Co-sedimentation Assay for the Detection of Direct Binding to F-actin

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[Abstract] This protocol describes measurement of direct protein-protein interactions by actin co-sedimentation assay. The actin co-sedimentation assay is a well-established technique and has been commonly used to demonstrate binding of proteins that interact directly with actin filaments (Ahrens *et al*., 2012; Mehta and Sibley, 2010; Schuler *et al.*, 2005; Singh *et al.*, 2011). We and others have previously shown that the damaged cell-recognition molecule C-type lectin 9A (Clec9A) recognises a conserved component within nucleated and non-nucleated cells that is exposed only when the cell membrane is damaged (Srivastava and Barber, 2008; Zhang *et al.*, 2012). Here we use Clec9A as an example and present a detailed procedure for demonstrating the direct binding of purified recombinant Clec9A ectodomain to actin filaments.

Materials and Reagents

1. Rabbit muscle G-actin (Cytoskeleton, catalog number: AKL95-C)
2. Recombinant Clec9A ectodomain was expressed and purified by affinity chromatography and size-exclusion chromatography as described previously 5
3. Tris
4. $CaCl_2$
5. ATP
6. β-mercaptoethanol
7. KCl
8. $MgCl_2$
9. NuPAGE Novex 4-12% Bis-Tris gel (Life Technologies, Invitrogen™, catalog number: NP0327BOX)
10. Laemmli reducing SDS-PAGE sample buffer (Laemmli 2x concentrate) (Sigma-Aldrich, catalog number: S3401)

11. Precision Plus Protein molecular weight marker (Bio-Rad Laboratories, catalog number: 161-0373)
12. Coomassie Brilliant Blue
13. 1x G-buffer (see Recipes)
14. 10x F-buffer (see Recipes)

Equipment

1. Superdex S200 10/300 GL column (GE Healthcare)
2. AKTA Fast Protein Liquid Chromatography (FPLC) system (GE Healthcare)
3. TLA 100 rotor and Beckman Coulter Optima TL Ultracentrifuge (Beckman Coulter)
4. Polycarbonate centrifuge tubes (7 x 20 mm, 230 µl) (Beckman Coulter, catalog number: 343775)
5. GS-800 calibrated densitometer and Quantity one image analysis software (Transmission densitometer) (Bio-Rad Laboratories, catalog number: 1707980)

Procedure

A. Preparation of actin
   1. Reconstitute vial of lyophilised powder of rabbit muscle G-actin in water (2 mg/ml) and incubate protein for 1/2 h at room temperature (RT).
      Note: If making up a large stock of actin, freeze in small, single use aliquots at -80 °C.
   2. Pre-equilibrate a Superdex S200 10/300 GL column with a minimum of 35 ml of G-buffer.
      Note: G-buffer is used as both pre-elution and elution buffer.
   3. Load 0.5 ml of actin (2 mg/ml) onto the Superdex S200, and run column in G-buffer at a flow rate of 0.5 ml/min, and collect column fractions corresponding to elution volumes of 12-15 ml for analysis.
   4. Analyse fractions reflecting the peak of actin protein (G-actin molecular mass is 42-43 kDa) by SDS-PAGE on a NuPAGE Novex 4-12% Bis-Tris gel under reducing conditions, and visualise proteins by Coomassie Brilliant Blue staining to confirm purity of G-actin.
   5. Pool fractions reflecting the peak of G-actin.
   6. Polymerise G-actin (pooled monomeric G-actin from step 1e) to F-actin by adding 1/10 volume of 10x F-buffer and incubating for 1 h at RT.

B. Assay for actin filament binding
   7. Use the actin filaments generated in step A-6 to set up a series of reaction tubes in polycarbonate ultracentrifuge tubes with a range of concentrations of actin filaments (0, 1,

2, 5 μM) and add a fixed amount of potential actin-interacting protein (Clec9A; 5 μM) or a control protein that does not interact with actin (5 μM) in a total volume of 50 μl. As a control, also include 1 tube of actin alone.

Note: The concentration of actin filaments used for each reactions should be based on the initial concentration of G-actin in step A-5.

8. Incubate reactions for 1 h at RT.
9. Sediment actin filaments and associated proteins by ultracentrifugation at 100,000 *x g* using a fixed angle rotor for 1 h at RT.
10. Transfer supernatant to a fresh tube.
11. Wash pellet by adding 50 μl of 1x F-buffer (diluted in G-buffer) and sediment actin filaments and associated proteins by ultracentrifugation at 100,000 *x g* for 1 h at RT.
12. Remove supernatant and resuspend pellet in 1x Laemmli reducing SDS-PAGE sample buffer (equal volume to supernatant).
13. Set up tubes containing an equal volume of supernatant (from step B-4) with SDS-PAGE sample buffer, or resuspended pellet in SDS-PAGE sample buffer (from step B-6) for each of the reactions of actin +/- actin binding proteins, then incubate samples at 95 °C for 5 min.
14. Analyse samples by SDS-PAGE and visualise proteins by Coomassie Brilliant Blue staining.
15. Quantitate the amount of Clec9A in the supernatant and pellet fractions by densitometry analysis using a GS-800 calibrated densitometer.

    Note: It is important to note that as the samples are being analysed under reducing conditions, the sizes of the bands do not change on SDS-PAGE. What changes is the shift of the bands from supernatant to pellet as a result of F-actin cosedimentation.
16. Calculate the intensity of the Clec9A bands in the pellet, and the intensity of the total amount of Clec9A (pellet + supernatant). Then calculate the proportion of Clec9A co-sedimenting with F-actin as the ratio of Clec9A in the pellet relative to the total Clec9A (Clec9A in the Supernatant + Pellet), that is, the intensity of Clec9A in pellet divided by the intensity of Clec9A in (supernatant + pellet). The proportion of Clec9A co-sedimenting with F-actin relative to a nonspecific binding control is interpreted as the specific binding to F-actin.

Recipes

1. 1x G-buffer
   20 mM Tris (pH 8)
   0.1 mM $CaCl_2$

0.1 mM ATP

5 mM β-mercaptoethanol

2. 10x F-buffer

   1 M KCl

   20 mM ATP

   20 mM $MgCl_2$

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