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Identification of associated proteins by coimmunoprecipitation

Many protein-protein associations that exist within the cell remain intact when a cell is lysed under nondenaturing conditions. Thus, if protein *X* is immunoprecipitated, then protein *Y*, which stably associated with *X*, may also precipitate. Coimmunoprecipitation is most commonly used to test whether two proteins of interest are associated *in vivo*, but it can also be used to identify interacting partners of a target protein. In both cases, the cells, labeled with [^{35}S]methionine, are collected and lysed under conditions that preserve protein-protein interactions. The target protein is specifically immunoprecipitated from the cell extracts, and the immunoprecipitates are fractionated by SDS-PAGE. Coimmunoprecipitated proteins are detected by autoradiography and/or by western blotting with an antibody directed against that protein. The identity of interacting proteins may be established or confirmed by Edman degradation of tryptic peptides. Some early examples of this method include the use of antibodies to viral antigens to determine the host cellular proteins that interact with these viral transforming oncoproteins. Two interacting proteins of particular note are the tumor suppressor proteins p53 and pRB[1–3]. This protocol was used to identify pVHL-associated proteins; conditions should be optimized for the protein of interest.

PROCEDURE

Preparation and precipitation of the lysate

1| Wash thirty 10-cm plates of the appropriate cells (a total of ~6×10^7 cells) in phosphate-buffered saline. Scrape each plate of cells into 1 ml of ice-cold EBC lysis buffer (50 mM Tris (pH 8), 120 mM NaCl, 0.5% NP-40, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF; ▲ CAUTION), 0.2 mM sodium orthovanadate, 100 mM sodium fluorine (▲ CAUTION)).

2| Transfer each milliliter of cell suspension into a centrifuge tube, and spin the tubes at maximum speed at 4 °C for 15 min in a centrifuge.

3| Pool the supernatants (~30 ml) and add 30 μg of the appropriate antibody (that will precipitate the target protein). Rock the immunoprecipitate at 4 °C for 1 h.

4| Add 0.9 ml of protein A–Sepharose slurry. Rock the immunoprecipitate at 4 °C for another 30 min.

5| Wash the protein A–Sepharose mixture in NETN buffer (20 mM Tris (pH 8), 1 mM EDTA, 900 mM NaCl, 0.5% NP-40). Repeat this wash five more times. Finally, wash the mixture once in NETN buffer containing 100 mM NaCl.

6| Remove the liquid portion of the mixture by aspiration. Add 800 μl of 1× SDS gel-loading buffer to the beads and boil for 4 min.

Analysis of the immunoprecipitate

7| Load the sample into a large well of the discontinuous SDS-PAGE gradient gel and run the gel overnight at 10 mA constant current.

8| Visualize the protein bands by staining with Coomassie blue, excise the band of interest from the gel and place it in a centrifuge tube. Wash the gel slice twice for 3 min each in 1 ml of 50% acetonitrile (▲ CAUTION).

9| Digest the protein with trypsin while it is still in the gel, and electroelute the peptides.

(**i**) Remove the gel slice to a clean surface, and allow it to partially dry.

(**ii**) Add 5 µl of trypsin digestion buffer and 2 µl of trypsin solution.

(**iii**) After the gel absorbs the trypsin solution, add 5-µl aliquots of trypsin digestion buffer until the gel slice regains its original size.

(**iv**) Place the gel slice in a centrifuge tube, immerse it in trypsin digestion buffer and incubate it for 4 h at 30 °C. Stop the reaction by addition of 1.5 µl of 0.1% trifluoroacetic acid.

10| Fractionate the peptides by narrow-bore high-performance liquid chromatography. Subject the collected peptides to automated Edman degradation sequencing on an ABI 477A or 494A machine.

SOURCE

This protocol was adapted from "Identification of associated proteins by coimmunoprecipitation" in *Molecular Cloning: A Laboratory Manual* (eds. Sambrook, J. & Russell, D.W.) 18.60–18.68 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2001). The protocol was provided by Peter D. Adams (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA) and Michael Ohh (Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts, USA).

1. Lane, D.P. & Crawford, L.V. T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**, 261–263 (1979).
2. Yee, S.P. & Branton, P.E. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. *Virology* **147**, 142–153 (1985).
3. Harlow, E., Whyte, P., Franza, B.R. Jr. & Schley, C. Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* **6**, 1579–1589 (1986).