Characterisation of viral proteins that inhibit TLR signal transduction

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Abstract

Toll-like receptor (TLR) signalling involves five TIR adapter proteins, which couple to downstream protein kinases that ultimately lead to the activation of transcription factors such as nuclear factor-κB (NFκB) and members of the interferon-regulatory factor (IRF) family. TLRs play a crucial role in host defence against invading microorganisms, and highlighting their importance in the immune system is the fact that TLRs are targeted by viral immune evasion strategies. Identifying the target host proteins of such viral inhibitors is very important because valuable insights into how host cells respond to infection may be obtained. Also, viral proteins may have potential as therapeutic agents. Luciferase reporter gene assays are a very useful tool for the analysis of TLR signalling pathways, as the effect of a putative viral inhibitor on a large amount of signals can be examined in one experiment. A basic reporter gene assay involves the transfection of cells with a luciferase reporter gene, along with an activating expression plasmid, with or without a plasmid expressing a viral inhibitor. Induction of a signalling pathway leads to luciferase protein expression which is measured using a luminometer. Results from these assays can be informative for deciding which host proteins to test for interactions with a viral inhibitor. Successful assays for measuring protein-protein interactions include co-immunoprecipitations (Co-IPs) and GST-pulldowns. Co-IP experiments involve precipitating a protein out of a cell lysate using a specific antibody bound to protein A/G sepharose. Additional molecules complexed to that protein are captured as well and can be detected by western blot analysis. GST pulldown experiments are similar in principle to Co-IPs, but a bait GST-fusion protein complexed to glutathione sepharose beads is used to pull down interaction partners instead of an antibody. Again, complexes recovered from the beads are analysed by western blotting.

1. Introduction

Accumulating evidence shows that many TLRs are involved in the detection of viruses, and viral proteins and nucleic acids have been shown to activate anti-viral signalling pathways via TLRs, leading to cytokine and interferon induction (reviewed in 1). Important examples of such anti-viral signalling pathways include activation of MAP kinases such as ERK, p38 and JNK, and of transcription factors such as nuclear factor κB (NFκB) and interferon regulatory factors (IRFs). Highlighting the importance of TLRs in anti-viral immunity, a number of proteins which viruses employ against TLR signalling have been identified, and these represent anti-immunity viral subversion and evasion strategies (1). For example, the Toll-IL-1 Receptor (TIR) domain-containing signalling adapter molecule, TRIF, is a target of the hepatitis C virus protease, NS3/4A. The NS3/4A serine protease causes specific proteolysis of TRIF, reducing its abundance and inhibiting dsRNA signalling through the TLR3-TRIF pathway to IRF3 and NFκB (2). NS3/4A can also target IPS-1 (3), thus HCV can disable both the TLR3 and RIG-I detection pathways with a single protein. This potentially limits the expression of multiple host defence genes, thereby promoting persistent infections with this medically important virus (2). Likewise, vaccinia virus (VACV) encodes a number of proteins which interfere with TLR signalling, for example A46 and A52 (4). In fact A46 was the first viral protein identified to inhibit TLR signalling. It was initially identified based on the fact that it contained a signature TIR domain. Upon infection of cells by VACV, A46 is expressed by the virus and can interact with the TIR signalling adaptor molecules Mal, MyD88, TRAM and TRIF, via its TIR domain (5). This allows A46 to block multiple signalling pathways emanating from TLR complexes (5). A46 can also inhibit IL-1 signalling, since IL-1 also utilises MyD88. Further, deletion of the *A46R* gene led to an attenuated phenotype in a murine intranasal model of infection (5). Thus, targeting TLR signalling pathways in infected cells confers an advantage on VACV *in vivo*.

As more viral inhibitors of TLR signalling are being identified (for example by bioinformatics or by using functional screens with cloned viral genes), it is important to be able to characterise their mechanism of action. There are a number of reasons for this. Studying the effects of viral proteins on TLR signalling will generate important insights into how host cells respond to infection. Novel signalling proteins (targeted by the virus)

may be identified. Viral proteins can be used as tools to probe the signalling pathways and examine the activities of host proteins. Further, viral proteins, or derivatives from them, may have potential as therapeutic agents. For example, a peptide derived from A52 was found to inhibit cytokine secretion in response to TLR-dependent signalling and to reduce bacterial-induced inflammation *in vivo* (6, 7). This peptide may have application in the treatment of chronic inflammation initiated by bacterial or viral infections.

Using A46 as an example, this chapter describes assays that can be used to analyse the effects of a cloned viral gene on TLR signalling pathways, and to identify the TLR signalling proteins which the viral inhibitor targets. A46, termed SalF9R in the Western Reserve strain of VACV (8), was cloned by PCR amplification from WR DNA and ligated into the multiple cloning site of the mammalian expression vector pRK5 (4). Firstly, cloned A46 is transfected into mammalian cells in order to measure its ability to modulate IL-1 and TLR signalling pathways by reporter gene assays. These reporter assays are mostly carried out in human embryonic kidney (HEK) 293 cells. However, a murine macrophage cell line, RAW264.7 cells, is also used. These cells express most TLRs (9) and are very useful for testing the effect of putative inhibitors on a wide variety of TLR agonists in a single experiment. A basic reporter gene assay involves the transfection of cells with a luciferase reporter gene (e.g. NFκB), a thymidine kinase (TK) renilla luciferase control plasmid along with other required activating expression plasmids (e.g. CD4-TLR4), with or without a plasmid expressing A46. Induction of a signalling pathway (i.e. NFκB activation induced by the transfection and expression of CD4-TLR4) leads to the expression of the firefly luciferase protein due to transcription of the reporter gene. Luciferase is then assayed by using a luminometer.

The Stratagene PathDetect System™ is used for MAP kinase assays. Each PathDetect *trans*-reporting system includes a unique fusion *trans*-activator plasmid that expresses a fusion protein. The fusion *trans*-activator protein consists of the activation domain of either the c-Jun, Elk1 or CHOP transcription activator fused with the DNA binding domain of the yeast Gal4 (residues 1-147). The transcription activators c-Jun, Elk1 and CHOP are phosphorylated and activated by c-Jun N-terminal kinase (JNK), ERK1/2 or p38 kinase, respectively, and their activity reflects the *in vivo* activation of these kinases and the corresponding signal transduction pathways. The pFR-luciferase

reporter plasmid contains a synthetic reporter with five tandem repeats of the yeast Gal4 binding sites that control expression of the firefly luciferase gene. The DNA binding domain (DBD) of the fusion *trans*-activator protein binds to the reporter plasmid at the Gal4 binding sites. Phosphorylation of the transcription activation domain of the fusion *trans*-activator protein will activate transcription of the luciferase gene from the reporter plasmid. Similar to the NFκB reporter assays, the amount of luciferase expressed reflects the activation status of the signalling events.

Trans-reporting systems for measuring IRF3, IRF5, IRF7 and p65 activation have also been developed (10, 11, 12). Similar to the Stratagene PathDetect System™, IRF3, IRF5, IRF7 and p65 fusion *trans*-activator proteins are used in conjunction with the pFR-luciferase reporter plasmid. The activity of upstream signalling events which lead to the phosphorylation of the transcription activation domains can be quantified by measuring the level of luciferase gene transcription from the reporter plasmid. In this way, a wide variety of IL-1R and TLR signals can be tested using a single end-point (luciferase expression), both by overexpressing signalling molecules and by stimulation of cells. Results from these assays will then be informative for deciding which host proteins to test for interaction with the viral inhibitor. For example, the observation that A46 blocked all IL-1 (NFκB and MAP kinase) and TLR4 signals (NFκB, MAPK and IRF3) tested suggested it was acting close to the receptor complex (5). Co-immunoprecipitation (Co-IP) and GST-pulldown experiments then demonstrated an interaction between A46 and Mal, MyD88, TLR4, TRAM and TRIF (5).

Immunoprecipitation is the technique of precipitating an antigen out of solution using an antibody specific to that antigen. Co-IP experiments are a standard method to assess protein-protein interaction. By precipitating one protein believed to be in a complex, additional members of the complex are captured as well. The protein complexes, once bound to the specific antibody, are removed from the whole cell lysate by capture with an antibody-binding protein attached to a solid support such as an agarose bead. These antibody-binding proteins (Protein A, Protein G) were initially isolated from bacteria and recognise a wide variety of antibodies. Following the initial capture of a protein or protein complex, the solid support is washed several times to

remove any proteins not specifically and tightly bound through the antibody. After washing, the precipitated protein(s) are eluted and analysed by western blotting.

GST pulldown experiments are another common method of examining interactions between a probe protein and a putative interaction partner. The probe protein is a GST fusion, whose coding sequence is cloned into an isopropyl-β-D-thiogalactoside (IPTG)-inducible expression vector. This protein is expressed in bacteria and purified by affinity chromatography on glutathione-sepharose beads. Cell lysates expressing the target protein and the GST fusion protein conjugated to the glutathione-sepharose beads are incubated together and then washed to eliminate non-specific pulldown. Complexes recovered from the beads are analysed by western blotting.

2. Materials

2.1. Cell culture

1. Human embryonic kidney (HEK) 293T cells
2. RAW 264.7 cells, a murine macrophage cell line
3. Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS and 2 mM glutamine with 50 μg/ml Gentamycin.
4. Trypsin-EDTA solution: 0.5 g/ml Trypsin, 0.2 g/ml EDTA.
5. T75 cell culture flasks.
6. Sterile cell culture 10 cm dishes, 6 well plates and 96 well plates

2.2. Transfection

1. Expression plasmids (*See* **Section 2.3**)
2. Sterile water
7. Serum-free DMEM
8. GeneJuice™ transfection reagent (Merck), a proprietary nontoxic formulation of a cellular protein and a small amount of a novel polyamine.
9. Sterile 1.5 ml eppendorfs
10. V-bottomed 96 well plates
11. Flat-bottomed 96 well plates
12. TLR ligands (*See* **Section 3.3.2**)

2.3. Expression Vectors

1. Empty vector, pcDNA3.1 (Invitrogen).
2. pRL-TK vector (Promega). This vector is used as an internal control reporter and can be used in combination with any experimental reporter vector. It provides low to moderate levels of *Renilla* luciferase expression in co-transfected mammalian cells. This protein functions as a genetic reporter immediately following translation.
3. NFκB-luciferase reporter gene construct (4).
4. IFN-β–luciferase reporter gene construct (13).
5. IL-8 promoter reporter gene luciferase construct (14).
6. RANTES promoter reporter gene luciferase construct (15).
7. ISRE-luciferase reporter gene construct (Stratagene).
8. PathDetect™ CHOP, Elk-1 and c-Jun *trans*-reporting system (pFA-CHOP, pFA-Elk-1, pFA-c-Jun, pFR-Luc, pFC-MEK3, pFC-MEKK and pFC-MEK1; Stratagene).
9. Gal 4-IRF3 (10).
10. Gal 4-IRF5 (11).
11. Gal4-IRF7 (10).
12. Gal4-p65 (12).
13. Flag tagged hTLR3 (11).
14. CD4-TLR4 (16).
15. HA-tagged full-length hMal (17).
16. AU1-tagged full-length hMyD88 (18).
17. Flag-tagged full-length hTRAM (10).
18. Flag- tagged full-length hTRIF (19).

19. Flag-tagged full-length hSARM (20).

2.4. Luciferase Assays

1. Luminometer capable of reading 96-well plates (Reporter™ microplate luminometer, Turner designs).
2. 5X Passive lysis buffer (Promega). Store at -20°C. Dilute to 1X using distilled water immediately prior to use.
3. Luciferase Assay Mix: 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 M EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, 530 mM ATP. 100-500 ml of this can be made at a time and aliquoted into smaller volumes (10-50 ml). Store in the dark at -20°C and thaw to room temperature before use.
4. 1 mg/ml Coelentrazine (Insight Bio) in 100% ethanol, stored at -20°C. Coelentrazine is the substrate for the Renilla enzyme.
5. White opaque 96 well plates.

2.5. Co-Immunoprecipitations

1. NP-40 Lysis buffer: 50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) NP-40 (IGEPAL) containing 10 µl/ml aprotinin, 1 mM PMSF and 1 mM sodium orthovanadate. PMSF is unstable in aqueous solutions and will need to be refreshed every 25 min during the lysis step.
2. 50% slurry of either Protein-A or Protein-G conjugated sepharose beads (Sigma; *See* **Note 1**).
3. 3X Laemmli sample buffer (For 10 ml): 3 ml glycerol, 0.6 g SDS, 30 mg bromophenol blue, 1.875 ml 1 M Tris-HCl pH 6.8, 5.125 ml dH_2O. Add 150 µl 1 M DTT per ml just before use. Prior to addition of DTT, 3X sample buffer can be stored at room temperature.

2.6. SDS –Polyacrylamide Gel Electrophoresis

1. Resolving Gel buffer: 1.5M Tris-HCl, pH 8.8.
2. Stacking gel buffer: 1 M Tris-HCl, pH 6.8.

3. 30% (v/v) acrylamide/bis solution (Protogel®; National Diagnostics). The unpolymerised solution is a neurotoxin, so gloves should be worn at all times.

4. N, N, N, N'-Tetramethyl-ethylenediamine (TEMED) (Sigma)

5. Ammonuim persulphate (APS). Make a 10% solution in water. For reliable results, it is best to make up this solution fresh each time as APS is quite unstable.

6. Isopropanol

7. Resolving Gel: 6 ml 30% (v/v) bisacrylamide mix, 3.75 ml 1.5 M Tris pH 8.8, 150 µl 10% (w/v) SDS, 150 µl 10% (w/v) APS, 15 µl TEMED made up to 15 ml with dH_2O. This is the volume required to pour two 0.75 mm 12% mini gels, and can be altered as required.

8. Stacking Gel: 1 ml 30% bisacrylamide mix, 0.75 ml 1 M Tris-HCl pH 6.8, 60 µl 10% (w/v) SDS, 60 µl 10% (w/v) APS and 6 µl TEMED made up to 6 ml with dH_2O. This is enough to pour the stacking gel for two 0.75 mm mini gels

9. 10X Running buffer pH 8.3: 250 mM Tris, 1.92 M glycine, 10% (w/v) SDS. Dissolve 30 g Tris, 144 g glycine and 10 g SDS in 1 litre of distilled water. Check pH but do not alter due to the presence of SDS.

10. Prestained molecular weight marker. (e.g. NEB Broad Range molecular weight markers)

2.7. Western blotting

1. 10X Transfer buffer: 250 mM Tris, 1.92 M glycine. Dissolve 144 g of glycine and 30.3 g Tris in 1 litre of water. Store at room temperature. Prior to use dilute to 1X with the addition of water and add methanol to 20%. At this stage the buffer may be stored at 4°C.

2. Polyvinylidene fluoride (PVDF) membrane (Millipore).

3. Phosphate buffered saline (PBS) –Tween. For convienience prepare a 10X solution of PBS: 687 mM NaCl, 39 mM NaH_2PO_4, 226 mM Na_2HPO_4 . Dissolve 85 g NaCl, 4.68 g anhydrous NaH_2PO_4 and 32.2 g anhydrous Na_2HPO_4 in 1 litre of water. Prior to use dilute in water to 1X and add Tween 20 to 0.1% (v/v).

4. Blocking buffer: 5% (w/v) non fat dry milk in PBS-Tween.

5. Antibody diluent: 5% (w/v) non fat dry milk in PBS-Tween.

6. Primary antibody raised against viral protein of interest. For example, here we use anti-A46 rabbit polyclonal antibody raised against a purified, bacterial-expressed A46-GST fusion protein. Alternatively if the protein is epitope tagged, an antibody against the epitope can be used (e.g. Flag, Myc or HA).

7. Anti-rabbit IgG conjugated horse radish peroxidase secondary antibody (Sigma) for polyclonal primary antibodies or anti-mouse IgG conjugated horse radish peroxidase secondary antibody (Sigma) for monoclonal primary antibodies.

8. Enhanced chemiluminescent (ECL) substrate. Commercial (e.g. Pierce) or homemade ECL substrate can be used. Lyophilised Luminol and p-Coumaric acid can be purchased from Sigma and used to make your own ECL substrate. 250 mM Luminol stock: 0.44g luminol in 10 ml DMSO. Store at -20°C in 1 ml aliquots, wrapped in tinfoil. 90 mM p-Coumaric acid stock: 0.15g p-Coumaric acid in 10 ml DMSO. Store at -20°C in 0.44 ml aliquots, wrapped in tinfoil. Solution A: 1 ml Luminol stock; 0.44 ml p-Coumaric acid; 10 ml 1 M Tris-HCl pH 8.5; make up to 100 mls with dH_2O; store wrapped in tinfoil at 4°C. Solution B: 10 ml 1 M Tris-HCl pH 8.5; make up to 100 mls with dH_2O; store wrapped in tinfoil at 4°C. 30% H_2O_2 solution is also required.

9. Stripping buffer: We use Re-Blot Plus Strong 10X solution (Chemicon). Dilute to 1X with water (*See* **Note 2**).

2.8. GST Pulldown Assays

1. *E. coli* BL21 competent cells transformed with either pGEX or pGEX-A46.

2. Luria-Bertani (LB) broth: to 900 ml dH_2O add: 10 g Tryptone, 5 g Yeast Extract and 5 g NaCl. Shake until the solutes dissolve. Make up to 1 litre with dH_2O. Sterilise by autoclaving.

3. Terrific Broth (TB): to 900 ml dH_2O add 12 g Tryptone, 24 g Yeast Extract and 4 ml glycerol. Shake until the solutes dissolve. In 90 ml dH_2O dissolve: 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4. Adjust the volume to 100 ml with dH_2O. Sterilise both solutions by autoclaving. Allow to cool and then mix the two together.

4. Ampicillin. Make 100 mg/ml stock in dH_2O, filter sterilise and aliquot in the hood. Store at -20°C . Use at 100 µg/ml, i.e. 1/1000 dilution.

5. NETN extraction buffer: 300 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 8.0, 0.5% NP-40 (v/v) plus 1 µl/ml leupeptin (stock @ 10 mg/ml), 5 µl/ml aprotinin, 10 µl/ml PMSF (100 mM stock) and 1 mg/ml lysozyme.

6. Glutathione sepharose (GSH) beads (Sigma; *See* **Note 1**).

7. Coomassie blue stain: 50% (v/v) methanol, 10% (v/v) glacial acetic acid, 2.5 g/L Coomassie blue dye. This can be reused many times, until the blue colour has faded and does not stain as efficiently or the solution gets a bit dirty.

8. Destain solution: 50% (v/v) methanol, 10% (v/v) glacial acetic acid. This can be regenerated by charcoal treatment. This is done by adding 10g charcoal (Sigma) to 1 litre of used destain solution. Shake the bottle and then filter using a funnel and filter paper. The filtrate can be used again immediately.

3. Methods:

3.1. Growth and maintenance of HEK 293T and RAW 264.7 cell lines

1. The HEK 293T and RAW 264.7 cell lines are cultured in DMEM and maintained at 37°C in a humidified atmosphere of 5% CO_2. For continuing cell culture, cells are seeded at 1 x 10^5 cells/ml (15 ml per T75 flask) and sub-cultured two or three times a week when cells were at 50-80% confluency.

2. HEK 293T cells are removed from the surface of the flask by incubation with 5 ml of trypsin-EDTA (0.05 mg/ml) for 1 minute. Complete medium (10 ml) is then added to the cells. The contents of the flask are then transferred to a 30 ml tube and centrifuged at 110 x g for 5 min.

3. RAW 264.7 cells are removed from the surface of the flask using a cell scraper. The entire contents of the flask are then transferred to a 30 ml tube and centrifuged at 110 x g for 5 min.

4. The supernatant is removed and the pellet resuspended in 1 ml of complete medium. Cells are counted using a haemocytometer and a bright light microscope.

3.2. Transient Transfection using GeneJuice

This is the basic protocol on which the transfections for co-immunoprecipitation and GST-pulldown experiments are based. Sections 3.4.2 and 3.6.2 contain details of how much of particular plasmids are to be used in these experiments.

1. For 96 well plate transfections, cells are seeded at 2 x 10^4 cells per well (HEK 293) or 4 x 10^4 cells per well (RAW 264.7) and grown overnight (*See* **Note 3**).
2. The plasmids, serum free medium and GeneJuice mixes are made up in sufficient quantities for 3.5 treatments, enough therefore to perform the experiment in triplicate.
3. Cells are transfected with 230 ng (HEK 293) or 200 ng (RAW 264.7) DNA per transfection (*See* **Note 3**). Reporter plasmids such as the NFκB-Luciferase reporter or the IFNβ–Luciferase reporter, TK renilla and plasmids expressing genes of interest such as A46, TLRs, the TIR adapters or other signalling molecules are mixed together in a V bottomed 96 well plate according to the experimental design. In all cases, the amount of DNA used per transfection is kept constant using the appropriate amount of relevant empty vector control, pcDNA3.1.
4. The appropriate amount of GeneJuice (0.8 μl per transfection for HEK 293 cells or 0.5 μl per transfection for RAW 264.7 cells) is mixed with either 9.2 μl (HEK 293) or 9.5 μl (RAW 264.7) serum-free DMEM per transfection and incubated at room temperature for 5 min (*See* **Note 3**).
5. 35 μl of this mixture is added to the triplicate amounts of DNA and incubated for 15 min at room temperature. 10 μl per well is then added to the cells, which are allowed to recover for 16 h at 37°C prior to stimulation.
6. For transfection of 10 cm plates, 8-12 μg DNA is used in combination with 15 μl GeneJuice and 235 μl serum-free DMEM.
7. All experiments are harvested at least 24 h after transfection.

3.3. Luciferase Gene Reporter Assay

3.3.1. Reporter Gene Assays

- For the NFκB and ISRE reporter gene assays, 60 ng of either κB-luciferase or ISRE-luciferase reporter genes are used.

- The Stratagene PathDetect System™ is used for MAP kinase reporter assays. CHOP (0.25 ng), c-jun (0.25 ng) or Elk1 (5 ng) Gal4 fusion vectors are used in combination with 60 ng pFR-luciferase reporter to measure p38, JNK and ERK1/2 activation respectively.
- For the p65, IRF3 and IRF7 transactivation assays, 1 ng p65-Gal4, 3 ng IRF3-Gal4 or 3 ng IRF7-Gal4 fusion vectors are used in combination with 60 ng pFR-luciferase reporter.
- The IL-8, IFN-β and RANTES assays are carried out using 60 ng of the IL-8, IFN-β or RANTES promoter luciferase reporter genes.
- In all assays, 20 ng of *Renilla*-luciferase internal control (Promega) is used.
- 50 ng CD4-TLR4-, 25 ng Mal-, 25 ng MyD88-, 25 ng TRAM- or 25 ng TRIF-expressing vectors are used to drive a wide variety of signals in reporter gene assays.
- 0.5 ng TLR3-expressing vector, in conjunction with stimulation of cells with poly(I:C), is used to examine TLR3 signals in reporter gene assays.
- 25 ng, 50 ng or 100 ng A46- and 100 ng SARM-expressing vectors are used in many different reporter gene assays to examine the effect of inhibitor proteins on TLR signalling pathways.

3.3.2. Stimulation of cells

HEK293s are responsive to IL-1, TNF-α and Phorbyl 12-myristate 13-acetate (PMA; Sigma). RAW 264.7 cells express all murine TLRs, and thus respond to Pam3Cys (Invivogen), MALP2 (Alexis Corporation), poly(I:C) (Amersham Biosciences), LPS (Alexis Corporation), Flagellin, R-848 and phosphothioate CpG DNA (Sigma), as well as IL-1, TNF-α and PMA.

- Cells are stimulated 18 h post transfection with the required TLR ligands according to your experimental plan.
- The concentrations of ligands used are as follows: 100 ng/ml IL-1, 100 ng/ml TNF-α, 125 nM MALP2, 5 mg/ml Pam3Cys, 1000 ng/ml LPS, 250 ng/ml Flagellin, 1 μM R-848, 5 mg/ml CpG or 10 ng/ml PMA.

3.3.3. Preparation of Cellular Lysates

1. Cells are transfected in 96 well plates as described in section **3.3**.
2. 6 h post-stimulation or 24 h post transfection (if no stimulation), medium is removed from HEK 293T cells and the cells are lysed for 15 min on a rocking platform at room temperature with 50 µl Passive Lysis Buffer.
3. The RAW 264.7 cell line, however, are centrifuged at 1,000 x g for 5 min before removal of the medium. Cells are then lysed as above.
4. 20 µl aliquots of these lysates are transferred into two opaque white plastic 96 well plates.

3.3.4. Measurement of Luciferase Activity

1. Firefly luciferase activity is assayed by the addition of 40 µl of luciferase assay mix to the 20 µl aliquots in one of the white 96 well plates.
2. Coelenterazine is diluted in PBS to give a 2 µg/ml final concentration. *Renilla* luciferase is assayed by the addition of 40 µl of this solution to the 20 µl aliquots in the other white 96 well plate.
3. Luminescence is read using either the Reporter™ (Turner designs) or Luminoskan™ (ThermoElectron Corp) microplate luminometer. Expression (or activity) levels of the Firefly luciferase gene reflect the activation status of the signalling events. Expression (or activity) levels of *Renilla* luciferase reflect the levels of transfection efficiency in the experiment.
4. Firefly luminescence readings are normalised for transfection efficiency by dividing the figures with the relevant *Renilla* values and are expressed as fold stimulation over unstimulated empty vector control.
5. The white opaque 96 well plates can be reused almost indefinitely if washed thoroughly after each use.

3.4. Immunoprecipitation and Immunoblotting

3.4.1. Antibody Precoupling

1. The relevant antibodies for immunoprecipitation are first precoupled to either protein A- (polyclonal antibodies and Flag monoclonal antibody) or protein G-sepharose (all other monoclonal antibodies). This is done by incubation with 30 µl of 50% protein A/G slurry per immunoprecipitation sample on a roller, overnight at 4^{o}C. This is done by placing the 50% protein A/G slurry/antibody mixture -containing eppendorfs in a 50 ml tube and then putting that tube on a roller mixer which is located in either a cold room or a cold cabinet. 2 µl of the A46 antibody was used for each immunoprecipitation sample. For all other antibodies, 2 µg of antibody per immunoprecipitation sample should be used.
2. Just before use, the beads are washed three times to remove uncoupled antibody. This is done by centrifuging the 50% protein A/G slurry/antibody mixture at 3,000 rpm for 3 min and removing the supernatant. 1 ml PBS is then pipetted into the eppendorf, and the tube is inverted a number of times to ensure thorough separation of beads. The slurry is again centrifuged at 3,000 rpm for 3 min and the supernatant removed. After the final wash, the beads are resuspended in the appropriate volume of PBS to ensure a 50% slurry once more.

3.4.2. Immunoprecipitation and Immunoblotting

1. HEK 293T cells are seeded into 10 cm dishes (1.5 x 10^6 cells per dish, in 15 ml medium) 24 h prior to transfection.
2. 4 µg of each construct is transfected using GeneJuice™ as previously described Where only one construct is expressed, the total amount of DNA (8 µg) is kept constant by supplementation with empty vector, pcDNA3.1 (*See* **Note 4**).
3. Cells are harvested 24 h post-transfection (*See* **Note 5**). The cells are washed with 1 ml room-temperature PBS to remove any medium and are then removed from the plate by scraping in 1 ml ice-cold PBS. The samples are centrifuged at 16,100 x g for 5 min and the supernatant discarded.

4. The pellet of cells is lysed in 850 µl NP-40 lysis buffer for 30 min (*See* **Note 5**). The samples are then centrifuged for 10 min at 16,100 x g.

5. 400 µl of lysate is added to the relevant precoupled antibodies and rolled overnight at 4^{o}C. This is done by placing the cell lysate-containing eppendorfs in a 50 ml tube and then putting that tube on a roller mixer which is located in either a cold room or a cold cabinet.

6. 25 µl 3X Laemlli sample buffer is added to the remaining 50 µl of lysate, which is then boiled for 4 min and frozen immediately.

7. The next day the immune complexes are washed three times with 1 ml lysis buffer. This is done by centrifuging the immune complexes at 3,000 rpm for 3 min and removing the supernatant. 1 ml NP-40 lysis buffer is then pipetted into the eppendorf, and the tube is inverted a number of times to ensure thorough separation of beads. The tubes are again centrifuged at 3,000 rpm for 3 min and the supernatant removed. After the final wash, all supernatant is removed (*See* **Note 6**) and beads are resuspended in 30 µl 3X Laemmli sample buffer. The samples are boiled for 4 min and either frozen immediately or analysed by SDS-PAGE and subsequent western blot.

8. 18 µl of the lysates are blotted for control purposes, 18 µl of the immune complex is immunoblotted for co-precipitating protein, and 10 µl is blotted for the protein directly recognised by the immunoprecipitating antibody (*See* **Note 4**).

3.5. Western Blot Analysis

3.5.1. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Samples are resolved on a sodium dodecylsulphate (SDS) polyacrylamide gel using a constant voltage of 150 V.

2. Samples are first run through a stacking gel to concentrate protein and then resolved according to size using 10-15% polyacrylamide gels (*See* **Note 7**).

3. Samples are run with pre-stained protein markers as molecular weight standards.

3.5.2. Transfer of Proteins to Membrane

1. The resolved proteins are transferred to Immobilon-P polyvinylidene diflouride (PVDF) membrane (Sigma) using the mini Trans-blot, a wet transfer system from Bio-Rad.

2. The PVDF membrane is cut to the required size (i.e. the size of the gel) and activated by placing in 100% methanol for a few seconds. It is then briefly rinsed in dH_2O.

3. All components (sponges, filter paper, PVDF membranes and gels) are soaked beforehand in transfer buffer.

4. The Western Blot "sandwich" is prepared as follows. A piece of sponge is placed on the black portion of the cassette. A layer of filter paper is laid on the sponge. The gel is placed on the filter paper and overlaid with the PVDF membrane. A second piece of filter paper is placed on the membrane, followed by a second sponge.

5. The cassette is placed in the transfer rig in the correct orientation (black-to-black and red-to-red), the chamber filled with transfer buffer and a constant voltage of 100 V was applied for 1 hour (*See* **Note 8**).

3.5.3. Antibody Blotting

1. Membranes are blocked for non-specific binding by incubation in blocking buffer at 4°C overnight or at room temperature for 1 hour.

2. The membrane is washed for 5 min in PBS-Tween.

3. The membrane is then incubated for 1 hour at room temperature or at 4°C overnight with the primary antibody of interest at 1:100 to 1:1000 dilutions depending on the antibody in question (*See* **Note 9**).

4. The membrane is washed five times for 5 min in PBS-Tween and incubated with the appropriate secondary horseradish peroxidase linked enzyme for 1 hour at room temperature.

5. Again the blots are washed five times. The blots are then washed in PBS without Tween for 2 min.

6. Blots are developed by enhanced chemiluminescence (ECL) using home-made ECL substrate. Just before use, equal volumes solutions A and B are mixed as required (1 ml of each per mini blot) and 0.61 µl 30% H_2O_2 per ml solution B is

added. The solution is mixed gently by pipetting and then added dropwise to the blots. The blots are incubated with the ECL substrate for a minute or so. The excess solution is removed before placing the blots in a cassette for developing in the darkroom.

7. If required, blots are stripped by shaking in 10 ml 1X Re-Blot Plus (Chemicon) for 15 min. The blots are then re-blocked and probed with the desired antibodies.

3.6. Glutathione-S-Transferase (GST) Fusion Protein Interaction Assay

3.6.1. Preparation of GST-Fusion Proteins

1. 20 ml LB broth (containing Ampicillin) are inoculated with a single colony of *E. coli* BL21 cells transformed with pGEX-A46R or pGEX and incubated overnight at 37°C, 200 cycles/minute.

2. The next day, the overnight cultures are diluted 1:50 (i.e. 10 ml into 500 ml broth) in Ampicillin-containing Terrific broth (TB) (pGEX-A46R) or LB broth (pGEX).

3. The cultures are incubated at 37°C, 200 cycles/minute for approximately 2 h or until $O.D._{600}$ of the culture is ≈ 0.5.

4. The cultures are then moved to a 30°C incubator and allowed to acclimate for 20 min.

5. IPTG is added to a final concentration of 70 μM and the cultures are incubated for a further 5 h (*See* **Note 10**).

6. The cultures are centrifuged using at 7,750 x g for 10 min, 4°C in 250 ml tubes. The supernatant is decanted and the pellets frozen until required at -70°C.

7. The pellets are left to thaw on ice and then resuspended in 15 ml NETN.

8. The resuspended pellets are transferred to 30 ml centrifuge tubes and each pellet is sonicated on ice with 20 second pulses followed by 10 second pauses to prevent over heating of the preparation, 12 times.

9. The suspensions are cleared of insoluble material by centrifugation 14,500 x g for 30 min, 4°C, and the supernatants pooled in a 50 ml tube.

10. 500 μl glutathione sepharose (GSH) beads (50% slurry), that had previously been washed three times in 1 ml NETN plus inhibitors but without lysozyme, are added and the mixture is incubated for 2 h at 4°C on a roller.

11. The mixture is then centrifuged at 1000 x g for 10 min at 4°C and the supernatant discarded.

12. 25 ml ice-cold NETN (plus inhibitors) is added to the beads, and the tube is rolled on a roller mixer for 5 min at 4°C before being centrifuged at 1000 x g for 5 min. This washing step is repeated a further four times (five washes in total).

13. After the final wash, the beads are resuspended in the appropriate volume of NETN (plus inhibitors) to make a 50% slurry, transferred to an eppendorf and stored overnight at 4°C.

14. The protein-containing slurry is then analysed by SDS-PAGE and Coomassie staining for the expression of the fusion protein (*See* **Note 11**). Resolved gels are soaked in Coomassie blue stain for at least 1 hour and then washed several times with destain solution until bands appear.

3.6.2. Affinity Purification of Complexes using GST-Fusion Proteins

1. HEK 293T cells are seeded into 10 cm dishes (1.5 x 10^6 cells) 24 h prior to transfection. For GST pulldown experiments, 8 μg of expression vector is transfected using GeneJuice as previously described. The cells are harvested 24 h post-transfection in an identical fashion as that described in Section **3.4.2**.

2. 25 μl 3X sample buffer is added to 50 μl of lysate, which was then boiled for 4 min and frozen immediately. 800 μl of lysate is added to 50 μl of a 50% slurry prewashed GST-fusion protein or GST alone as a control and incubated for 2 h on a roller at 4^oC as previously described.

3. The samples are washed three times with 1 ml NP-40 lysis buffer.

4. All supernatant is removed and beads are resuspended in 50 μl 3X sample buffer.

5. The samples are boiled for 4 min and analysed by Western blot.

4. Notes

1. Protein A, Protein G and glutathione sepharose beads arrive from their supplier in an ethanol suspension. Before use, swirl or agitate the container to resuspend the beads in their liquid. It is very useful to have stock of P200 tips with their pointed ends cut off for the purpose of handling the sepharose beads as the beads will block uncut tips. Remove an amount approximate or slightly more than you require and place in an eppendorf. Centrifuge the beads gently (1000 x g) for 1 minute and remove the supernantant. Resuspend the beads in 1 ml PBS and centrifuge again. Remove supernatant, thus removing the last of the ethanol. Using your own judgement, assess the volume of beads in the eppendorf and add the same volume of PBS. You now have a 50% slurry of Protein A or Protein G sepharose which is stable for storage at 4°C for a number of weeks.
2. 10 ml 1 x solution of Re-Blot Plus is sufficient for the stripping of one blot. If stored at 4°C, the solution can be reused a number of times (until the solution takes on a cloudy appearance).
3. Before attempting transfections in different cells to those used in these experiments, it is important to optimise cell numbers, GeneJuice volumes and DNA amounts. Too much GeneJuice or DNA can be toxic to the cells. However, too little of either can result in poor transfection efficiency. Different combinations of reporter plasmids may be worth trying in other cell lines also. Cell density is another factor to optimise which is critical to transfection efficiency.
4. For any interaction experiments, stringent controls are vital. It is very important to demonstrate that the interaction is real, and not just an artefact, for example due to non-specific antibody binding. For an experiment where you wish to see if protein X interacts with protein Y, the following experimental set-up is recommended. Transfect three 10 cm dishes as follows: X alone (4 µg), Y alone (4 µg), and X and Y together (4 µg of each). Lyse in 850 µl NP-40 lysis buffer as previously described. Incubate 400 µl of each lysate with Protein A/G sepharose beads conjugated to an antibody for X (i.e. IP: X). Incubate the other 400 µl of each lysate with Protein A/G sepharose beads conjugated to an antibody for Y (i.e. IP: Y). Then load two gels in the following manner and analyse by SDS-PAGE and subsequent Western Blot. Gel

1: 18 µl lysates 1-3, 18 µl IP: X 1-3, 10 µl IP: Y. Probe this blot with the antibody for Y. Gel 2: 18 µl lysates 1-3, 18 µl IP: Y 1-3, 10 µl IP: X. Probe this blot with the antibody for X. In each of these two gels, lanes 1,2 and 3 show expression of either X or Y in the appropriate samples and lanes 7,8 and 9 show specific pulldown of either X and Y by their relevant antibodies. Lane 6 in both gels is where you will see an interaction or not, and you should not see a band in lanes 4 or 5 if your antibody pulldowns are specific.

5. There are a number of variations that can be attempted when optimising your immunoprecipitation experiments. You can vary the length of time for which the cells are transfected from 24 to 48 or even 72 h. For membrane-bound proteins which can be difficult to extract, cells can be lysed for longer times such as 1 hour or perhaps more. Other lysis buffers such as RIPA or modified RIPA may also be tried. However, we would advise against using too stringent a lysis buffer as you may end up disrupting your interaction!

6. When removing the final supernatant from your immunoprecipitation samples, it is extremely convenient to use gel-loading tips to remove the final traces of liquid. These tips have finely pointed ends of over 3cm in length and are too finely bored to suck up the sepharose beads.

7. The heavy chain, or indeed the light chain, of the immunoprecipitating antibody can be a major annoyance when doing immunoprecipitation experiments if your protein of interest is of a similar or identical size to either of these proteins, as the chains appear as very strong bands on your resultant immunoblots. This “masking” of interesting bands can be circumvented in a number of ways. Firstly, try and separate your band from the antibody band as much as possible. In the case of the heavy chain, this can be achieved by slowly running your samples on a low percent gel (8%). If it is the light chain that is the problem, running your samples on a very high percent gel (15 or 18%) may help. Also, there are antibodies which recognise either the heavy or light antibody chain only, and these can be very useful. For example, anti-mouse IgG, Fab specific (Sigma) or anti-mouse IgG, Fc specific (Sigma). It is also possible to covalently bind your antibody to the sepharose beads using commercially available reagents.

8. Depending on the size of your protein of interest you may want to vary the blotting conditions. For large proteins of over 80 kDa, increasing your blotting time over the recommended 60 min may be useful. When blotting for very long time periods, it is important to prevent the apparatus from overheating. Replacing your cooling ice-block midway through the process or carrying out the procedure in the cold room are ways of maintaining optimal temperatures. For very small proteins, blotting for 30 min may mean greater retention of your protein on the membrane and better detection when developing your blots.

9. The basic factor that can be altered when optimising antibody conditions is primary antibody concentration. Optimal antibody dilutions can vary from 1/100 to 1/10,000. A good starting point is usually 1/1000. The concentration of secondary antibody is also very important. If too much secondary antibody is used, non-specific bands may mask your results. On the other hand, blank blots or very faint bands may result if too little secondary antibody is used. Other variables are the blocking agent (powdered milk or BSA), diluent (PBS-Tween or TBS-Tween), secondary antibody concentration and temperature of use.

10. It is a good idea to optimise your fusion protein expression conditions before attempting large scale protein purification for experiments. Simple changes to temperature (18°C, 25°C, 30°C or 37°C) and IPTG concentration (0.2 to 1mM) may make a huge difference to protein yield and quality.

11. When you have analysed your GST-fusion protein production by SDS-PAGE, it may not be as clean as you would have hoped i.e. there are a lot of other bands visible on the gel. By increasing your NaCl concentration in the NETN extraction buffer to 500 mM or even 750 mM and washing for longer, you will find that your extract is much purer. For future experiments, pre-clearing the bacterial lysates with sepharose beads that are not conjugated to glutathione may also improve matters.

5 References

1. Bowie, A.G. (2007). Translational mini-review series on Toll-like receptors: recent advances in understanding the role of Toll-like receptors in anti-viral immunity. *Clin Exp Immunol* **147**, 217-26.
2. Li, K., Foy, E., Ferreon, J.C., Nakamura, M., Ferreon, A.C., Ikeda, M., Ray, S.C., Gale, M. and Lemon S.M. (2005). Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor adapter protein TRIF. *Proc Natl Acad Sci U S A* **102**, 2992-7.
3. Li, X.-D., Sun, L., Seth, R.B., Pineda, G. and Chen, Z.J. From the cover: hepatitis C virus protease NS3/4Acleaves mitochondrial anti-viral sinalling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci U S A* **102**, 17717-22.
4. Bowie, A., Kiss-Toth, E., Symons, J. A., Smith, G. L., Dower, S. K. and O'Neill, L. A. (2000). A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. *Proc Natl Acad Sci U S A* **97**, 10162-7.
5. Stack, J., Haga, I. R., Schroder, M., Bartlett, N. W., Maloney, G., Reading, P. C., Fitzgerald, K. A., Smith, G. L. and Bowie, A. G. (2005). Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *J Exp Med* **201**, 1007-18.
6. McCoy, S. L., Kurtz, S. E., Macarthur, C. J., Trune, D. R. and Hefeneider, S. H. (2005). Identification of a peptide derived from vaccinia virus A52R protein that inhibits cytokine secretion in response to TLR-dependent signaling and reduces in vivo bacterial-induced inflammation. *J Immunol* **174**, 3006-14.
7. Tsung, A., McCoy, S.L., Klune, J.R., Geller, D.A., Billiar, T.R. and Hefeneider, S.H. (2007). A novel inhibitory peptide of Toll-like receptor signalling limits lipopolysaccharide-induced production of inflammatory mediators and enhances survival in mice. *Shock* **27**, 364-369.
8. Smith, G.L., Chan, Y.S. and Howard, S.T. (1991). *J. Gen. Virol.* **72**, 1349-1376.
9. Applequist, S. E., Wallin, R. P. and Ljunggren, H. G. (2002). Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int Immunol* **14**, 1065-74.

10. Fitzgerald, K. A., Rowe, D. C., Barnes, B. J., Caffrey, D. R., Visintin, A., Latz, E., Monks, B., Pitha, P. M. and Golenbock, D. T. (2003). LPS-TLR4 Signaling to IRF-3/7 and NF-KB Involves the Toll Adapters TRAM and TRIF. *J Exp Med* **198**, 1043-55.
11. Schoenemeyer, A., Barnes, B. J., Mancl, M. E., Latz, E., Goutagny, N., Pitha, P. M., Fitzgerald, K. A. and Golenbock, D. T. (2005). The interferon regulatory factor, IRF5, is a central mediator of TLR7 signaling. *J Biol Chem* **280**, 17005-17012.
12. Jefferies, C., Bowie, A., Brady, G., Cooke, E. L., Li, X. and O'Neill, L. A. (2001). Transactivation by the p65 subunit of NF-kappaB in response to interleukin-1 (IL-1) involves MyD88, IL-1 receptor-associated kinase 1, TRAF-6, and Rac1. *Mol Cell Biol* **21**, 4544-52.
13. Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S., Tanaka, N. and Taniguchi, T. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* **13**, 539-48.
14. Kiss-Toth, E., Guesdon, F.M., Wyllie, D.H., Qwarnstrom, E.E. and Dower, S.K. (2000). A novel mammalian expression screen exploiting green fluorescent protein-based transcription detection in single cells. *J Immunol Methods* **239**, 125-35.
15. Lin, R., Heylbroeck, C., Genin, P., Pitha, P.M. and Hiscott, J. (1999). Essential role of interferon regulatory factor 3 in direct activation of RANTES chemokine transcription. *Mol Cell Biol* **19**, 959-66.
16. Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-7.
17. Fitzgerald, K. A., Palsson-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A. and O'Neill, L. A. (2001). Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. *Nature* **413**, 78-83.

18. Muzio, M., Ni, J., Feng, P. and Dixit, V. M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612-5.
19. Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K. and Akira, S. (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* **171**, 4304-10.
20. Liberati, N. T., Fitzgerald, K. A., Kim, D. H., Feinbaum, R., Golenbock, D. T. and Ausubel, F. M. (2004). Requirement for a conserved Toll/interleukin-1 resistance domain protein in the Caenorhabditis elegans immune response. *Proc Natl Acad Sci U S A* **101**, 6593-8.