum of 8.5 cm length with characteristics similar to the gel described in 3.1.1, except that the separation gel contains 12% acrylamide.
2. Denature the WCE obtained in step 2.6 by adding 1:4 (v/v) 5x loading buffer (125 mM Tris-HCl pH 6.8 (RT), 10% SDS, 20% (p/v) glycerol, 0.0005% bromophenol blue and 25% β-mercaptoethanol in ddH₂O) followed by heating at 100 °C for 10 min. Quick spin the tubes to bring down the condensation that forms in the cap.
Caution! β-mercaptoethanol is toxic by inhalation. Wear protective gloves and manipulate under a fume hood.
3. Mount the gels in the migration apparatus. Fill the upper and lower chambers with running buffer containing 25 mM Tris-Base, 0.1% SDS and 192 mM glycine in distilled water (dH₂O).
4. Load 14 μl of molecular weight standard in one well of each 16 cm gel and 7 μl of molecular weight standard in one well of the 8.5 cm gel. Load 30 μg of denatured WCE (prepared as described in step 3.2) per well of the two gels prepared in step 3.1.1 for IRF3 forms detection. Load 8 - 10 μg of denatured WCE (prepared as described in step 3.2) per well on the gel prepared in step 3.1.2 for SeV analysis.
5. Run the gels at 30 mA constant current until the migration front reaches the bottom of the gel.
NOTE: Migration typically lasts approximately 3 hr for a 16 cm gel and 45 min for an 8.5 cm gel.
6. Proceed to the transfer onto nitrocellulose membranes (step 5).

4. Analysis of IRF3 Dimerization by Native-PAGE

NOTE: This method was originally described by the group of Dr. T. Fujita²⁷.

1. Prepare the upper (-) and lower (+) chamber electrophoresis buffers. The upper chamber buffer consists of 25 mM Tris-HCl pH 8.4 (RT), 192 mM glycine and 1% sodium deoxycholate (DOC) in ddH₂O. The lower chamber buffer contains 25 mM Tris-HCl pH 8.4 (RT) and 192 mM glycine in ddH₂O.
NOTE: The upper and lower chamber electrophoresis buffers can be stored at 4 °C until use. However, make sure that they are pre-warmed to RT before performing the electrophoresis.
2. Pour a non-denaturing resolving gel of a minimum of 8.5 cm length containing 7.5% acrylamide/bis-acrylamide (37.5:1), 375 mM Tris-HCl pH 8.8 (RT), 1% ammonium persulfate and 0.1% TEMED in ddH₂O.
3. Pre-run the gel at 40 mA constant current for 30 min on ice. Press the running apparatus into the ice approximately to the level of the lower chamber electrophoresis buffer. It is important that the gel is not in the ice.
4. During pre-run, mix WCE kept on ice with 2x native-PAGE loading buffer 1:1 (v/v) containing 125 mM Tris-HCl pH 6.8 (RT), 30% glycerol and 0.1% bromophenol blue in ddH₂O.
5. Load 8 - 10 μg WCE (prepared as described in step 4.4) immediately at the end of the pre-run.
6. Run the gel at 25 mA constant current on ice, as described above for the pre-run, until the migration front reaches the bottom of the gel (approximately 40 min).

5. Immunoblot Analysis of IRF3 Species

1. Prepare the transfer buffer containing 25 mM Tris-Base and 192 mM glycine in ddH₂O. Refrigerate the transfer buffer at 4 °C before use.
2. Wet three pieces of nitrocellulose membrane cut to a size slightly larger than the gels in a plastic/glass box containing the transfer buffer. Indicate the orientation of the membrane by cutting one corner. The transfer buffer can be reused 3 times. Store the buffer at 4 °C between uses.
3. Uncast the gels (SDS-PAGE and native-PAGE) and cut one corner of each gel for proper orientation.
 1. For native-PAGE, incubate the gel at RT with gentle agitation for at least 30 min in the SDS-PAGE running buffer to remove the DOC before transferring to the transfer buffer.
4. Incubate the gels (SDS-PAGE and native-PAGE) in the transfer buffer for 5 - 10 min.
5. Mount a transfer sandwich per gel in a transfer cassette with the membrane towards the positive electrode (foam pad/filter paper/membrane/gel/filter paper/foam pad). Be careful to remove all the bubbles in between the layers of the sandwich.
6. Perform the transfer as recommended by the manufacturer for the transfer apparatus used.
NOTE: Perform all incubations and washes in the next steps on a rocking or orbital shaking platform.
7. At the end of the transfer time, incubate the membranes for 15 min in the fixation solution containing 7% acetic acid, 40% ethanol and 3% glycerol in ddH₂O. Wash the membranes 3x 5 min in PBS (137 mM NaCl, 2.7 mM KCl, 10.2 mM Na₂HPO₄ and 1.8 mM KH₂PO₄ in dH₂O).
Caution! Acetic acid is toxic, irritant and flammable. Wear protective gloves and manipulate under the fume hood.
NOTE: The fixation solution can be reused multiple times.
8. For the native-PAGE membrane proceed directly to step 5.11.

9. Rinse the three SDS-PAGE membranes quickly in dH₂O before incubating them for 1 min in red ponceau solution containing 6.57 mM red ponceau and 1% acetic acid in ddH₂O.
NOTE: The red ponceau solution can be reused several times.
10. Rinse the membranes in dH₂O until the background is white enough to see the protein bands stained in red. Note the markers with a pencil and cut the excess membrane around the proteins. Destain the membranes by incubation for 5 min in PBS under agitation.
11. Incubate the membranes for 1 hr at RT or O/N at 4 °C in the blocking solution (PBS containing 0.05% Tween 20 and 5% non-fat dry milk (PBS-T-milk). Wash the membranes 3x 5 min in PBS-T.
NOTE: The PBS-T wash is optional and only strictly required when applying antibodies diluted in PBS-T containing 5% bovine serum albumin (PBS-T-BSA) (**Figure 1 and Table 1**) in the next step.
12. Incubate the four membranes (from SDS-PAGE and native-PAGE) with the primary antibodies according to the sequential order detailed in **Figure 1 and Table 1**. Perform 5x 5 min washes in PBS-T.

Figure 1. Sequence of Immunoblot Analyses. The schematic describes the individual SDS-PAGE and native-PAGE gels required to detect the various IRF3 phosphorylated and monomeric/dimeric forms. The specific order of antibodies applied to the membrane resulting from each gel in the immunoblot procedure is described. Note that the anti-actin antibodies are used first to ensure equal loading of the samples before applying any other specific antibodies. The alternate sequence, with anti-actin antibodies being applied after the anti-phospho-IRF3 antibodies, also works. Stripping is used between anti-actin and anti-SeV antibodies, or between anti-phospho-IRF3 and anti-IRF3 antibodies because of overlapping size of the signals. [Please click here to view a larger version of this figure.](#)

Primary antibodies	Dilution	Dilution buffer	Incubation	Secondary antibodies	Comments
Anti-Actin	1/10,000	PBS-T-BSA	15 min at RT	Anti-mouse	Use after SDS-PAGE. Diluted antibodies can be reused several times if stored at 4 °C in the presence of 0.02 % Sodium Azide.
Anti-IRF3-P-Ser386	1/200	PBS-T-BSA	O/N at 4 °C	Anti-rabbit	Use after Native-PAGE. It is not recommended to reuse the diluted antibody.
Anti-IRF3-P-Ser396	1/10,000	PBS-T-BSA	O/N at 4 °C	Anti-rabbit	Use after SDS-PAGE. It is not recommended to reuse the diluted antibody. Anti-IRF3-P-396 is also available from Cell Signaling. Optimal dilution was defined as 1/1,000 for this antibody, but may vary between lots.
Anti-IRF3-P-Ser398	1/10,000	PBS-T-BSA	O/N at 4 °C	Anti-rabbit	Use after SDS-PAGE. It is not recommended to reuse the diluted antibody.
Anti-IRF3 full length	1/7,500	PBS-T-milk	3 hr at RT	Anti-rabbit	Use after SDS-PAGE. Anti-IRF3 full length antibody can be used after native-PAGE, but it is less sensitive to detect the monomer. Diluted antibodies can be reused several times if stored at 4 °C in the presence of 0.02 % Sodium Azide.
Anti-IRF3-NES	0.5 µg/ml	PBS-T-milk	3 hr at RT	Anti-rabbit	Use after Native-PAGE. Diluted antibodies can be reused several times if stored at 4 °C in the presence of 0.02 % Sodium Azide.
Anti-SeV	1/14,000	PBS-T-BSA	3 hr at RT	Anti-rabbit	Use after SDS-PAGE. Diluted antibodies can be reused several times if stored at 4 °C in the presence of 0.02 % Sodium Azide.

NOTE: Dilution and buffer used for HRP-coupled secondary antibodies need to be optimized as it varies from one company to the other.

Table 1. Specifications of Antibodies used in the Immunoblotting Procedure.

- Incubate the membranes with the horseradish peroxidase (HRP)-conjugated secondary antibodies as detailed in **Figure 1** and **Table 1**. Wash the membranes 5x 5 min in PBS-T, followed by 2x 5 min in PBS to fully remove traces of Tween.
- Incubate the membranes for 1 min in a volume of enhanced chemiluminescence reagent sufficient to fully cover the membranes. Dry the membranes using filter paper.
- Place the membranes in a luminescent image analyzer to visualize the immunoreactive bands.
 - Alternatively, perform the detection of immunoreactive bands using sensitive X-Ray films.
- Wash the membranes 3x 5 min in PBS.

NOTE: At this step the membranes can be kept dry. However, if further stripping is required, it is better to perform the stripping before drying the membrane. Membranes can also be stored for short-term in PBS.
- For the membranes that do not require stripping between incubation with antibodies (see **Figure 1**) proceed directly to step 5.20.

18. When stripping is required between antibodies (see **Figure 1**), incubate the membranes in pre-warmed stripping solution containing 2% SDS, 62.5 mM Tris-HCl pH 6.8 (RT) and 0.7% β -mercaptoethanol in ddH₂O at 50 °C for 20 min under regular agitation. Wash the membranes 3x 5 min in PBS.
NOTE: Agitation during the stripping procedure is key. Stripping can be performed in a hybridization oven. Alternatively, stripping can be performed using membranes sealed in plastic bags and immersed in a water bath. In this case, it is important to agitate the membranes 4 - 5 times during the incubation.
19. Incubate the membranes for 1 hr at RT or O/N at 4 °C in PBS-T-milk.
20. Repeat steps 5.12 to 5.16 according to **Figure 1**.

Representative Results

Figure 2 shows a typical immunoblot image of IRF3 detected with IRF3 total antibodies and IRF3-phosphospecific antibodies against Ser396 and Ser398 after resolution of WCE by high-resolution SDS-PAGE. In unstimulated A549 cells, IRF3 is detected as two bands at 50 and 53 kDa on the SDS-PAGE corresponding to the non-phosphorylated (form I) and the hypophosphorylated (form II) species of IRF3. Exposure of A549 cells to SeV for 3 - 9 hr results in a time-dependent shift to slowly migrating hyperphosphorylated forms III and IV, which are well distinguished from the forms I and II. The hyperphosphorylated bands migrate at 55-57 kDa. The forms III and IV are also specifically immunodetected using the phosphospecific antibodies against Ser396 and Ser398. The immunodetection of actin serves as a control of equal loading between the lanes. A control of SeV infection is shown by the accumulation of the virus nucleocapsid protein (N), which migrates at 60 kDa.

In **Figure 3**, the profile of detection of IRF3 obtained from WCE resolved by native-PAGE followed by immunoblot using anti-IRF3-NES antibodies and phosphospecific antibodies against Ser386 is shown. In unstimulated A549 cells, IRF3 is detected as a single band corresponding to the monomeric form. Upon infection with SeV for 3 - 9 hr, the levels of IRF3 monomer decrease with a concomitant accumulation of a slowly migrating band that corresponds to the dimeric form of IRF3. The phosphospecific antibodies against Ser386 only detect IRF3 dimer species.

Figure 2. Detection of Distinct IRF3 Phosphorylated Species (form I-IV) Induced by SeV Infection in A549 Cells. A549 cells were left uninfected or infected with SeV for the indicated times. WCE were analysed by high-resolution SDS-PAGE followed by immunoblot (IB) using anti-IRF3-Ser396 (IRF-3-P-Ser396) and anti-IRF3-Ser398 (IRF-3-P-Ser398) phosphospecific antibodies and anti-IRF3 full length protein antibodies. The infection was monitored using anti-SeV antibodies (the nucleocapsid N protein is shown). Anti-actin antibodies were used as a loading control. [Please click here to view a larger version of this figure.](#)

Figure 3. Monitoring of Phosphorylated/dimerized IRF3 Induction by SeV in A549 Cells. A549 cells were left uninfected or infected with SeV for the indicated times. WCE were resolved by native-PAGE and revealed by immunoblot using anti-IRF3-Ser386 (IRF-3-P-Ser386) phosphospecific antibodies and anti-IRF3-NES antibodies. [Please click here to view a larger version of this figure.](#)

Discussion

The protocol we describe here consists of a combination of high-resolution SDS-PAGE and native-PAGE coupled to the use of several phosphospecific antibodies to distinguish the monomeric/dimeric and phosphoforms I-IV of IRF3. Appropriate detection of these IRF3 species is essential to fully characterize IRF3 activation in a specific setting. For instance, LPS stimulation of activated macrophages leads to the formation of dimeric, Ser396/398 phosphorylated IRF3 that exhibits a hypophosphorylated (form II), but not hyperphosphorylated (form III and IV), pattern in SDS-PAGE¹⁵. Using the described protocol, this profile can be distinguished from virus-induced hyperphosphorylated forms that require additional Ser339 phosphorylation in addition to Ser386 and Ser396 phosphorylation¹⁰. Importantly, this also allows discriminating between Ser396 phosphorylated species that do not exhibit dimerization, but are transcriptionally active¹⁰. Importantly, it needs to be noted that the form I-IV pattern of IRF3 phosphoforms applied to human IRF3. Murine IRF3 does not exhibit a similar pattern and therefore activation of IRF3 is characterized only by the use of phosphospecific antibodies in immunoblot after SDS-PAGE and by native-PAGE.

The achievement of a high resolution separation of the distinct phosphorylated species of IRF3 by SDS-PAGE requires specific parameters. For appropriate resolution, it is very important to use gels that have a minimum length of 16 cm. Even with this length of separation gel, it is key to let the migration front reach the bottom of the gel to obtain an appropriate separation of the different IRF3 forms. Also, the stacking gel must extend to a minimum of 1 cm below the comb to obtain well-defined bands. This is important, as the hyperphosphorylated forms of IRF3 migrate very close to one another. Poorly focused bands will result in lack of distinction between phosphorylated forms impairing their respective quantification. Additionally, the concentration of ammonium persulfate and TEMED vary from one SDS-PAGE protocol to another. Variation in these concentrations from the ones indicated here was found to significantly impair the resolution in the separation of the IRF3 species.

Detection of IRF3 dimer formation through the native-PAGE was originally described by the group of Dr. T. Fujita. This protocol uses buffer containing DOC that allows the dissociation of the IRF3 dimer from CBP/p300²⁷. It is highly recommended to proceed to native-PAGE immediately after cell lysis as storage of the WCE at -80 °C was found to result in a significant alteration of the IRF3 monomer/dimer ratio. Additionally, it is very important that the pH of the upper and lower chamber buffers is adjusted to pH 8.4 at RT, and not at 4 °C, after mixing all components to achieve proper separation of the monomer vs dimer. A separation gel with a minimum length of 8.5 cm allows appropriate separation of IRF3 monomer and dimer. The ideal volume of sample plus loading buffer is around 8 µl for a 5 mm well as the resolution is better when the volume is kept to a minimum. Thus, it is important to use the lowest volume of lysis buffer to obtain WCE with a high concentration. However, it should be taken into account that efficient protein extraction requires lysis in at least 5x the volume of the cell pellet. If the volume of the samples exceeds the limit indicated above, this would result in poor resolution of the monomer/dimer. This can be solved by adding a stacking gel containing 4% acrylamide, 62.5 mM Tris-HCl pH 6.8 (RT), 1% ammonium persulfate and 0.1% TEMED in ddH₂O. It is very important to load the samples without disturbing banding. Loading slowly with a gel-loading tip close to the bottom of the well gives very good results.

Here, we describe the *in vivo* detection of phosphorylation at specific residues using currently available phosphospecific antibodies against Ser386⁹, Ser396¹⁹ and Ser398¹⁵. The use of IRF3 mutants and mass spectrometry analyses have confirmed that these residues are phosphorylated upon virus infection or overexpression of IKKε/TBK1 kinases and are critical for IRF3 activation^{6,9,10,19,21}. The immunodetection of phospho-Ser396, phospho-Ser398 and total IRF3 after SDS-PAGE requires the use of two identical gels (**Figure 1**). Indeed, significant loss of sensitivity is observed when phosphospecific antibodies are used after the stripping of the membrane. Therefore, it is highly recommended to use phosphospecific antibodies first. Note that alternative primary and secondary antibodies may also work, but the specific dilutions and sequence of usage should be optimized. For IRF3 analysis, 30 µg of WCE is appropriate to obtain a significant detection of IRF3 phosphorylation by immunoblot using the indicated antibodies when using 4 mm width wells. Although in many cell types infected with various viruses 30 µg was found to be appropriate, this amount might need to be increased depending on cell type and stimulation. It is preferable to use fresh extracts for SDS-PAGE, but the detection of IRF3 phosphorylation using the phosphospecific antibodies described in this paper is stable enough to allow a round of storage of the WCE at -80 °C before analysis. The concentrations of phosphatase inhibitors described here were found to be sufficient when using A549 cells. Higher concentrations were not found to yield better results. However, these concentrations might need to be increased when studying IRF3 activation in cell types that exhibit higher phosphatase activity levels. Importantly, the phosphorylation of IRF3 and the underlying mechanisms are still far from being understood. Therefore, a full panel of Ser/Thr- and Tyr-phosphatase inhibitors is used to

block possible yet uncharacterized activities that could affect the detection of IRF3 phosphorylation, dimerization and degradation. For detection of IRF3 monomer and dimer after most virus infections and in most cell types, 8 - 10 μ g WCE was found to be sufficient. However, higher amount of proteins might be required for stimulation activating IRF3 less efficiently. The phosphospecific antibodies against Ser386 used in this study are recommended to be used in native-PAGE as the sensitivity in denaturing SDS-PAGE is very weak. Moreover, the phosphorylation at Ser386 is proposed to correlate with the dimeric form of IRF3⁹, thus making the use of these antibodies in native-PAGE an appropriate strategy to confirm this concept in a specific setting. Of note, alternative antibodies are available from various companies and are claimed to efficiently detect phospho-Ser386 after SDS-PAGE. Phosphospecific antibodies against Ser396 have also been efficiently used to detect IRF3 phosphorylation after native-PAGE²⁰. Importantly, Ser402 in the C-terminal cluster of phosphoacceptor sites was also found to be important for IRF3 activation and the phosphorylation of Ser402 was observed through mass spectrometry analysis of IKK ϵ /TBK1-phosphorylated IRF3^{10,21,28}. However, despite two different attempts (NG, unpublished), no phosphospecific antibodies are currently available to follow the phosphorylation of this specific residue *in vivo*. Note that phosphospecific antibodies against Thr157 and Ser339 have also been described^{11,13}. These additional phosphospecific antibodies could be used in a procedure of high-resolution SDS-PAGE similar to the one described here. In this case, additional large SDS-PAGE gels would be required for each antibody and processed the same way as gel #2 (**Figure 1**). To our knowledge no phosphospecific antibodies against Ser173 or Thr390 have yet been described, but they could be easily added to the protocol described here once they become available^{14,20}.

IRF3 activity is terminated by proteasome-mediated degradation of hyperphosphorylated forms^{11,17}. The protocol described here also permits the monitoring of IRF3 degradation when the kinetic of stimulation is prolonged and is coupled to the use of proteasome inhibitors (MG132, lactacystin or bortezomid). Typically, in A549 cells infected with SeV at 40 HAU/10⁶ cells, IRF3 phosphorylation starts as soon as 2 hr post-SeV infection and IRF3 degradation starts after 12 hr. Proteasome-mediated degradation will be concluded from the observed diminution of forms III and IV levels at late time points that is reversed in conditions with proteasome inhibitors. This will also translate on native-PAGE in a diminution of IRF3 dimer levels that are recovered upon proteasome inhibitor treatment²⁹. Importantly, the high-resolution technique presented here allows differentiating between the degradation process and a lack of activation. Indeed, both degradation and lack of activation of IRF3 result in the absence of detection of dimer/phospho-Ser386/396. However, lack of activation is associated with the detection of IRF3 monomer and of forms I and II, while these IRF3 species are not detected when IRF3 is degraded³⁰.

Immunoprecipitation coupled with mass spectrometry-based analysis is a method of choice to detect the *in vivo* phosphorylation of IRF3 at distinct sites. This technique was used to confirm the phosphorylation of IRF3 at Ser173, Ser175, Ser386, Thr390, Ser396 and Ser402 residues^{10,20,21}. However, mass spectrometry is not yet an affordable/benchside technique that can be easily used for daily analysis of IRF3 activation following several stimulations at different time points. Therefore, the procedure described here using SDS-PAGE coupled to native-PAGE in combination with immunoblot using total and phosphospecific antibodies constitutes a practical, affordable and sensitive approach to detect all currently defined forms of activated IRF3.

Disclosures

The authors declare that they have no competing financial interests.

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