Video Article

Co-immunoprecipitation Assay for Studying Functional Interactions Between Receptors and Enzymes

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Abstract

Receptor-associated enzymes are the major mediators of cellular activation. These enzymes are regulated, at least in part, by physical interactions with cytoplasmic tails of the receptors. The interactions often occur through specific protein domains and result in activation of the enzymes. There are several methods to study interactions between proteins. While co-immunoprecipitation is commonly used to study domains that are required for protein-protein interactions, there are no assays that document the contribution of specific domains to activity of the recruited enzymes at the same time. Accordingly, the method described here combines co-immunoprecipitation and an on-bead enzymatic activity assay for simultaneous evaluation of interactions between proteins and associated enzymatic activation. The goal of this protocol is to identify the domains that are critical for physical interactions between a protein and enzyme and the domains that are obligatory for complete activation of the enzyme. The importance of this assay is demonstrated, as certain receptor protein domains contribute to the binding of the enzyme to the cytoplasmic tail of the receptor, while other domains are necessary to regulate the function of the same enzyme.

Video Link

The video component of this article can be found at https://www.jove.com/video/58433/

Introduction

Catalytic receptors and receptor tyrosine kinases are transmembrane proteins in which binding of an extracellular ligand causes enzymatic activity on the intracellular side[1]. Some receptors possess both receptor and enzymatic functions, while others recruit specific enzymes such as kinases and phosphatases to their cytoplasmic tails. Recruitment of an enzyme to the receptor's tail and the subsequent catalytic action of this enzyme are two separate processes that are not always regulated by the same protein domains[2]. Unfortunately, there are no specific tools to assess both the interaction and enzymatic activity simultaneously. The functional co-immunoprecipitation assay described here is a useful method to dissect the recruitment of an enzyme to the tail of a receptor from its activation. This assay utilizes immunoprecipitation of tagged receptors by antibody-coated beads. Subsequently, both an enzymatic activity assay and western blot analysis on beads are performed. The overall goal of this method is to uncover which protein domains are necessary for interactions between receptors and enzymes (assessed by western blot analysis) and which domains are obligatory for complete activation of the enzymes (measured by on-bead enzymatic activity assay). It is significant to develop tools for studying the separate functions of receptor-associated enzymes due to their involvement in the pathogenesis of human diseases. Moreover, further understanding the mechanisms of action of these proteins may help the design of novel therapeutic interventions.

Programmed death-1 (PD-1) is an inhibitory receptor on the surface of T cells and is required for limiting excessive T cell responses. In recent years, anti-PD-1 antibodies have been implicated in the treatment of multiple malignancies[1,2]. PD-1 ligation restrains numerous T cell functions, including proliferation, adhesion, and secretion of multiple cytokines[3,4,5]. PD-1 is localized to the immunological synapse, the interface between T cells and antigen-presenting cells[6], where it colocalizes with the T cell-receptor (TCR)[7]. Subsequently, the tyrosine phosphatase SHP2 [Src homology 2 (SH2) domain containing tyrosine phosphatase 2] is recruited to the cytoplasmic tail of PD-1, leading to dephosphorylation of key tyrosine residues within the TCR complex and its associated proximal signaling molecules[3,4,5,8,9]. The cytoplasmic tail of PD-1 contains two tyrosine motifs, an immunoreceptor tyrosine-based inhibitory motif (ITIM), and an immunoreceptor tyrosine based-switch motif (ITSM)[10]. Both motifs are phosphorylated upon PD-1 ligation[9,10]. Mutagenesis studies have revealed a primary role for the ITSM in SHP2 recruitment, as opposed to the ITIM, whose role in PD-1 signaling and function is not clear[4].

SHP2 adopts either a closed (folded), inhibited conformation or an open (extended), active conformation[11]. The contribution of each tail domain of PD-1 to SHP2 binding or activation has not yet been elucidated. To answer this question, we developed an assay that enables parallel testing of the recruitment of SHP2 to the tail of PD-1 and its activity[12]. We employed co-immunoprecipitation and an on-bead phosphatase activity assay

to test both the interaction and enzymatic activity in parallel. Using this assay, we show that the ITSM of PD-1 is sufficient to recruit SHP2 to the tail of PD-1, while the ITIM of PD-1 is needed to fully extend and activate the enzyme.

There are many receptors that have several adjacent domains in their cytoplasmic tails. The functional co-immunoprecipitation assay can uncover the role of specific domains that are necessary for either protein recruitment or enzymatic activation.

Protocol

1. Transfection of Cells

1. Seed HEK 293T cells into twelve 10 cm plates (5 x 10^6 cells per plate) the day before transfection (**Figure 1**). Perform the cell counting using a hemocytometer. For each plate, use 10 mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Incubate the cells at 37 °C in 5% CO_2.
2. Once the cells are 80-90% confluent, transfect 5 HEK 293T plates using a lipid-based transfection reagent with one of the following plasmids: a SHP2-expressing vector, a PD-1-GFP-expressing vector [a wild type (WT) version of PD-1], an ITIM-mutated version (Y223F) of a PD-1-GFP-expressing vector, and an ITSM-mutated version (Y248F) of a PD-1-GFP-expressing vector (**Figure 1**).
   NOTE: Two plates will be transfected with the WT PD-1-GFP-expressing vector. One additional plate will serve as a non-transfected cells control (**Figure 1**).
3. Perform the transfection as per the manufacturer's protocol for adherent cells in 10 cm plates.
   NOTE: In the mutated versions of PD-1, the tyrosine (Y) in each motif was replaced by phenylalanine (F) that cannot be phosphorylated.
4. Transfect a second identical set of five plates and one additional plate serving as a non-transfected control for the measurement of expressed protein amount [input; also known as whole cell lysate (WCL)] before the immunoprecipitation (**Figure 1**).
5. Incubate the transfected cells for 48 h in 37 °C, 5% CO_2 in a tissue culture incubator.
   NOTE: To ensure that transfection has successfully occurred, examine the cells for GFP expression using a fluorescent microscope.

2. Promoting Phosphorylation in Transfected Cells

1. Following incubation, prepare fresh pervanadate by mixing 50 µL of sodium-orthovanadate (from 100 mM stock) with 50 µL of 30% H_2O_2.
   NOTE: The mixture should change into a yellowish color. Pervanadate is a cell-permeable phosphatase inhibitor that promotes phosphorylation of tyrosine residues[13]. In this assay, it is used to robustly induce phosphorylation of the tails of PD-1.
2. To phosphorylate the tagged versions of PD-1, remove the media from the PD-1-GFP-transfected cells and add 10 mL of plain DMEM (without serum or antibiotics) together with 10 µL of pervanadate (step 2.1) to each 10 cm plate (resulting in eight plates expressing different versions of PD-1) (**Figure 1**). Place the plates at room temperature in the dark for 15 min.
   NOTE: The SHP2-transfected cells (two plates; **Figure 1**) and the two non-transfected control plates are not treated with pervanadate, since these plates will serve as the source for the active SHP2 enzyme.
3. Wash the cells with 5 mL of ice-cold 1x PBS. Repeat this step twice.

3. Immunoprecipitation

NOTE: The following steps should be performed on ice or at 4 °C.

1. Supplement the lysis buffer (50 mM Tris-HCl at pH 7.2, 250 mM NaCl, 0.1% NP-40, 2 mM EDTA, 10% glycerol) with protease inhibitors (dissolve 1 tablet in 10 mL of lysis buffer) and with 1 mM sodium orthovanadate. Add 500 µL of ice-cold lysis buffer to the cells and remove and collect the cells from the plates immediately using a cell scraper.
   NOTE: It is important to add the sodium orthovanadate only to the lysis buffer that will be used for the PD-1-GFP-transfected plates, since the SHP2-transfected plates and non-transfected control plates must retain phosphatase activity.
2. Transfer the lysates into 1.5 mL cold tubes and rotate them at 0.005 x *g*, 4 °C for 30 min.
3. To collect the post nuclei supernatant (PNS) from the lysates, spin down the lysates for 10 min at 10,000 x *g* at 4 °C and transfer the supernatants into new tubes. Discard the pellet. Store the supernatants of the second set of six plates (**Figure 1**) on ice for later WCL analysis.
4. **Preparation of the anti-GFP beads for immunoprecipitation of PD-1-GFP from the PD-1-GFP-transfected lysates of the first set (four plates) (Figure 1).**
   1. To prevent settling of the beads, gently shake the bottle with the beads before opening. Remove 40 µL of the anti-GFP beads from the slurry per each condition.
   2. Spin at 500 x *g* for 3 min at 4 °C and remove the supernatant to wash the beads.
      NOTE: It is important to minimize the contact between the pipette plastic tips and agarose beads to prevent loss. It is recommended to cut the edge of a 200 µL tip before transferring the beads to another tube.
   3. Resuspend the beads in 80 µL of lysis buffer (per sample).
5. Add the washed beads directly to the cell lysate (PNS) from the PD-1-GFP-expressing cells of the first set (four plates) (step 3.3). Rotate at 0.005 x *g* for 30 min at 4 °C to immunoprecipitate the GFP-tagged proteins.
6. Wash the beads with 1 mL of cold lysis buffer (without orthovanadate) 3 times. Centrifuge the tube at 2,500 x *g* for 10 s.
   NOTE: When adding the lysis buffer to the beads, add it directly onto the beads without touching them with the tips. There is no need to pipette up and down during the washes.
7. Equally divide the lysate of the active SHP2 from the first set (one plate; step 3.3) and add it to three tubes of the washed PD-1-GFP-containing beads (the WT PD-1-GFP and the two different phosphdeficient mutations, Y223F and Y248F). Add one-third of the volume from the lysate of the non-transfected cells to the second tube of the WT PD-1-GFP beads. Discard the remaining two-thirds.

8. Incubate the beads for 4 h at 4 °C with gentle rotation (0.005 x *g*).
9. Wash the beads twice with 1 mL of cold lysis buffer (without pervanadate or orthovanadate), as reported in step 3.6.
10. Add 80 µL of lysis buffer (without pervanadate or orthovanadate) per sample ensuring that the total volume in each tube is 100 µL (20 µL of beads and 80 µL of lysis buffer). Mix gently and transfer 50 µL from each tube into two fresh 1.5 mL tubes.
    NOTE: Following this step, there will be two tubes filled with a mixture that contains beads and lysis buffer: one tube will be used for testing the co-immunoprecipitated SHP2 by western blotting, and the second will be used for the phosphatase activity assay.

4. Phosphatase Activity Assay

1. Wash the beads once with phosphatase wash buffer (30 mM Hepes at pH 7.4 and 120 mM NaCl). Remove the supernatant completely.
2. Add 100 µL of assay buffer (30 mM Hepes at pH 7.4, 120 mM NaCl, 5 mM DTT, 10 mM p-nitrophenylphosphate) to the beads, and incubate at 30 °C for 30 min under gentle agitation. NOTE: Incubation time may vary, but wait until the buffer turns yellow upon dephosphorylation.
3. To terminate the reaction, add 50 µL of 1 M NaOH when the buffer turns yellow.
4. Spin down at 2,500 x *g* for 10 s and transfer 50 µL of the supernatant to two wells (duplicates with 50 µL per well) of half-area in a 96-well plate. Read the absorbance at 405 nm.
5. Express the results as relative optical density (OD) over the control wild-type version of PD-1-GFP.

5. SHP2 Western Blot Analysis

1. Spin down the beads and remove the supernatant.
2. Add 20 µL of 2x Laemmli buffer (see **Materials Table**) to the beads and boil at 95 °C for 5 min.
3. Measure the protein concentration of the input controls (second set) using a BCA kit (see **Materials Table**). Transfer 30 µL of the most diluted sample to a new tube. Dilute the rest of the input controls with lysis buffer to the same concentration as the most diluted sample, and transfer 30 µL from each of them to a new tube.
   NOTE: Following this step, there will be 4 tubes with 30 µL each, at similar protein concentrations, representing the input controls lysates.
4. Add equal volumes of 2x Laemmli buffer to the lysates and boil at 95 °C for 5 min. Spin down the beads and load the supernatant on 4–20% SDS-PAGE Tris-based gel (see **Materials Table**) for western blot analysis. In addition, load 50 µL from each sample of the second set.
5. Continue western blot analysis according to the protocol previously described[12].

Representative Results

While the contribution of the ITSM of PD-1 to SHP2 binding is established, the role of the ITIM of PD-1 is less clear. Because SHP2 has two SH2 domains that can bind to two sequential phosphotyrosines on PD-1 (one tyrosine in the ITSM and another in the ITIM), we hypothesized that the ITSM of PD-1 anchors SHP2 to PD-1, while the ITIM of PD-1 facilitates SHP2 activity by stabilizing its open conformational state[11,14]. To test this, we developed a combined co-immunoprecipitation and enzymatic activity assay for the parallel assessment of receptor-enzyme interactions and activation. Wild type GFP-PD-1, GFP-PD-1 Y223F (ITIM mutant), or GFP-PD-1 Y248F (ITSM mutant) were expressed in HEK 293T cells that were subsequently treated with pervanadate (**Figure 1**), leading to phosphorylated PD-1 tails. PD-1 proteins were collected using anti-GFP coated beads. These beads were used for SHP2 co-immunoprecipitation from lysates of cells overexpressing SHP2. The levels of SHP2 bound to each version of PD-1 and its enzymatic activity were recorded.

Unsurprisingly, SHP2 failed to bind to PD-1 when the ITSM was mutated (Y248F; **Figure 2A**). Remarkably, the mutant version of the ITIM (Y223F) inhibited SHP2 binding only to a limited extent (**Figure 2B**). Nevertheless, the SHP2 phosphatase activity assay revealed that the ITIM and ITSM were equally indispensable for the enzymatic activity (**Figure 2C**). Since PD-1 immunoprecipitates may contain other phosphatases, in addition to SHP2, we used a control condition (**Figure 2B** and **2C,** blue) in which SHP2 was not overexpressed.

Hence, a two-step activation model is revealed in which SHP2 is folded into an auto-inhibited conformation under resting conditions (**Figure 2D**, left). Upon activation of PD-1, SHP2 is recruited to the phosphorylated ITSM (**Figure 2D**, middle). However, the ITIM must also be phosphorylated to unfold SHP2 into its active conformation (**Figure 2D**, right).

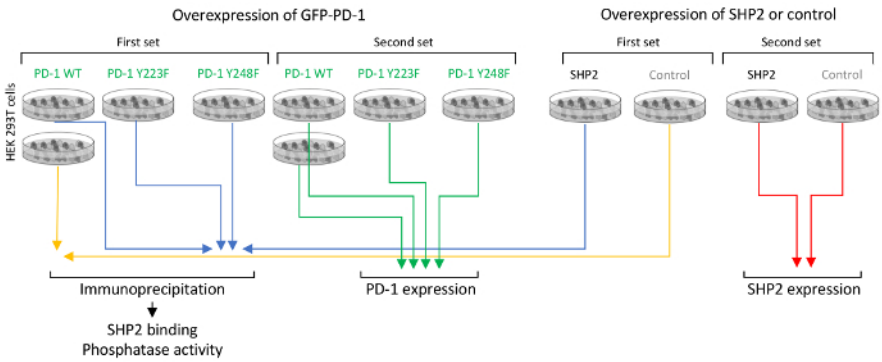

Figure 1: Experimental conditions and strategy. GFP-PD-1 WT (wild type), GFP-PD-1 Y223F (ITIM mutant), or GFP-PD-1 Y248F (ITSM mutant) were expressed in HEK 293T cells that were subsequently treated with pervanadate. Phosphorylated GFP-PD-1 proteins collected by GFP immunoprecipitation were mixed with lysates from cells overexpressing SHP2, and the levels and activity of SHP2 bound to each version of PD-1 were recorded. Please click here to view a larger version of this figure.

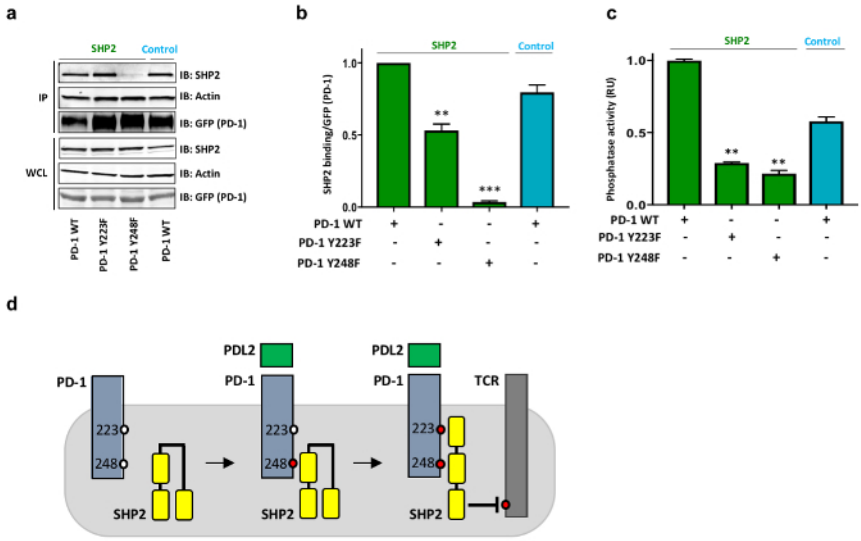

Figure 2: The ITIM of PD-1 is necessary for SHP2 activity. HEK 293T cells were transfected with the indicated versions of GFP-PD-1, followed by pervanadate treatment and immunoprecipitation using anti-GFP mAb-agarose (**a**). SHP2 levels bound to precipitated GFP-PD-1 were quantified (**b**) and subjected to a phosphatase activity assay (**c**). Values of pulled-down SHP2 were normalized to GFP expression levels. All values are fold-change compared with the intensity of precipitated SHP2 by the WT GFP-PD-1 (wild type). Phosphatase activity values are fold-change compared with the activity of co-immunoprecipitated SHP2 by the WT GFP-PD-1. RU = relative units. (**d**) The two-step activation model. First, SHP2 is recruited to the ITSM, and only then does the second SH2 domain bind to the ITIM of PD-1, which extends the catalytic domain of SHP2 to the fully active conformation. Data are presented as mean ± SEM. Asterisks represent significant differences between the denoted group and the WT PD-1 (b and c): ***p* <0.01, ****p* <0.001, unpaired t-test, n = 3. Permission to use data from Peled *et al.* (2018)[12] was granted. Please click here to view a larger version of this figure.

Discussion

Receptor-enzyme interactions are crucial for intracellular signaling. Many enzymes are recruited to receptors through SH2 domains binding to phosphorylated tyrosines that decorate tails of the same receptors. However, enzymes are often folded into closed inactive conformations, and activation requires a conformational change[11] that can be mediated by other domains of the same receptor. The assay described here measures the interactions between receptors and enzymes as well as the activity induced by these interactions.

We used a colorimetric assay that utilizes p-nitrophenylphosphate (pNPP) as substrate for SHP2. pNPP is a non-selective substrate and phosphor donor that is released by a broad number of enzymes[15]. Phosphorylated recombinant peptides can serve as alternative substrates (*e.g.*, those similar to ones used in the malachite green assay). These peptides are more specific in terms of the phosphatase activity; however, it is costlier, and due to its sensitivity, it can only be performed in phosphate-free solutions. Another approach to circumvent the lack of specificity towards pNPP is to include a control cell line in which the phosphatase in question is knocked out. In these cells, overexpression of SHP2 will be the only source for the enzyme, and any increase in phosphatase activity should be attributed specifically to the over-expressed phosphatase. A third approach is to use a purified epitope-tagged SHP2 protein and purified epitope-tagged PD-1 instead of WCL supplemented by phosphopeptides as the substrate. An alternative to using a phosphopeptide as the substrate is to use a phosphoprotein instead, and then the dephosphorylation of the protein may be detected by a phospho-specific antibody or phos-tag SDS-PAGE.

Importantly, the method described here can shed light on the biology of other receptor-enzyme interactions. For example, it can uncover the function of tyrosine residues in SLAM family receptors, which have been found to be negligible for interactions with SHP2[16] but may actually be necessary for activation of the same enzyme. This method can also be applied to other phosphatases as well as receptor-interacting kinases.

While this method is relatively straightforward, it is important to note when phosphatase inhibitors should be avoided. These are necessary in the initial step of phosphorylation induction of PD-1, but later they must be removed in order to test the phosphatase activity of SHP2.

Disclosures

The authors have nothing to disclose.

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