

Biochemical Analysis of Caspase-8-dependent Proteolysis of IRF3 in Virus-infected Cells

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[Abstract] Interferon regulatory factor 3 (IRF3) is a transcription factor, which is critical for the antiviral response against a wide range of viruses (Hiscott, 2007; Ikushima *et al.*, 2013). It gets activated in virus-infected cells via Toll like receptors (TLRs), RIG-I (retinoic acid inducible gene 1) like receptors (RLRs), cyclic GMP-AMP synthase (cGAS) – stimulator of interferon genes (STING), which are sensors of viral components in the cells (Chattopadhyay and Sen, 2014a; 2014b; Hiscott, 2007). IRF3 is a cytoplasmic protein, upon activation by virally activated sensors it gets phosphorylated, translocated to the nucleus and binds to the interferon-sensitive response element (ISRE) of the gene promoters to induce their transcription (Hiscott, 2007). IRF3 has other functions, including direct stimulation of apoptosis in virus-infected cells. In this pathway, the transcriptional activity of IRF3 is not required (Chattopadhyay *et al.*, 2013b; Chattopadhyay *et al.*, 2016; Chattopadhyay *et al.*, 2010; Chattopadhyay and Sen, 2010; Chattopadhyay *et al.*, 2011). These pathways are negatively regulated by host factors as well as by viruses. Our studies indicate that IRF3 can be proteolytically processed by caspase-8-dependent cleavage (Sears *et al.*, 2011). A specific site in IRF3 is targeted by caspase-8, activated in RNA or DNA virus-infected and dsRNA-stimulated cells (Sears *et al.*, 2011). The direct involvement of caspase-8 was confirmed by *in vitro* cleavage assay using recombinant proteins and *in vivo* by virus activated caspase-8. The proteolytic cleavage of IRF3 can be inhibited by chemical inhibition or genetic ablation of caspase-8. The cleavage of IRF3 removes the activated pool of IRF3 and thus can be used as a pro-viral mechanism (Figure 1). Using a C-terminally epitope-tagged human IRF3, we analyzed the cleavage of IRF3 in virus-infected cells. Moreover, we used recombinant proteins *in vitro* to conclude that IRF3 is a substrate of caspase-8 (Sears *et al.*, 2011). In the current protocol, we have outlined a simple and detailed procedure to biochemically analyze the proteolysis of IRF3 in virus-infected cells and the specific role of caspase-8 in this process.

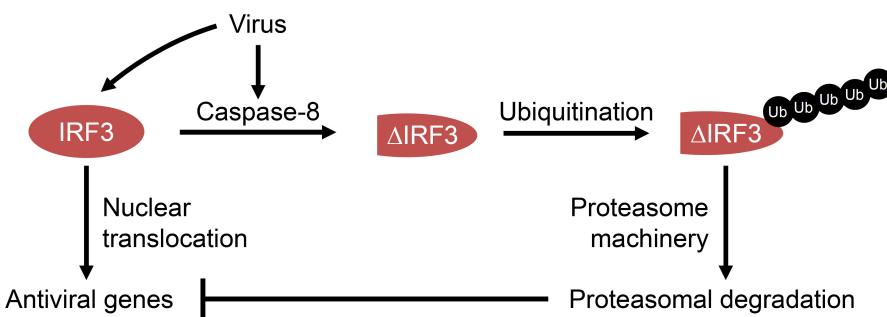


Figure 1. Model for virus-induced caspase-8-dependent proteolysis of IRF3. In virus-infected cells, IRF3 can be proteolytically cleaved by caspase-8, which gets activated during infection. The cleaved IRF3 is subjected to poly-ubiquitination (Ub) and degradation by proteasome machinery. The degradation of IRF3 inhibits dsRNA-induced antiviral gene induction.

[Background] IRF3 functions as a transcription factor as well as a pro-apoptotic factor in virus-infected cells. The two properties of IRF3 determine its overall antiviral functions. Our studies have revealed a number of additional regulatory mechanisms of IRF3 for both of its functions. Using genetically modified cells and mice, we have shown how the two functional branches of IRF3 protect against virus infections (Chattopadhyay *et al.*, 2013a; Chattopadhyay *et al.*, 2016; Chattopadhyay *et al.*, 2011). Dysregulated IRF3 activation is undesirable for normal physiological functions and, therefore, negative regulatory mechanisms are in place to control continuous activation of IRF3. In virus-infected cells, IRF3 can be proteolytically cleaved at a specific site by caspase-8, which gets activated by TLR or RLR signaling. The proteolytic cleavage of IRF3 prevents unregulated expression of dsRNA-dependent genes (Sears *et al.*, 2011). We speculate that such mechanisms are used by viruses to promote their replication and to downregulate IRF3 responses. Therefore, the proteolysis of IRF3 represents a critical step, which both the viruses and the host can regulate to their favorable outcomes. This protocol provides a method to biochemically analyze the proteolysis of IRF3 in virus-infected cells.

Materials and Reagents

A. Materials

1. Autoradiography film (Denville Scientific, catalog number: E3012)
2. Tissue culture plates (Corning)
3. 1.5 ml Eppendorf tubes (USA Scientific)
4. Centrifuge tubes (Thermo Fisher Scientific)
5. 26-gauge needle
6. PVDF membranes

B. Cells

1. HT1080 fibrosarcoma cells

Note: These cells are maintained in DMEM containing 10% FBS, 100 international units of penicillin, 100 µg/ml streptomycin (complete DMEM).

2. 1080.10 cells (IRF3 overexpressing HT1080 cells [Elco et al., 2005])

Note: These cells are maintained in complete DMEM containing 1 µg/ml puromycin.

3. P2.1/IRF3 cells (P2.1 cells expressing C-terminally Flag-tagged human IRF3, Figure 2A)

Notes:

- a. *These cells were generated by lentiviral transduction of the P2.1 cells with cDNA of Flag-tagged human IRF3. After lentiviral transduction, the cells were selected in puromycin (1 µg/ml)-containing complete medium. Use the selected pool of cells for the experiments.*
- b. *HT1080 cells were described previously (Chattopadhyay et al., 2016) and the other cell lines (1080.10 and P2.1/IRF3) were generated in the authors' laboratory.*

C. Viruses

1. Sendai virus (SeV) Cantell strain (Charles River laboratories) – this strain was originally obtained from ATCC (ATCC, catalog number: VR-907TM)
2. Adenovirus Ad5 strain (a kind gift from Thomas Shenk, Princeton University, New Jersey)

D. Reagents

1. DMEM (Lerner Research Institute Central Cell Services, catalog number: 11-500p)
2. PolyI:polyC (Poly [I:C]) (GE Healthcare, catalog number: 27-4732-01)
3. Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, InvitrogenTM, catalog number: 11668030)
4. Opti-MEM I reduced serum medium (Thermo Fisher Scientific, InvitrogenTM, catalog number: 31985070)
5. Phosphate-buffered saline (PBS) (Lerner Research Institute Central Cell Services, catalog number: 123-1000p)
6. Recombinant caspase-8 (EMD Millipore, catalog number: CC123)
7. Protein assay dye (Bio-Rad Laboratories, catalog number: 5000006)
8. 10x SDS-PAGE running buffer (AMRESCO, catalog number: 0783)
9. Antibodies:
 - anti-Flag (used at 1:2,500) (Sigma-Aldrich, catalog number: F3290)
 - anti-IRF3 (dilution used at 1:10,000) (Active Motif, catalog number: 39033)
 - anti-actin (used at 1:10,000) (Sigma-Aldrich, catalog number: A5441)
 - HRP-conjugated secondary antibodies (used at 1:5,000) (Rockland Immunochemicals, catalog numbers: 611-103-121, 611-103-122)
10. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11550)
11. Proteasome inhibitor MG132 (EMD Millipore, catalog number: 474790)
12. Phosphatase inhibitor (PhosSTOP) (Roche Diagnostics, catalog number: 04906837001)
13. cOmplete EDTA-free protease inhibitor (Roche Diagnostics, catalog number: 11873580001)

14. Caspase inhibitors:
 - z-VAD (MBL, catalog number: 4800-520)
 - z-WEHD (MBL, catalog number: BV-1100-3)
 - z-IETD (MBL, catalog number: 4805-510)
15. Anti-Flag-agarose beads (Sigma-Aldrich, catalog number: A2220)
16. FLAG peptide (Sigma-Aldrich, catalog number: F3290)
17. SDS-PAGE loading buffer (Laemmeli) (Bio-Rad Laboratories, catalog number: 161-0737)
18. Tris buffered saline with Tween-20 (TBS-T) (AMRESCO, catalog number: K873)
19. Nonfat dry milk (Bio-Rad Laboratories, catalog number: 1706404XTU)
20. Pierce™ ECL2 (Thermo Fisher Scientific, Fisher Scientific, catalog number: PI80196)
21. Tris buffer (pH 7.4) (Affymetrix, catalog number: 22639)
22. Sodium chloride (NaCl) (Affymetrix, catalog number: 75888)
23. Triton X-100 (Sigma-Aldrich, catalog number: T9284)
24. Sodium orthovanadate (NaVO₄) (Sigma-Aldrich, catalog number: S6508)
25. Sodium fluoride (NaF) (Sigma-Aldrich, catalog number: S7920)
26. β-glycerophosphate (Sigma-Aldrich, catalog number: G9422)
27. Sodium pyrophosphate (Na₂H₂P₂O₇) (Sigma-Aldrich, catalog number: P8135)
28. HEPES (Sigma-Aldrich, catalog number: H3375)
29. CHAPS hydrate (Sigma-Aldrich, catalog number: C3023)
30. EDTA (Affymetrix, catalog number: 15694)
31. Glycerol (Affymetrix, catalog number: 16374)
32. 1,4-dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 10197777001)
33. 10x transfer buffer (AMRESCO, catalog number: 0307)
34. Cell lysis buffer (see Recipes)
35. Caspase-8 *in vitro* cleavage assay buffer (see Recipes)

Equipment

1. Tissue culture incubator (at 37 °C with 5% CO₂) (Thermo Fisher Scientific)
2. Biosafety cabinet
3. Table top centrifuge (Eppendorf)
4. Water bath (Benchmark)
5. Heating block (at 95 °C) (Benchmark)
6. SDS-PAGE and transfer apparatus (Bio-Rad Laboratories, model: Mini PROTEAN-II Cell)
Note: This equipment is no longer available at manufacturer.
7. Rocker (Benchmark)
8. Rotator (Labnet)
9. Refrigerator
10. Autoradiography film processor (Kodak)

11. Spectrophotometer
12. Vortex (Thermo Fisher Scientific)

Software

1. ImageJ (freely available from National Institutes of Health, <https://imagej.nih.gov/ij/>)

Procedure

A. Proteolysis of IRF3 in virus-infected cells (Figure 2B)

1. For virus-induced IRF3 cleavage, the HT1080 (expressing endogenous IRF3) or 1080.10 (expressing higher levels of human IRF3) cells are used.
2. Seed 500,000 cells/well (in 6-well plates) in complete DMEM for virus infection ([Sendai virus, multiplicity of infection, moi: 10] or [Adenovirus, moi: 10]) or in antibiotic-free DMEM for poly (I:C) transfection as described below. After 24 h, use these cells for induction of proteolysis of IRF3 by virus infection or poly (I:C) transfection as described below.
3. Induction of IRF3 proteolysis
 - a. Virus infection: Wash the cells in serum-free DMEM and infect with SeV (moi: 10) in 2% serum containing DMEM for 1 h, with occasional gentle shaking. At the end of infection, wash the cells with PBS and place in 10% serum containing DMEM. For Ad5 infection (moi: 10), use the above infection protocol in serum-free DMEM.
 - b. Poly (I:C) transfection: Transfect with poly (I:C) (2 μ g/ml) using Lipofectamine 2000 (at 1:2 ratio of poly [I:C] and Lipofectamine 2000 in opti-MEM).
4. At the desired time post infection (0-12 h for SeV infection, 0-96 h for Ad5 infection and 8 h for poly (I:C) transfection (Sears *et al.*, 2011), harvest the cells in cold PBS and lyse in 30 μ l of lysis buffer by incubating on ice for 30 min with occasional vortexing. Centrifuge at 12,000 \times g for 15 min and collect the soluble cell lysates.
5. Measure protein concentrations by adding 1 μ l of the cell lysates in 1 ml of pre-diluted (1:5) Bio-Rad protein assay dye and measuring the absorbance at 595 nm after 5 min of incubation at room temperature.
6. Analyze equal amounts (20 μ g) of protein from the cell lysates by SDS-PAGE followed by Western blot with anti-Flag antibody to detect the full length and cleaved forms of IRF3.

B. Involvement of caspase-8 in proteolysis of IRF3 (Figure 2C)

1. Seed 500,000 P2.1/IRF3 cells in 6-well plate in complete DMEM and for poly (I:C) transfection, seed the cells in antibiotic-free DMEM (in 10% FBS).
2. After 24 h, pre-treat the cells with various inhibitors of caspases (z-VAD: pan caspase inhibitor, z-WEHD: caspase-1 inhibitor and z-IETD: caspase-8 inhibitor) at 10 μ M for 1 h.
3. Induce proteolysis of IRF3 by adding poly (I:C) to the culture medium (100 μ g/ml, TLR3

activation, T in Figure 2C) or transfection of poly (I:C) using Lipofectamine 2000 (2 μ g/ml or poly [I:C], RLR activation, R in Figure 2C).

4. Harvest cells at 8 h post poly (I:C) treatment in cold PBS and lyse in 30 μ l of lysis buffer by incubating on ice for 30 min, with occasional vortexing. Centrifuge at 12,000 $\times g$ for 15 min and collect the soluble cell lysates.
5. Measure protein concentrations by adding 1 μ l of the cell lysates in 1 ml of pre-diluted (1:5) Bio-Rad protein assay dye and measuring the absorbance at 595 nm after 5 min of incubation at room temperature.
6. Analyze equal amounts (20 μ g) of protein from cell lysates by SDS-PAGE followed by Western blot with anti-Flag antibody (at 1:2,500) to detect the full length and cleaved forms of IRF3.

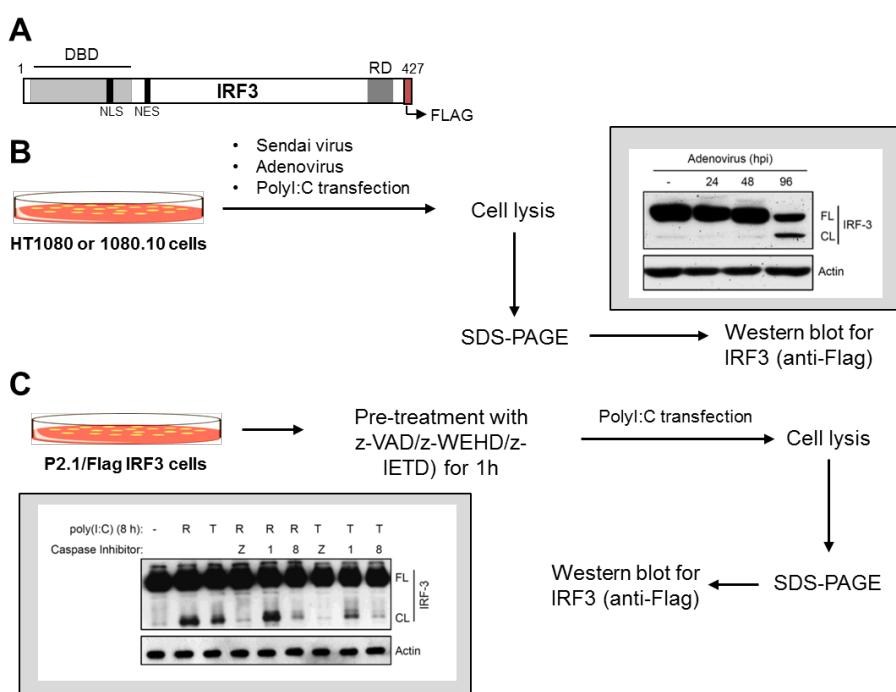


Figure 2. Virus-induced proteolysis of IRF3 and caspase-8 involvement. A. C-terminally Flag-tagged human IRF3 and its functional domains, DBD, DNA binding domain, NLS, nuclear localization signal, NES, nuclear export signal, RD, regulatory domain. B. Flow chart of virus infection and dsRNA (poly [I:C])-induced proteolysis of IRF3 (inset shows a representative result of virus-induced IRF3 cleavage). C. Flow chart of proteolysis of IRF3 in the absence or the presence of various caspase inhibitors (as indicated) (inset shows a representative result).

C. *In vitro* cleavage of IRF3 by caspase-8 (Figure 3)

1. Purification of Flag-IRF3

- Seed P2.1/IRF3 cells (10^6 cells/plate) in complete DMEM in twenty-five 10 cm tissue culture plates. When the plates are confluent, harvest the cells in cold PBS, centrifuge at 9,000 $\times g$ to collect the cell pellet. Lyse the cells in 2 ml of lysis buffer, incubate on ice for 30 min and

centrifuge at 12,000 $\times g$ to clear the lysate. Collect the supernatants as cell lysate to purify IRF3.

- b. Incubate the cell lysate with anti-Flag-agarose beads (100 μ l bead volume) for 16 h at 4 °C by placing on a rotator.
- c. Centrifuge at 2,000 $\times g$ for 1 min, discard the supernatant and wash the beads three times with 1 ml of cell lysis buffer.
- d. Elute the bound IRF3 from the beads using Flag peptide (at a final concentration of 25 μ g/ml) by placing the washed beads in elution buffer (Flag peptide in the cell lysis buffer) on a rotator. Confirm by Western blot that the eluted proteins contain the full-length IRF3 protein by using anti-Flag and anti-IRF3 antibodies.
- e. Use the eluted IRF3 as a substrate for *in vitro* proteolysis.

2. Incubate the purified Flag-IRF3 with various amounts (0.01-1 units) of recombinant active caspase-8 in the cleavage assay buffer at 37 °C for increasing lengths of time (0-3 h) in 50 μ l of total reaction volume in 1.5 ml Eppendorf tubes.
3. Terminate the reaction by adding SDS-PAGE loading buffer.
4. Analyze equal protein amount from the reaction mixture by SDS-PAGE followed by Western blot using anti-Flag antibody.
5. See Figure 3 for a representative result of *in vitro* IRF3 cleavage.

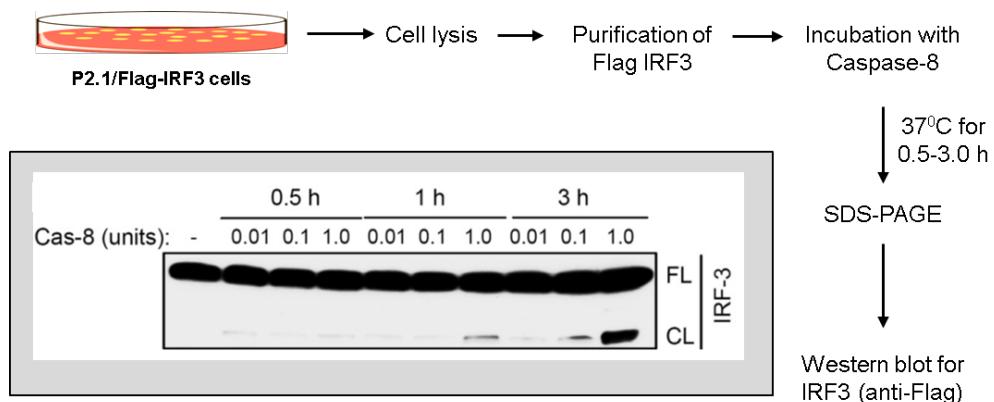


Figure 3. *In vitro* proteolysis of IRF3 by recombinant caspase-8. Flow chart of *in vitro* proteolysis of IRF3 by recombinant caspase-8 (inset shows a representative result of caspase-8-induced *in vitro* cleavage of IRF3).

D. SDS-PAGE and Western blot

SDS-PAGE and Western blot procedures were followed from the standard protocols described elsewhere (Towbin *et al.*, 1979; Renart *et al.*, 1979). Briefly, follow the steps below:

1. Load equal amounts of protein from the cell lysates or *in vitro* reactions on 10% SDS gels and electrophoresis at 100 V for 2 h to separate the proteins.
2. At the end of the electrophoresis, transfer the separated proteins to the PVDF membranes using a Bio-Rad wet transfer apparatus at 300 mA for 1 h.

3. Block the transferred proteins on the membranes in TBS-T containing 5% nonfat dry milk (blocking buffer) for 1 h at room temperature on a rocker.
4. Incubate the membranes with primary antibodies diluted (as described in the procedures) in the blocking buffer for 16 h at 4 °C on a rocker.
5. Wash the membranes three times with TBS-T and incubate with the HRP-conjugated secondary antibodies, diluted in blocking buffer, for 1 h at room temperature on a rocker.
6. Wash the membranes with TBS-T for three times and incubate in ECL2 reagents and develop on autoradiography films using automated the film processor.

E. Quantification of IRF3 cleavage

Use the autoradiograph of the Western blots to analyze IRF3 cleavage by ImageJ computer program. Using ImageJ, measure the band intensities of the full length (higher molecular weight band, ~55 kDa) and the cleaved (lower molecular weight band, ~45 kDa) forms of IRF3. Percentage cleavage of IRF3 is calculated as:

$$\text{Percent Cleavage} = \frac{\text{Amount of Cleaved IRF3}}{\text{Total IRF3}} \times 100$$

Percent cleavage of IRF3 is presented as a representative of multiple independent experiments. The quantitative estimation of IRF3 cleavage has been reported previously (Sears *et al.*, 2011).

Notes

Special care should be taken to separate the cleaved (~45 kDa), and the full length (~55 kDa), forms of IRF3, by electrophoresing the SDS gel (10%) at 100 V for longer time (when the protein ladders indicate clear separation of the desired sizes).

Recipes

1. Cell lysis buffer
 - 50 mM Tris buffer, pH 7.4
 - 150 mM NaCl
 - 0.1% Triton X-100
 - 1 mM sodium orthovanadate
 - 10 mM sodium fluoride
 - 10 mM glycerophosphate
 - 5 mM sodium pyrophosphate
2. Caspase-8 *in vitro* cleavage assay buffer
 - 50 mM HEPES
 - 50 mM NaCl
 - 0.1% CHAPS
 - 10 mM EDTA

- 5% glycerol
- 10 mM DTT
- 3. Poly (I:C) preparation
 - Re-suspend poly (I:C) in PBS (at a final concentration of 1 mg/ml)
 - Pass through a 26-gauge needle several times to shear

Acknowledgments

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