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Co-culture Activation of MAP Kinase in *Drosophila* S2 Cells

Josefa Steinhauer

Abstract

Intercellular communication often involves phosphorylation of signal transduction proteins, including mitogen-activated protein kinases (MAPKs). Immunological detection of phosphorylated MAPK can be used to monitor signaling in vivo, identify novel pathway components, and assess ligand activity. In this chapter, I describe a cell co-culture method to assess activity of cell-bound extracellular ligands that result in phosphorylation of the ERK (extracellular signal-regulated kinase) MAPK in *Drosophila*. This protocol may be adaptable to other pathways and/or model systems.

Keywords

MAP kinase; ERK; dp-ERK; S2 cells; Co-culture; Western blot

1 Introduction

Signal transduction cascades convey extracellular signals to the cytoplasm and the nucleus, resulting in behavioral and fate changes within the receiving cell. Mitogen-activated protein kinases (MAPKs) are key components of many signaling cascades. Upstream signal transduction results in activation of MAPK via phosphorylation, and activated MAPK in turn phosphorylates potentially hundreds of cytoplasmic and nuclear targets [1–6]. Thus, MAPK phosphorylation represents a critical node in the signal response pathway and can be used as a measure of pathway activity [7, 8].

Much knowledge has been gained from studying signal transduction in *Drosophila melanogaster*. *Drosophila* possesses five MAPKs. The canonical MAPK encoded by the *rolled* gene, which is homologous to mammalian ERK-1 and ERK-2 (extracellular signal-regulated kinases), is activated by receptor tyrosine kinases (RTKs), including the Epidermal Growth Factor Receptor (EGFR) and Insulin Receptor (InR) [9–12]. Antibodies for the dually phosphorylated form of ERK (diphosphorylated-ERK, dp-ERK) allow specific detection of activated ERK, enabling visualization of pathway activity [13]. These antibodies have been used for immunohistochemistry, revealing spatial and temporal signaling patterns in vivo [14–16], and for Western blotting of tissue culture experiments, where RNAi has been used to identify novel genes required for maximal pathway activation [9, 17–19]. Tissue culture and Western blotting have also been used to investigate receptor ligand activity [20].

Extracellular ligands for EGFR family receptor tyrosine kinases are often soluble diffusible proteins, but, in several cases, cell-bound ligands also can activate receptors [20–24]. In order to examine EGFR activation by cell-bound ligands, I developed a cell co-culture method that utilizes phosphorylated ERK MAPK as the readout [20]. This protocol could be adapted for other signal transduction pathways as well, where phospho-epitope specific antibodies for pathway components are available.

2 Materials

1. Complete *Drosophila* S2 cell culture medium: Gibco Schneider medium containing 10 % fetal calf serum (heat-inactivated at 65 °C for 30 min), 50 U/mL penicillin, 50 μg/mL streptomycin. Filter sterilized.
2. D2F cells: S2 cells stably transfected with *Drosophila* EGFR, from B. Shilo [25].
3. S2 cells [26].
4. 10 mM $CuSO_4$ stock solution.
5. G418/Geneticin.
6. Qiagen Effectene Transfection Reagent (Qiagen).
7. Six-well plates.
8. 15- and 50-mL Falcon tubes, microcentrifuge Eppendorf tubes.
9. PBS (Phosphate-buffered saline, standard recipe).
10. RIPA buffer: 150 mM NaCl, 50 mM Tris–HCl pH 7.5, 1 mM NaF, 1 % NP40. Add one tablet of cOmplete™ Mini EDTA-free protease inhibitor (Roche) per 10 mL of RIPA. RIPA buffer can be stored at 4 °C for 1 week. Add fresh Na_3VO_4 phosphatase inhibitor to a final concentration of 1 mM immediately before each use (from 200 mM stock).
11. 2× Sample loading buffer: 160 mM Tris–HCl pH 6.8, 4 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 0.005 % bromophenol blue. Freeze in aliquots.
12. SDS-PAGE and Western blotting equipment and reagents.
13. Primary antibodies: mouse anti-dp-ERK (Sigma-Aldrich, M8159) and rabbit anti-ERK (Sigma-Aldrich, M5670), used at 1:2000 and 1:50,000 dilutions, respectively.
14. Secondary antibodies: IRDye 800CW donkey anti-mouse (Li-cor) used at 1:10,000 dilution, IRDye 800CW donkey anti-rabbit (Li-cor) used at 1:10,000 dilution, and HRP-conjugated donkey anti-mouse and anti-rabbit antibodies (Jackson Immunoresearch), both used at 1:4000 dilution.
15. Odyssey scanner and software (Li-cor).
16. Substrate and developer kit for enhanced chemiluminescence (ECL, e.g., ThermoScientific SuperSignal West Pico Chemiluminescent Substrate).

3 Methods

In this method, ligand-expressing S2 cells are mixed with EGFR-expressing D2F cells and co-cultured. Cell lysates are then prepared, electrophoresed, and blotted with anti-ERK and anti-dp-ERK antibodies (Fig. 1).

3.1 Culture Preparation

3.1.1 Day 1—Grow cells to a confluence of 1–2 × 10^6 cells/mL.

1. Plate S2 cells in six-well plates at 2 × 10^6 cells/well in complete medium. Use a volume of 1.6 mL/well.
2. Transfect S2 cells with DNA constructs using Qiagen Effectene Transfection Reagent, following the manufacturer's protocol (*see* Notes [1] and [2]).
3. Plate D2F cells in six-well plates at 2 × 10^6 cells/mL in complete medium. Use a volume of 1 mL/well. Include antibiotic for selection (150 μg/mL of G418). Prepare 2 × 10^6 D2F cells (i.e., one well) for each test condition.

3.1.2 Day 2—Induce expression of inducible transfected constructs (e.g., constructs under control of *metallothionein* promoter typically require 700 μM $CuSO_4$).

3.1.3 Day 3, a.m

1. Induce EGFR expression in the D2F cells with 60 μM $CuSO_4$ (*see* Note [3]).
2. Allow EGFR to express for 3 h.

3.2 Co-culture

3.2.1 Day 3, p.m

1. Collect cells and transfer to 50-mL Falcon tubes. D2F cells can be pooled.
2. Pellet cells by centrifugation at 800 rpm in a table-top swing-bucket centrifuge (~130 × *g*) for 5 min.
3. Discard supernatant.
4. Resuspend cells in room temperature PBS to wash.
5. Repellet cells and discard wash supernatant.
6. Resuspend S2 cells in 500 μL of complete medium per well.

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[1] If using *UAS* constructs, cotransfect *actin-GAL4* to induce expression.

[2] I have used *UAS-GFP* as a negative control and *UAS-sSpi* as a positive control in each experiment. *UAS-GFP* encodes cytoplasmic green fluorescent protein. *UAS-sSpi* encodes a constitutively secreted form of the *Drosophila* EGFR ligand Spitz (Spi) that lacks a transmembrane domain but is tethered to the cell membrane via N-terminal palmitoylation [25, 27]. Other EGFR ligands assayed by this technique include chimeric proteins in which Spi was fused to various transmembrane proteins as well as mutant Spi proteins that retained their endogenous transmembrane domains. Both type I and type II transmembrane proteins have been assayed with this protocol [20].

[3] *Metallothionein*-driven constructs are typically induced with 700 μM $CuSO_4$. The low $CuSO_4$ concentration and short duration of induction used here are recommended by Schweitzer et al. to minimize EGFR auto-activation [25]. These induction conditions could be modulated as necessary.

7. Resuspend D2F cells in half the original volume of complete medium (add 150 μg/mL G418 and 60 μM $CuSO_4$).
8. In 15-mL Falcon tubes, mix 500 μL of D2F cells with each 500 μL sample of transiently transfected S2 cells.
9. Rock co-cultures in Falcon tubes at room temperature for 1 h.

3.3 Cell Lysis

1. During co-culture, chill PBS and RIPA buffer on ice.
2. Following co-culture, pellet cells, as before. Discard supernatant.
3. Resuspend cells in ice-cold PBS to wash.
4. Pellet cells, discard wash supernatant.
5. Resuspend cells in 100 μL of ice-cold RIPA buffer per sample and move to microcentrifuge tubes. Pipet up and down vigorously to lyse cells (~30 times).
6. Vortex each sample for 30 s. Do not rock or pipet samples after this step.
7. Immediately spin samples at 13,000 × *g* for 10 min at 4 °C.
8. Transfer supernatant to fresh microcentrifuge tubes. Add 100 μL of Sample loading buffer.
9. Heat samples at 95 °C for 5 min.
10. Place samples on ice and load immediately on an SDS-PAGE gel or freeze them at −20 °C until electrophoresis.

3.4 SDS-PAGE and Western Blot

1. Prepare SDS-PAGE gel according to standard protocol.
2. Run two sets of the entire experiment, complete with molecular weight marker and each co-culture condition. Use the same volume per well for each set.
3. Follow standard Western blotting protocol.
4. Following transfer, cut the nitrocellulose membrane to separate the two experimental sets.
5. Blot one experimental set with anti-dp-ERK and the other with anti-ERK primary antibodies. Use fluorescent secondary antibodies (e.g., from Li-cor).
6. Scan blots with Li-cor Odyssey.
7. Quantify pixel intensity of each band using Odyssey software (*see* Notes 4 and 5).

[4]For quantification, compare dp-ERK signal intensity in each lane to total ERK signal intensity in the equivalent lane. Then normalize each ratio to the dp-ERK:ERK ratio of the negative control (e.g., *UAS-GFP*).
[5]It is useful to repeat the experiment at least three times. Quantifications can be averaged, graphed, and evaluated for statistical significance.

8. Wash the membranes and reprobe overnight with HRP-conjugated secondary antibodies.

9. Develop with ECL (*see* Note 6).

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[6]The purpose of the reblot with HRP-conjugated secondary antibodies is purely cosmetic; the ECL development gives a better image for publication than the Odyssey scan.

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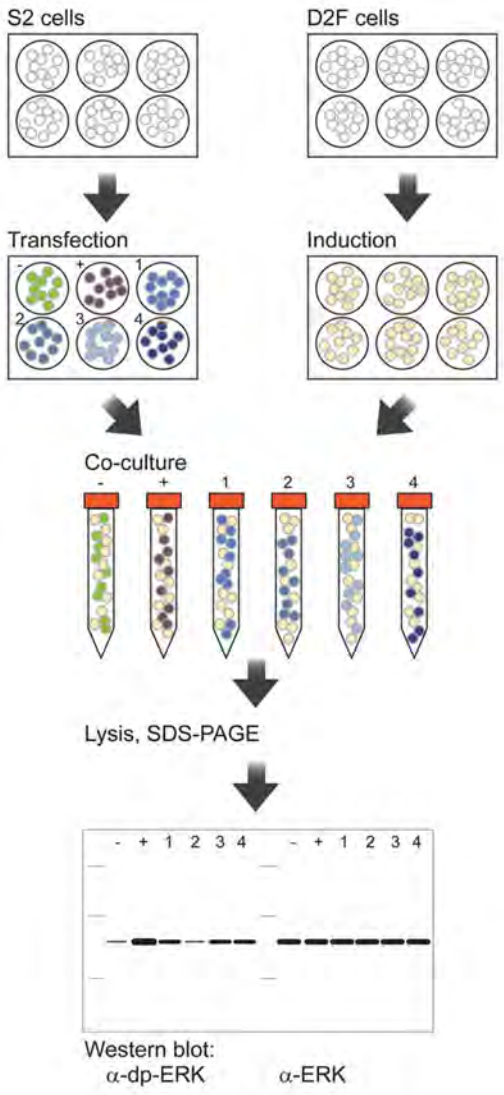

Fig. 1.

Diagram of the experiment. S2 cells and D2F cells are plated separately. S2 cells are transfected with a negative control (*green*, −), a positive control (*purple*, +), or experimental constructs (*different shades of blue* indicate different experimental constructs, 1–4). EGFR expression (*yellow*) is induced in D2F cells. Each set of transfected S2 cells is mixed with an equal number of EGFR-expressing D2F cells. Following co-culture, cells are lysed, and lysates are electrophoresed and subjected to Western blotting. Each lysate is simultaneously blotted with anti-dp-ERK and anti-ERK antibodies. A ratio of dp-ERK:ERK is then calculated from the band intensities in the Western blots and normalized to the negative control