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***In Vitro* Signaling by MAPK and NF κ B Pathways Inhibited by *Yersinia* YopJ**

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Abstract

A procedure for an *in vitro* signaling assay is described for the MAPK and NF κ B pathways. The method uses a membrane-cleared lysate that contains all the soluble components required for activating these signaling cascades. The pathways can be activated by variety of molecules, including kinases, G-proteins, and E3 ligases. We demonstrate that YopJ inhibits downstream of all these activators. The *in vitro* signaling assay is ideal for initial biochemical studies on activators and inhibitors of the MAPK and NF κ B pathways.

1. Introduction

Yersinia pestis is the infectious agent that caused the Black Death in the Middle Ages, and *Y. pseudotuberculosis* and *Y. enterocolitica* (two closely related food-borne pathogens) are causal agents of gastrointestinal disorders (Cornelis, 2000). *Yersinia* spp. harbor a 70-kb plasmid that encodes a type III secretion system (TTSS) that secretes Yops (*Yersinia* outer proteins, also referred to as virulence factors or effectors) (Cornelis, 2002). The type III secretion system is a transport vehicle used by the microbial pathogen to deliver effectors from the bacterial cytosol to the target host cytosol (Viboud and Bliska, 2005). Upon translocation of the effectors into the target host cell, these molecules cripple the target cell by (1) blocking phagocytosis, (2) destroying the host defense system, and (3) inducing apoptosis (Orth, 2002). Each effector appears to mimic or capture the activity of a eukaryotic protein, which alters the signaling machinery in the target cell and gives an advantage to the pathogen during infection. Paradoxically, the effectors remain in a quiescent state inside the pathogen due to the lack of a substrate or an activator or possibly the presence of a chaperone (Feldman and Cornelis, 2003; Orth, 2002).

Early studies on one of the *Yersinia* effectors, YopJ, demonstrate that this 32-kDa effector inhibits cytokine production and induces apoptosis in a target cell during infection (reviewed by Orth, 2002). YopJ, with no obvious homology to any known eukaryotic protein domain, possesses the remarkable capacity to block multiple signaling pathways including the MAPK and the NF κ B pathways. Interruption of these signaling pathways disrupts expression of TNF α and IL-8 by the infected target cell (Monack *et al.*, 1997; Palmer *et al.*, 1998; Ruckdeschel *et al.*, 1998).

Later studies revealed that YopJ interacted specifically with the family of mitogen activated protein kinase kinases (MKKs), and did not interact with downstream kinases (MAPKs: ERK, JNK and p38) or with upstream kinases (B-Raf) (Orth *et al.*, 1999). Although the MAPK and NF κ B pathways are distinct, insights into how YopJ could inhibit both of these signaling systems are gained when the pathways are viewed in parallel as depicted in (Fig. 24.1) (Orth *et al.*, 1999). Herein, a representative MAPK pathway is shown that includes a cascade of three kinases (Raf, MKK, and MAPK) resulting in the phosphorylation of the MAPK. The NF κ B pathway is activated by a number of stimuli that converge on the activation of the I κ B kinase (IKK) complex. When the IKK complex is activated, I κ B from

the NF κ B:I κ B complex is phosphorylated, which provides a signal for its own ubiquitination and subsequent degradation. Removal of I κ B exposes the NLS on the NF κ B transcription factor, resulting in translocation of NF κ B to the nucleus where it activates transcription (see Fig. 24.1). Studies demonstrating that YopJ binds IKK β , the MKK equivalent in the NF κ B pathway, support the hypothesis that YopJ blocks the pathways at a similar point. In total, these observations identified a common critical point in the MAPK pathways and the NF κ B pathway that is targeted for disruption by a single *Yersinia* virulence factor, YopJ (Orth *et al.*, 1999).

Further studies revealed that YopJ contains a catalytic triad with a cysteine, histidine, and aspartate, similar to those found in hydrolases. Mutation of the catalytic cysteine results in a mutant of YopJ (C172A) that is unable to inhibit the signaling pathways. It was discovered that YopJ uses its catalytic site to acetylate serine and threonine residues on the activation loop of MKKs, thereby directly competing with the post-translational modification phosphorylation (Mukherjee *et al.*, 2006) (Fig. 24.2). The finding of this newly discovered post-translational modification on how YopJ inhibited these signaling pathways was made, in part, by development of the *in vitro* signaling assay described in this article.

2. The *in Vitro* Signaling Assay

The cell-free signaling system uses concentrated, membrane-free, soluble lysate, an ATP regenerating system, and various partially purified or purified proteins to activate the various signaling pathways. The readout for this assay is the production of phosphorylated proteins: for the MAPK pathways the readout is the phosphorylated MAPKs, including ERK, p38, and JNK; for the NF κ B pathway the readout is phosphorylated I κ B. Each of these phosphorylated proteins can be detected by specific antibodies in immunoblot analysis.

3. Materials and Methods

3.1. Materials

3.1.1. Plasmids and reagents—Expression constructs used in this study are pSFFV-YopJ-Flag and pSFFV-YopJ-Flag-C172A (Orth *et al.*, 1999), pGEX-TEV-YopJ (Mukherjee *et al.*, 2006), pRK7-Flag-NIK (kindly provided by Zhijian J. Chen), and pDCR-HA-RasV12 (kindly provided by Mike White).

Antibodies for phospho-ERK1/2, phospho-MKK, phospho-I κ B, phospho-IKK, and phospho-MKK6 and MKK1/2, are purchased from Cell Signaling; for IKK β and aldolase, Santa Cruz Biotechnology; for anti-HA antibody, Covance; and for anti-FLAG antibody, SIGMA.

Recombinant B-Raf is purchased from Upstate. EGF and Okadaic acid are purchased from Alexis; anti-Flag M2 resin from SIGMA; and protease inhibitor cocktail tablet from Roche.

3.1.2. Solutions

Phosphate buffered saline (PBS): 8 mM Na₂HPO₄, 1.1 mM NaH₂PO₄, 150 mM NaCl

HTX: 10 mM HEPES, pH 7.4, 0.5% Triton-X 100, 10 mM MgCl₂, 1 mM MnCl₂, 0.1 mM EGTA

HNT: 10 mM HEPES, pH 7.4, 50 mM NaCl, and 1% TritonX-100

10 $\times$ ATP Regenerating Solution (ARS): 10 mM ATP, 350 mM creatine phosphate, 20 mM Hepes, pH 7.2, 10 mM MgCl₂, 500 μ g/ml creatine kinase

5 $\times$ SDS-PAGE sample buffer

3.1.3. Cell culture—HEK293 cells are used in all experiments. The cells are cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% cosmic calf serum (Gemini) in 5% CO₂. Large-scale transfections are performed with cells cultured on 150-mm plates with 10 μ g of plasmid DNA using calciumphosphate-based transfection method (Russell, 2006). In brief, cells are transfected in the morning, media is changed 8 h after transfection, and then 24 h later, cells are harvested for NF κ B experiments, or cells are starved with serum-free media overnight and then harvested for MAPK experiments.

3.2. Methods

3.2.1. Membrane-cleared lysates—The protocol for lysate preparation is a modified version of a protocol developed by Sturgill and colleagues (Dent *et al.*, 1993).

1. One 150-mm plate of HEK293 cells, transfected with pSFFV and pSFFV-YopJ-Flag or pSFFV-YopJ-Flag-C172A as described above, is placed on ice and gently washed with 2.5 ml of cold PBS + 1 mM EDTA. After removing the cold wash, cells are popped loose from the bottom of the plate by tapping sharply on the side of the dish. Scraping off cells from the plate is avoided during harvesting to avoid shearing of cells. Loose cells are harvested by gentle washing of the plate with 5 ml of cold PBS + 1 mM EDTA, followed by centrifugation at 400 $\times$ *g* for 5 min at 4 $^{\circ}$.
2. Each cell pellet is gently resuspended in 1 ml of cold PBS and transferred into a 1.5-ml eppendorf tube. It is important to keep the volume of cells in each tube below ~250 μ l so that the cells are efficiently lysed after step 3.
3. Cells are recentrifuged at 400 $\times$ *g* for 5 min, and after removal of the liquid above the cell pellet, a volume of HTX lysis buffer (10 mM HEPES, pH 7.4, 0.5% Triton-X 100, 10 mM MgCl₂, 1 mM MnCl₂, 0.1 mM EGTA, and protease inhibitor cocktail tablet [Roche]) equal to the volume of the cell pellet is added to the eppendorf tube. The cells are gently resuspended and then incubated on ice for 20 to 60 min. Close to the end of the incubation period the cells are dounced with an eppendorf pestle.
4. The cell lysate is subjected to differential centrifugation. At first it is centrifuged for 5 min at 800 $\times$ *g* to separate the nuclear fraction. The cloudy liquid above the pellet is transferred to a prechilled eppendorf (if more than one 150-mm plate has been used to prepare a large lysate preparation, the lysates can be combined at this step into a single eppendorf). The lysate is next spun at 16,000 $\times$ *g* for 10 min to remove various cellular organelles such as lysosomes and mitochondria. To clear the lysate of membranes, the supernatant is transferred to a prechilled 1.5-ml polyallomer microfuge tube (Beckman) for centrifugation at 100,000 $\times$ *g* for 30 to 60 min in a table-top Beckman ultracentrifuge (TL-100).
5. In the final step, the supernatant is transferred to a prechilled 1.5-ml eppendorf tube. The protein concentration of the lysate is measured using Coomassie Plus Protein Assay reagent (Pierce). Protein concentration should be ~10 mg/ml. For long-term storage, the lysate is snap-frozen on dry ice and transferred to a -80 $^{\circ}$ freezer.

Stability of cell lysates can be sensitive to freeze/thaw. Background levels of phosphorylated MAP kinases may increase with samples that are freeze/thawed multiple times. Based on our experience with this assay, the NF κ B signaling pathway is less sensitive to freeze/thaw storage.

3.2.2. Isolation of EGF- and RasV12-activated membranes—The preparation of stimulated membranes is essentially the same as the preparation of lysate with the following exceptions (Dent *et al.*, 1993):

1. For RasV12-activated membranes, one 150-mm plate of HEK293 cells is transfected with 10 μ g of pDCR-HA-RasV12 plasmid DNA using calcium-phosphate-based transfection method (Russell, 2006) in the morning, media is changed 8 h after transfection, and then 24 h later cells are harvested after overnight starvation with serum-free media.

For EGF-stimulated membranes, HEK293 cells are plated at 80% confluency and are starved with serum-free media overnight. Cells are then incubated with 50 ng/ml EGF (2 ml/150-mm plate) for 5 min at room temperature.

For both RasV12- and EGF-treated cells, one 150-mm plate of transfected HEK293 cells is placed on ice and gently washed with 2.5 ml of cold PBS + 1 mM EDTA. After removing the cold wash, the cells are popped loose from the bottom of the plate by tapping sharply on the side of the dish. Scraping off cells from the plate is avoided during harvesting to avoid shearing of cells. Loose cells are harvested by gentle washing of the plate with 5 ml of cold PBS + 1 mM EDTA, followed by centrifugation at 400 $\times$ *g* for 5 min at 4°.

2. Follow steps 2 to 4 in section 3.2.1.
3. The supernatant after the 100,000 $\times$ *g* spin is discarded and the membrane pellet is resuspended in 100 μ l of HTX buffer, forming a crude suspension of membranes containing activated signaling components. Protein concentrations can be estimated using the Coomassie Plus protein assay reagent (Pierce).

3.2.3. Purification of recombinant TRAF6—pFast-Bac-T6RZC virus (kindly provided by Zhijian J. Chen and Junichiro Inoue) was used to purify the recombinant TRAF6 protein from Sf9 cells using Ni²⁺ affinity purification (Qiagen). Infected cells were lysed using Emulsiflex C-5 cell homogenizer (Avastin) in lysis buffer containing 20 mM Tris (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 10 mM NaF, 40 mM EGTA, 0.05% β -mercaptoethanol, and a protease inhibitor cocktail tablet (Roche). Cell lysate was allowed to bind to Ni-NTA resin for 1 h at 4°, and then eluted from beads using 250 mM imidazole-containing buffer.

3.2.4. Isolation of activated FLAG-NIK beads

1. One 100-mm plate of HEK293 cells, at 60 to 70% confluency, is transfected with 3.5 μ g PRK7-FLAG-NIK using Fugene transfection reagent (Roche) and harvested 24 h post-transfection.
2. Cells are placed on ice and gently washed with 2 ml of cold PBS. After removing the cold wash, the cells are incubated with 1 ml of HNT lysis buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 1% TritonX-100, 1 mM DTT, 20 mM NaF, 20 mM β -glycerophosphate, 0.5 mM sodium vanadate, 0.5 mM EGTA and protease inhibitor cocktail tablet) for 10 min on ice.
3. Cells are scraped from the plate using a scraper, and the lysate is centrifuged at 16,000 $\times$ *g* for 10 min at 4° to get rid of the cell debris.
4. The cleared lysate is subjected to immunoprecipitation using anti-FLAG M2 resin (30 μ l beads for 1 ml lysate) for 1 h at 4°, following which the beads are washed three times with HNT buffer. The beads are next used in the assay described in the following section to activate the NF κ B pathway.

3.2.5. In Vitro signaling assays

3.2.5.1. In Vitro signaling assay for activation of ERK/MAPK signaling pathway: The results of the following assay are shown in Fig. 24.3, where control lysates and YopJ lysates have been activated by the addition of RasV12-activated membranes. The MAPK pathway in control lysates is activated as demonstrated by the appearance of phosphorylated ERK/MAPKinase (sample 3). By contrast, the MAPK pathway in the YopJ lysates is inhibited, as demonstrated by the lack of phosphorylated ERK/MAPKinase (sample 4).

Materials used in this assay include:

Membrane-cleared lysates for MAPK signaling from cells transfected with pSFFV (V) and pSFFV-YopJ-Flag (J). All lysates should be adjusted to 10 mg/ml protein as measured by the Coomassie Plus protein assay reagent (Pierce).

RasV12-activated membranes

10× ARS

HTX

Samples are prepared as described below in prechilled eppendorf tubes. Solutions are added in order as described in Table 24.1.

1. Reactions are initiated by addition of 2 μ l of RasV12 activated membranes to samples 3 and 4.
2. All samples are incubated at 37° for 10 min.
3. Reactions are terminated by the addition of 20 μ l of 5× SDS-PAGE sample buffer, followed by incubation at more than 90° for 5 min.
4. Samples are separated by SDS-PAGE and analyzed by immunoblot analysis with anti-phospho-ERK antibody followed by immunoblot analysis with anti-MKK antibody (for equal loading of lysate). The phosphorylated molecules should always be analyzed first as the post-translational modification appears labile after multiple immunoblot analyses.

Similar experiments can be designed with other activators, such as activated membranes from cells treated with EGF or recombinant B-Raf as demonstrated by Mukherjee *et al.* (2006). In this protocol, 2 μ l of the activator is added in step1, followed by steps 2 to 4 as described (see Fig. 24.3).

3.2.5.2. In Vitro signaling assay for activation of NF κ B signaling pathway by TRAF6—The results of the following assay are shown in Fig. 24.4, where control lysates and YopJ lysates have been activated by the addition of TRAF6. The NF κ B pathway in control lysates is activated as demonstrated by the appearance of phosphorylated I κ B. By contrast, the NF κ B pathway in the YopJ lysates is inhibited, as demonstrated by the lack of phosphorylated I κ B.

Materials used in this assay include:

Membrane-cleared lysates for MAPK signaling from cells transfected with pSFFV (V) and pSFFV-YopJ-Flag (J). All lysates should be adjusted to ~10 mg/ml protein as measured by the Coomassie Plus protein assay reagent (Pierce).

Recombinant TRAF6 (TRAF6)

10× ARS

HTX

Samples are prepared as described below in prechilled eppendorf tubes. Solutions are added in order as described in Table 24.2.

1. Reactions are initiated by addition of 2 μ l of TRAF6 (final 0.5 μ M) to samples 2, 4, and 6.
2. All samples are incubated at 37° for 10 min.
3. Reactions are terminated by the addition of 20 μ l of 5 $\times$ SDS-PAGE sample buffer, followed by incubation at more than 90° for 5 min.
4. Samples are separated by SDS-PAGE and analyzed by immunoblot analysis with anti-phospho-I κ B antibody followed by immunoblot analysis with antialdolase antibody or anti-IKK β (for equal loading of lysate).

The phosphorylated molecules should always be analyzed first as the post-translational modification appears labile after multiple immunoblot analyses.

Similar experiments can be designed by addition of activated FLAG-NIK beads, prepared as described above. In the protocol shown above, 2 μ l of a 1:1 bead slurry are added in step 1 instead of TRAF6. Steps 2 to 4 are then performed as described. The addition of equal amounts of activated FLAG-NIK beads is confirmed by immunoblot analysis with anti-FLAG antibody.

Alternatively, another recombinant kinase, such as MEKK1, can be added to start the *in vitro* signaling reaction, with confirmation that equal amounts of the kinase are added by performing Western blot analysis (see Fig. 24.4).

4. Conclusion

We have described an *in vitro* signaling assay for MAPK and NF κ B signaling pathways. Diverse types of activators, including kinases, activated G-proteins, and E3 ligases, are used to trigger the activation of signaling cascades within a concentrated lysate. The time course for the assay is relatively short (<10 min) and the readout for activation of a pathway is simply an immunoblot. Herein, we demonstrate that this assay can be used to study inhibitors, such as the *Yersinia* effector, YopJ. This *in vitro* signaling assay is ideal for initial studies on novel activators and inhibitors of the MAPK and NF κ B signaling pathways. Immunoblot analysis of intermediate components of the signaling pathway can be analyzed using phosphospecific antibodies (such as anti-phospho-MKK or anti-phospho-IKK). The observations from the *in vitro* signaling system are consistent with previous genetic, microbial and cellular studies on the inhibitory activity of YopJ on the MAPK and NF κ B signaling pathways. The *in vitro* signaling assay is a method for analyzing signaling pathways in a test tube and provides a means to manipulate the signaling system by the addition of extraneous proteins or chemicals.

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Figure 24.1.

MAPK and NF κ B signaling pathways. The MAPK pathway is activated *in vitro* by Ras or Raf and the phosphorylation of MAPKinase (P-MAPK) is observed to indicate activation of the pathway. The NF κ B pathway is activated by TRAF6, NIK, or MEKK1, and the phosphorylation of I κ B (P-I κ B) is observed to indicate activation of the pathway.

Figure 24.2. YopJ inhibits activation MAPK kinase by acetylation of serine and threonine residues, thereby preventing phosphorylation by upstream kinase (MKKK).

Figure 24.3.

In vitro activation of the ERK/MAPK pathways by RasV12-activated membranes. Lysates from cells transfected with a control plasmid (V) (lanes 1, 2) or YopJ expression plasmid (J) (lanes 3, 4) are incubated in the absence (lanes 1, 3) or presence (lanes 2, 4) of RasV12-activated membranes. Samples are assayed by immunoblot analysis with anti-phospho-ERK antibody (used as an indicator for activation of pathway) and anti-MKK1,2 antibody (used as a load control for lysates). (Note slightly more V lysate was added to sample #1, resulting in a slight increase in background for the anti-phospho-ERK blot.)

Figure 24.4.

In vitro activation of the NF κ B pathway by recombinant TRAF6. Lysates from cells transfected with a control plasmid (V) (lanes 1, 3) or YopJ expression plasmid (J) (lanes 2,4) are incubated in the absence (lanes 1, 2) or presence (lanes 3,4) of TRAF6. Samples are assayed by immunoblot analysis with anti-phospho-I κ B antibody (used as an indicator for activation of pathway) and anti-aldolase antibody (used as a load control for lysates).

Table 24.1
***In vitro* MAPK signaling assay**

Sample	1	2	3	4
V	16 μ l	—	16 μ l	—
J	—	16 μ l	—	16 μ l
10 $\times$ ARS	2 μ l	2 μ l	2 μ l	2 μ l
HTX	2 μ l	2 μ l	—	—

Table 24.2*In vitro* NF κ B signaling assay

Sample	1	2	3	4
V	16 μ l	16 μ l	—	—
J	—	—	16 μ l	16 μ l
10 $\times$ ARS	2 μ l	2 μ l	2 μ l	2 μ l
HNT	2 μ l	—	2 μ l	—